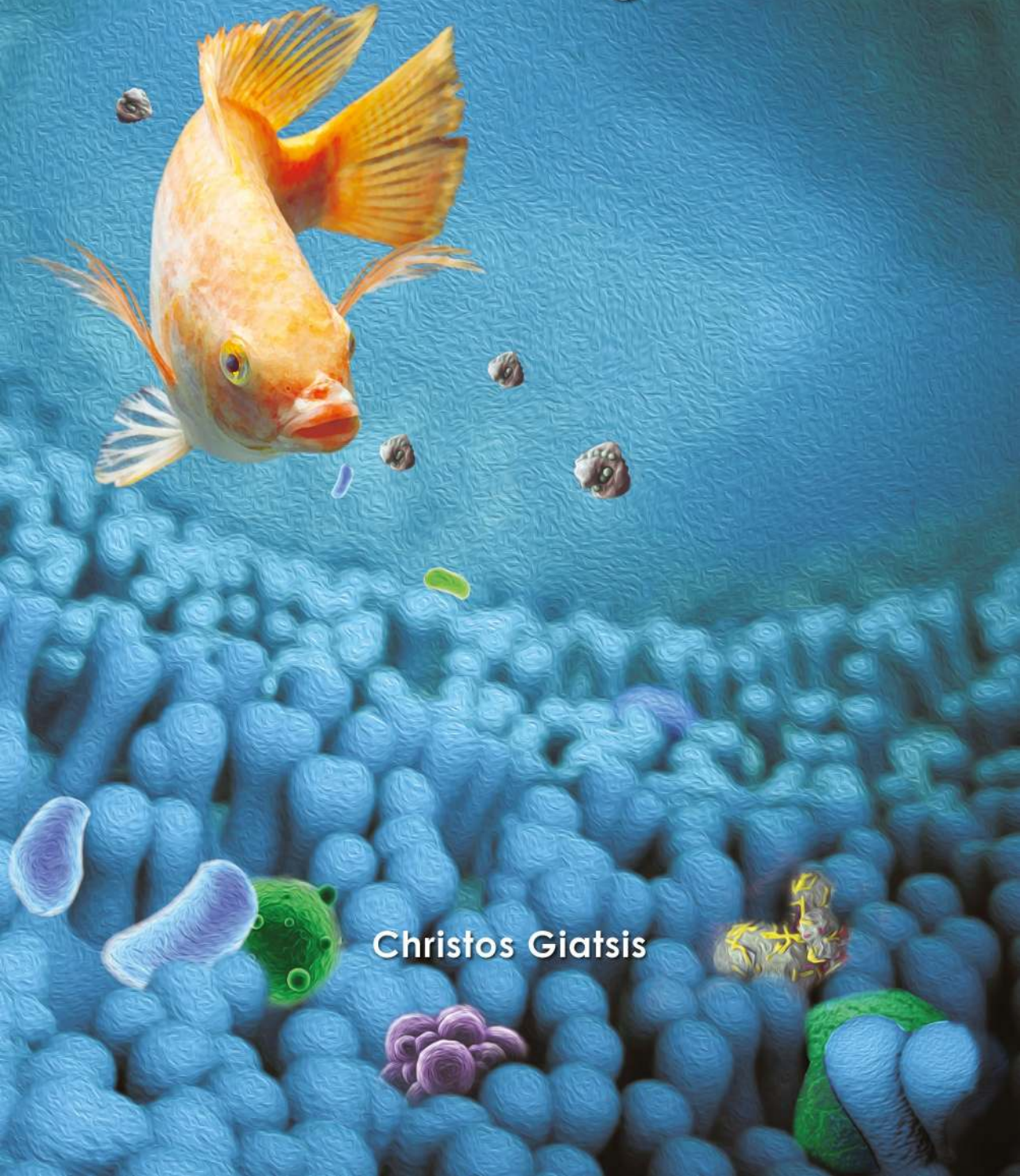


Microbial interactions in the fish gut



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Microbial interactions in the fish gut

Christos Giatsis

Thesis

submitted in fulfillment of the requirements for the degree of doctor
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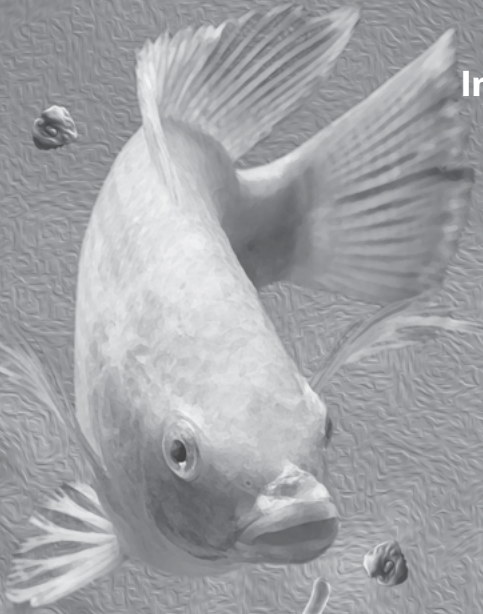
Fiat justitia - ruat caelum
-Ancient Roman proverb-

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Chapter 1

Introduction and thesis outline



1.1 Bottlenecks in aquaculture

Aquaculture is growing more rapidly than all other animal food producing sectors, with an average yearly growth rate of more than 6% over the last 20 years (FAO, 2014). Over the past years there has been a considerable progress in aquaculture mainly of empirical character, but partly also of fundamental nature. This progress together with the increasing demand of seafood has made aquaculture a profitable business.

Despite significant advances, the aquaculture still faces major bottlenecks related to suboptimal growth, inexplicable mortalities and diseases as well as malformations and variable quality of juveniles (Attramadal et al., 2012). Attempts in overcoming the aforementioned difficulties focused mainly on broodstock management and breeding, optimization of water quality and on species dietary requirements. However, fish performance (health and growth) still varies considerably between identically reared individuals, even when from the same sibling group. Fish-microbe interactions have been suggested to be a key factor in explaining this lack of reproducibility (Vadstein et al., 2013).

Gut microbiota influences a wide range of biological processes in humans (Rawls, 2007; Sekirov et al., 2010), domesticated terrestrial animals (Chaucheyras-Durand and Durand, 2010; McFall-Ngai et al., 2013) and fish (Nayak, 2010b; Sullam et al., 2012). Despite the significant contribution of several studies on fish gut microbiota, our understanding on the functional significance of microbial fluctuations lags well behind that of terrestrial vertebrates.

1.2 Fish in a microbial world

The nutrient rich environment of aquaculture tanks and ponds favors the proliferation of microbes. Exposed body surfaces of fish, including the gut, are quickly colonized right after hatching mostly due to the influence of water communities (Reitan et al., 1998; Verschuere et al., 2000). Feed microbiota becomes important later mostly from the time that active feeding occurs (Hansen and Olafsen, 1999; Ringo and Birkbeck, 1999). This dynamic community is subjected to changes related to the developmental stage of the host and constantly adapts to shifts in the nutritional and environmental situation (Nayak, 2010b; Romero and Navarrete, 2006; Navarrete et al., 2012; Li et al., 2012b). Fish gut microbiota vary within species due to factors such as season, salinity, pH, temperature, trophic level and feed (Nayak, 2010b; Sullam et al., 2012).

Although water and feed are the main sources of microbes, fish and their gut bacteria seem to have more complex relationships than one would expect from a simple reflection of the free-living microbial community in the environment (Sullam et al., 2012). For the ingested microbes, the gut provides different (micro) habitat niches leading to the

establishment of a very complex microbial community upon which the host has a great influence (Sommer and Bäckhed, 2013; Hansen and Olafsen, 1999).

1.3 Host-microbe interactions

Fish microbial communities are strongly influenced by, but also influence, their hosts. (Sullam et al., 2012). The beneficial role of the intestinal microbiota in fish is recently being explored, suggesting that microbiota stimulates nutrient metabolism, innate immune response and epithelial proliferation (Roeselers et al., 2011). Additionally, gut microbiota constrains the colonization of infection agents and, by interacting with the host, mediate the development, maintenance and effective functionality of the intestinal mucosa (Dimitroglou et al., 2011).

The majority of the ingested microbes are only transient in the gut (Sugita et al., 1996). For ingested bacteria to proliferate and persist as “resident” microbiota, they must be capable to adapt to the environmental conditions inside the gut, such as nutrient availability, pH and digestive enzymes (Hansen and Olafsen, 1999). Host dependent selective pressures impacting gut microbiota in fish, are mostly attributed to gut habitat (ecology) and host genotype (Yan et al., 2012; Rawls et al., 2006; Navarrete et al., 2012).

Nevertheless, insights in the roles of microbial communities and the factors that drive their diversity remain limited. Apart from a few pathogens, host-microbe interactions in fish are still poorly understood and unravelling composite relationships within the fish gut remains challenging mostly due to the ecological and functional complexity of this ecosystem.

1.4 Microbial community management

Lack of understanding on the driving forces governing microbial colonization in the gut makes it difficult to develop applications or to interfere in the process of microbial community assembly. The following strategies have so far mainly been applied for microbial control management in aquaculture.

Reduction of microbial load: This strategy mostly aims at the reduction of microbes in a non-selective manner. Such methods include the use of antibiotics or disinfectants either by administrating them directly to the host or by treating the surrounding environment. Besides the risk of promoting antibiotic resistant microbes, the main drawback of these methods is the proliferation of opportunistic microbes due to the large and non-selective reduction of microbial load (Hess-Erga et al., 2010).

Reduction of organic load: This is a strategy for semi-selective microbial reduction in the sense of a non-specific targeting of particular taxa. However, different microbes use different substrates and the removal of easily degradable organic matter selects against opportunistic heterotrophic bacteria. The easily degradable organic matter is removed from the water, thus indirectly decreasing the overall bacterial load in the system (Attramadal et al., 2012). However, rapid fluctuations of the organic load in the system should be avoided due to the unpredictable proliferation of opportunistic species.

Use of pro and prebiotics: The use of live microorganisms (probiotics) that confer a health benefit to the host appears promising in aquaculture (Pérez-Sánchez et al., 2014). Probiotics reduce infections caused by bacterial pathogens (Gatesoupe, 1999; Martínez Cruz et al., 2012) and are successfully used as immunostimulants (Cain and Swan, 2010; Lepage et al., 2012; Song et al., 2014) and growth promoters in fish and shrimp (Pérez-Zánchez et al., 2013; Merrifield et al., 2010). However, these strains seem to persist only temporarily in the gut after supplementation is stopped. The use of prebiotics as a nutritional substrate for the selective enrichment of specific probiotic species could facilitate the establishment/selection of beneficial bacteria.

Apart from the use of antibiotics and the implementation of hygiene protocols, microbial management techniques have limited fundamental scientific backing and there has been a rush to empirical solutions bypassing the need for more fundamental knowledge on host-microbial interactions in aquaculture species. Establishing a desired gut microbiota in fish larvae and assuring proper functionality is not straight forward. First colonization in particular, can be assumed to have long-term impact for larval health. This encourages the development of microbial management strategies to steer first colonization. Developing the proper tools for microbial assembly management implies gaining insight into the ecological mechanisms and processes that govern it, which is the main focus of this thesis.

1.5 Thesis aim and outline

All experimental chapters of this thesis focused on the early developmental stages (i.e. from first feeding to 6 weeks old) of Nile tilapia *Oreochromis niloticus* (Linnaeus) larvae. Tilapia larvae were chosen mostly because: a) early life stages are crucial for fish development and thus the base for a successful production cycle, b) tilapia generally feeds in shallow waters grazing on sediments, which creates a direct contact of the fish with a large and diverse microbial load and c) the impact of free-living bacteria on gut microbiota is more pronounced during early ontogenetic stages when the fish gut is not yet fully developed and the immune system is still immature.

This thesis starts from the concept that the fish gut microbial communities (MC) are influenced by the host itself and by the free-living MC of host's habitat. However, colonization of the fish gut is partially a stochastic process, with successful bacteria being those that are present at the right moment and place. Stochastic phenomena have been proposed as a cause of high inter-individual variation in gut microbiota (Fjellheim et al., 2007) which could in turn be associated with highly variable quality and health of reared animals.

To comprehend the full range of diversity of gut microbiota, it is very important to characterize and quantify the inter- and intra-individual variation in space and time. In fact, the characterization of the variation between identically reared individuals can serve as baseline to determine the contribution of stochastic factors to the overall variation. For this reason we initially characterized the variation between identically reared individuals to determine the contribution of stochastic factors to the overall variation. In **chapter 2** we quantified the spatio-temporal variation of water and gut microbiota of Nile tilapia larvae, reared in different production systems i.e. recirculating or active suspension systems (RAS vs. AS). The location effects on gut microbiota of newly hatched larvae were compared to individuals reared within the same or between replicate tanks, and between replicate and different systems for a period of 6 weeks.

Due to rapid shifts in the microbial community composition during early ontogenetic stages, it is difficult to relate changes in gut microbiota to alterations of a single environmental factor. By understanding the factors underlying the successful colonization of ingested microbes and the community assembly we could increase predictability and repeatability of gut microbiota interventions. Regarding the relative importance of water and feed microbiota in determining gut microbial composition, outcomes from experimental studies are controversial. There seems to be a general acceptance that diet is a major contributor of microbes for the gut of fish larvae (Nayak, 2010b; Reid et al., 2009), but this hypothesis is not supported across studies (Bakke et al., 2013). In order to provide first insights into the forces that structure fish gut communities and illustrate MC affinities to symbiotic and free-living communities, the similarity among gut bacteria of fish and the free-living bacteria present in their environments (i.e. water) was investigated. **Chapter 3** evaluates the relative contribution of water and feed microbiota to gut microbiota composition of larvae. Therefore, the composition of gut microbiota of tilapia larvae reared in either RAS or AS systems from the moment of first feeding was characterized at different time points. Better understanding of how microbiota in water, feed and gut interact will help improving the design, microbial management and nutrient cycling of fish rearing systems.

In chapters 2 and 3, input of bacteria via the feed was the common denominator (all treatments received the same commercial feed) whereas the impact of water MC on gut communities was evaluated by testing the effect of different rearing systems. In **chapter 4** the opposite scenario was investigated i.e. all larvae shared the same water (reared in

the same system), but were fed with different diets. We explored the potentials of steering gut communities during early life stages of tilapia via microbial manipulations of feed communities. We hypothesized that if gut microbial composition is strongly shaped by selective pressures in the gut, then similar gut microbial communities should develop regardless of the dietary treatment. Contrary, if the gut microbial community is mostly shaped by the composition of the microbial community present in the diet, this will result in a significantly different gut microbiota between treatments. To test our hypothesis fish larvae were fed four diets with microbiota derived from aerobic, methanogenic or denitrifying sludge reactors. All individuals were reared in the same system to avoid variation between treatments due to differences in water microbiota. We performed 16S rRNA gene sequence-based comparisons between gut microbial communities from different treatments and we associated them with the ones present in feed and water.

Probiotics have been widely applied in aquaculture for many years. However, probiotic strains often colonize the gut only transiently and quickly fall below detection limits (Gatesoupe, 1999; Grześkowiak et al., 2012). Currently, delivery of probiotics through feed in larval stages is the most common strategy for management of larval gut microbiota in aquaculture (Nayak, 2010a). The administration of the probiotic strain on early ontogenetic stage, when the gut microbiota still develops, could enhance gut-colonization of the probiotic itself and may therefore lead to the development of distinct gut communities. In **chapter 5** we tested whether an early contact of larvae with bacteria has a long lasting effect on gut microbial community composition on later life stages, i.e. an imprinting effect. We hypothesized that an early contact of fish larvae with the probiotic strain would have an impact on shaping gut microbiota even after the administration of the probiotic discontinues. To do this, we constrained the initial contact with microbes from the environment by producing both axenic hosts and hosts with a known microbial community (addition of a single strain probiotic). Subsequently, the imprinting hypothesis was tested by exposing the two host types to similar husbandry conditions.

Finally, **chapter 6** discusses the main results from all experimental chapters in the context of the existing literature, and overall conclusions and practical implications are presented. Furthermore, a meta-analysis of 16S rRNA gene sequence data from 12 different studies on gut microbiota of different fish species was performed. The meta-analysis incorporated datasets from the three studies included in this thesis, two unpublished datasets from our previous studies on tilapia larvae and seven publically available gut microbiome datasets from studies performed in trout, carp, zebrafish and tilapia. By examining the phylogenetic similarity among gut bacteria of the same and different fish species from different habitats, diets, facilities and developmental stage, we investigated the factors that primarily shape these communities. The role of host selectivity over environmental impact on gut communities was investigated by comparing phylogenetic distance between gut bacteria of the different fish species and studies.

Chapter 2

The colonization dynamics of the gut microbiota in tilapia larvae



This chapter has been published as:

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The gut microbiota of fish larvae evolves fast towards a complex community. Both host and environment affect the development of the gut microbiota; however, the relative importance of both is poorly understood. Determining specific changes in gut microbial populations in response to a change in an environmental factor is very complicated. Interactions between factors are difficult to separate and any response could be masked due to high inter-individual variation even for individuals that share a common environment. In this study we characterized and quantified the spatio-temporal variation in the gut microbiota of tilapia larvae, reared in recirculating aquaculture systems (RAS) or active suspension tanks (AS). Our results showed that variation in gut microbiota between replicate tanks was not significantly higher than within tank variation, suggesting that there is no tank effect on water and gut microbiota. However, when individuals were reared in replicate RAS, gut microbiota differed significantly. The highest variation was observed between individuals reared in different types of system (RAS vs. AS). Our data suggest that under experimental conditions in which the roles of deterministic and stochastic factors have not been precisely determined, compositional replication of the microbial communities of an ecosystem is not predictable.

2.1 Introduction

The gut of fish harbors a diverse microbial community. It provides niches for adherence, colonization and proliferation of mutualistic, benign commensal and pathogenic microbial species that affect many physiological and immunological functions of the host (Cahill, 1990; Gómez and Balcázar, 2008; Hansen and Olafsen, 1999). The microbial community in the gut changes with the developmental stage of the host and constantly adapts to the nutritional and environmental situation (Nayak, 2010b; Romero and Navarrete, 2006; Navarrete et al., 2012; Li et al., 2012b). Impacts on fish gut microbiota are more pronounced during early ontogenetic stages when the fish gut is not yet fully developed and the immune system is immature (Gatesoupe, 1999).

However, due to high inter-individual variation between fish and rapid changes in the microbial community composition during early life stages, it is difficult to relate changes in gut microbiota to alterations of a single factor. It has been suggested that inter-individual variation in gut microbial community composition both in humans (Walker et al., 2011) and animals (Haenen et al., 2013) might mask treatment effects. High individual variation was suggested as the reason for not detecting differences in gut microbiota in Atlantic salmon (*Salmo salar*) fed with different diets (Ringø et al., 2008). High inter-individual variation in quantity, diversity and richness of gut bacteria was also observed between individuals from the same tank in Bluefin tuna (Gatesoupe and Covès, 2012) as well as in cod larvae (Fjellheim et al., 2011).

Inter-individual variation in gut microbiota between individuals reared under the same conditions can be partially explained by stochastic processes (Fjellheim et al., 2007). However, “stochastic variation” cannot just be considered as “noise”. To comprehend the full range of genetic and metabolic diversity of gut microbiota, it is very important to characterize and quantify the inter- and intra-individual variation in space and time. In fact, the characterization of the variation between identically reared individuals can serve as baseline to determine the contribution of stochastic factors to the overall variation.

In this study we characterized and quantified the spatio-temporal variation of water and gut microbiota of Nile tilapia larvae, reared for six weeks in two replicate Recirculating aquaculture systems (RAS). The location effects on larvae gut microbiota were compared for individuals reared within the same or between replicate tanks, and between replicate RAS systems. To determine the generality of any pattern observed in the RAS, and to avoid any affinity of the results with the specific habitat (RAS), temporal and replication effects were also studied in replicate active suspension (AS) systems also known as zero-exchange activated sludge systems or biofloc systems (Crab et al., 2012).

2.2 Materials and methods

2.2.1 Ethics Statement

The experiment was approved by the Ethical Commission for Animal Experiments of Wageningen University (Project Name: Promicrobe; Registration code: 2009055d).

2.2.2 Experimental animals and set up

Three to four days old fertilized Nile tilapia eggs, obtained from TilAqua International (Velden, the Netherlands) were incubated at 27°C. Two different culture systems were used to rear the newly hatched larvae: a recirculating aquaculture system (RAS) with two replicates (Ra and Rb), and an active suspension (AS) system with five replicates (AS 1-5). Each RAS contained five 20-L tanks and the tanks were connected to the same water purification unit. The two replicate RAS systems were not connected to each other. The five 120-L AS tanks were independent units and they did not share the same water. Initially, all systems were filled with water from the same source. In addition, before the start of the experiment, water and filter materials from the two RAS were mixed. Water of the five AS systems was treated similarly. The larvae were incubated together in a common tank before stocking. In each tank, 100 randomly selected swim-up larvae (7 days post fertilization) were introduced before the first feed application. Feeding started 9 days post fertilization (referred to as day 0; D00) and was continued for 42 days. Each day, larvae were fed with 0.5 mm commercial starter tilapia diet (F-0.5 GR Pro Aqua Brut – Trouw Nutrition, France) until apparent satiation for maximum 30 minutes at 9.00, 12.30 and 16.00 hours. The same type of feed, originating from a common batch, was used throughout the 42 day experimental period. Feed pellets were introduced slowly while observing feeding behavior, and administration stopped when it took more than 15 seconds before fishes reacted to newly fed pellets. Just before the first feeding, water and gut samples were collected, to determine the “initial” microbiota. Other samples were taken before the first daily feeding on day 07, 14, 28 and 42 (Figure S2.1).

Water physicochemical characteristics were maintained at safe levels for Nile tilapia larvae (pH 6.6–8.5, temperature 26–28 °C, $\text{NH}_3\text{-N} < 0.2 \text{ mg L}^{-1}$, $\text{NO}_2\text{-N} < 1 \text{ mg L}^{-1}$ and $\text{DO} > 5 \text{ mg L}^{-1}$). The photoperiod was set to 12 hours light – 12 hours dark. During the experiment both RAS and AS operated as fully closed systems.

2.2.3 Collection of gut and water samples

On each sampling day ten larvae per tank were collected for microbial community analysis. The larvae were euthanized with 0.6 g L⁻¹ Tricaine Methanesulfonate (TMS,

Crescent Research Chemicals, Phoenix, Arizona, USA), buffered with 0.12 g L⁻¹ sodium bicarbonate in water originating from the corresponding rearing tank. Subsequently, larvae were rinsed with 70% ethanol and sterile water before dissecting out aseptically the gut under a dissection microscope. Whole gut samples were flash frozen in liquid nitrogen and stored individually at -80°C until subsequent analyses.

All tools and dissecting surfaces were disinfected with chloramine-T (Halamid-d, Veip BV, The Netherlands) and 70% ethanol between dissections. In addition, the tools were always held in a propane gas flame before use.

From each tank, 250 mL water samples were collected at the same time of gut sampling. The water was filtered through 0.45 µm (type HAWP) and 0.22 µm (type GTTP) membrane filters (Millipore - Isopore) using a vacuum apparatus.

The microbiota in the water and gut was analyzed using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal RNA (rRNA) gene fragments. One water and 3 gut samples were taken from each of the 15 tanks and analyzed by using PCR-DGGE on days 0, 7, 14, 28 and 42. In addition, 1 water and 3 gut samples were taken from 2 replicate tanks of each RAS and from 2 AS tanks. Those samples were analyzed by 454 pyrosequencing of partial 16S rRNA genes on days 7 and 42. Samples that were analyzed with 454 pyrosequencing were a subset of the sample set that was analyzed with PCR-DGGE.

2.2.4 Genomic DNA isolation

DNA was extracted from larval gut samples using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol with the following modifications: The gut samples were added to 180 µL enzymatic lysis buffer and incubated at 37°C for 1 hour. Furthermore, 40 µL proteinase K and 180 µL ATL buffer were added to improve cell lysis, and the mix was incubated for 1.5 h at 55°C. Cell-lysis was further optimized by performing an additional step in which gut tissue was homogenized in 200 µL AL buffer (Qiagen) with the aid of a custom bead mix (4 glass beads 2-3 mm, 0.5 g zirconia beads 0.1 mm) (MO-BIO Carlsbad, CA USA) and using the FastPrep instrument (QBioGene, Irvine, CA, USA) for 1 min at 6,000 rpm. The samples were eluted twice in 50 µL AE buffer. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE), and DNA samples were stored at -20°C until use.

For DNA extraction from water samples, the FastDNA SPIN kit for soil (MP Biomedicals, Ohio, USA) was used. The DNA was extracted from water membrane filters. Briefly, homogenization was achieved by addition of 978 µL sodium phosphate and 122 µL MT buffer, and the cell lysis in the lysing matrix was enhanced by a bead beating step of 40

s at 6000 rpm. DNA purification was achieved by addition of 1 mL silica binding matrix and 500 μ L SEWS-M (salt ethanol wash) followed by centrifugation at 14,000 g for 5 min. The DNA was eluted by the addition of 50 μ L DES (DNA elution solution ultra-pure water) and incubated at room temperature for 5 min. Subsequently, the DNA was collected by centrifugation at 14,000 g for 5 min. For more details see instructions given by the manufacturer.

2.2.5 PCR-DGGE analysis

Target fragments of the bacterial 16S ribosomal RNA gene were amplified from the extracted DNA by PCR by using the following cycling conditions: 95°C for 2 min, followed by 35 cycles consisting of 95°C for 30 s, 53°C for 40 s and 72°C for 1 min and then a final 5 min extension step at 72°C. Samples were cooled to 4°C. PCR for DGGE was performed by using primers L1401-R (5'-CGGTGTGTACAAGACCC-3') and U968-F (5'-CGCCGGGGCGCGC CCCGG GCGGGGCGGGGCACGGGGGAACGCGAAGAACCCTTAC-3') fitted with a GC-clamp (Nubel et al., 1996). The PCR reaction mixture consisted of Phusion HF buffer, 0.2 μ M of each primer, 200 μ M of each dNTP, and 1 unit of Phusion Hot Start II High Fidelity Polymerase. To the 50 μ L reactions 20-50 ng of DNA was added. Five μ L of all PCR products were visualized by gel electrophoresis using 1% agarose gel with ethidium bromide to check the quality. DGGE analysis of PCR amplicons was performed as described previously (Muyzer et al., 1993) using the DCode system (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gels consisted of 8% (vol/vol) polyacrylamide (37.5:1 acrylamide-bisacrylamide) in 0.5xTris-acetate-EDTA. A denaturing acrylamide containing 7M urea and 40% formamide was defined as 100%. The gels were poured from the top by using a gradient maker (Econopump; Bio-Rad, La Jolla, CA) and pumping the solution at a speed of 4.5 mL min⁻¹. A gradient from 30 to 60% was used for the separation of the PCR amplicons. Electrophoresis was performed for 16 h at 85 V in a 0.5xTris-acetate-EDTA buffer at a constant temperature of 60°C. Subsequently, gels were stained with AgNO₃ according to the method described by Sanguinetti et al. (Sanguinetti et al., 1994).

2.2.6 454 Pyrosequencing

For more detailed 16S rRNA gene-based microbial composition profiling, barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated by PCR using the 27F-DegS primer (Van den Bogert et al., 2011) that was appended with the titanium sequencing adaptor A and an 8 nucleotide sample-specific barcode (Hamady et al., 2008) at the 5' end. As a reverse primer, an equimolar mix of two primers 338R I and II (van den Bogert et al., 2013) was used that carried the titanium adaptor B at the 5' end. Extracted DNA was diluted to a concentration of 20 ng μ L⁻¹ based on Nanodrop readings. PCR was performed using a GS0001 thermocycler (Gene Technologies, Braintree, United Kingdom).

The PCR mix (100 μ L final volume) contained 20 μ L of 5 \times HF buffer (Finnzymes, Vantaa, Finland), 2 μ L 10 mM (each nucleotide) PCR-grade Nucleotide Mix (Roche Diagnostic GmbH, Mannheim, Germany), 1 μ L of Phusion hot start II High-Fidelity DNA polymerase (2U/ μ L) (Finnzymes), 500 nM of the reverse primer mix and the forward primer (Biolegio BV, Nijmegen, The Netherlands); 2 μ L (i.e. 40 ng) template DNA and 65 μ L nuclease free water. PCR was performed under the following conditions: 98°C for 30 s to activate the polymerase, followed by 30 cycles consisting of denaturation at 98°C for 10 s, annealing at 56°C for 20 s, and elongation at 72°C for 20 s, and a final extension at 72°C for 10 min. Twenty μ L of the PCR products were analyzed by 1% (w/v) agarose gel electrophoresis in the presence of 1 \times SYBR[®] Safe (Invitrogen, Carlsbad, CA, USA) and purified from gel using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) according to manufacturer's instructions. DNA concentrations of gel-purified amplicons were measured by a Nanodrop[®] ND-1000 spectrophotometer, and purified PCR products were mixed in equimolar amounts, run again on an agarose gel and subsequently excised and purified using a DNA gel extraction kit (Milipore, Billerica, MA, USA). Nucleotide sequences were generated by pyrosequencing using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany). Pyrosequencing data were deposited at the European Bioinformatics Institute in the sequence read archive under study accession number PRJEB4462 and sample accession numbers ERS343984 – ERS344037.

The 454 pyrosequencing analysis was paired to the DGGE data by using samples collected from 2 replicate tanks on day 07 and 42, for the 2 RAS and 2 AS. We used both complementary methods for the characterization of spatiotemporal variation in the microbial communities in order to evaluate whether the outcome was consistent and comparable between methods, allowing for more general statements regarding the consequences for study design. Although pyrosequencing provided also useful direct sequence information with respect to the composition and ecology of the microbial communities in the samples, this was beyond the scope of this study and will be addressed in a separate paper.

2.2.7 Normalization between DGGE gels

On every DGGE gel a standard reference marker consisting of an amplicon mix of 10 different cloned bacterial 16S rRNA genes was included at 3 different positions, for digital gel normalization. These 10 fragments of the reference marker produced a known distinctive pattern defined by the position of the bands. The designation of the inter-gel band classes was based upon their relative position on the profile compared with the standard reference used, as described above. An overall comparison of the reference markers between all gels showed that all markers from the 15 gels clustered together, with a similarity higher than 95% and regardless of the gels that they belonged to,

indicating that a valid comparison in community fingerprints was possible also between multiple gels. Using a standard reference marker to allow intra and inter-gel comparisons, has been suggested elsewhere (Joossens et al., 2011; Goodhead et al., 2013; Machiels et al., 2013; Thompson, 2014; Ercolini, 2004; Kowalchuk et al., 2006; Vanhoutte et al., 2004; Li et al., 2014b; Temmerman and Scheirlinck, 2003; Tourlomousis et al., 2010; Muyzer and Smalla, 1998). In addition to that, the inter-gel variation among profiles was tested at the beginning of the DGGE analysis. To this end, randomly selected samples from 2 or 3 different gels were selected and re-loaded into a single gel. DGGE gels (Figures S2.2, S2.3 and S2.4) showed that samples were clearly grouped based upon their band pattern and not upon the gel they belonged to, allowing for a fair inter-gel comparison.

2.2.8 Data handling and statistical analysis

DGGE patterns were analyzed with Bionumerics software 5.1 (Applied Maths, St-Martens-Latem, Belgium) following the manufacturer's instructions. The patterns were normalized and individual bands were initially marked automatically (5% minimum profiling), followed by visual inspection and manual correction whenever necessary. For automatic band matching the position tolerance of the fingerprints was set to 1% (percentage of the pattern length) maximum shift between two bands. Optimization for the best possible matching was set to a maximum allowable shift of 0.5%. The band-classes were arbitrarily generated in a global alignment of all entries (DGGE lanes) of combined DGGE gels, by tracing common bands across different profiles. The designation of the band-classes was based on their position in the profile compared with the reference marker used as a normalization standard, to ensure gel-to-gel comparability. The bands were furthermore inspected manually for consistency. As measure of relative abundance, relative intensity of each band within individual DGGE profiles was used. Subsequently, data were square root transformed to decrease the importance of the most dominant bands in the subsequent analysis (Legendre and Gallagher, 2001).

Pyrosequencing data were analyzed using the QIIME 1.5.0 pipeline (Caporaso et al., 2010), and quality filtering (de-noising) was performed as follows. Low quality sequences were removed using default parameters (i. reads with fewer than 200 or more than 1000 nucleotides; ii. reads with more than 6 ambiguous nucleotides, homopolymer runs exceeding 6 bases, reads with missing quality scores and reads with a mean quality score lower than 25; iii. reads with mismatches in the primer sequence), and operational taxonomic units (OTUs) were identified at the 97% identity level. Representative sequences from the OTUs were aligned using PyNAST (DeSantis et al., 2006). The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80% against the 12_10 Greengenes core set (Wang et al., 2007). Possible chimeric OTUs were identified using QIIME's ChimeraSlayer and removed from the initially generated OTU list, producing a final set of non-chimeric OTUs.

For the DGGE data, there were five possible factors in the experimental design: "origin" (two levels; gut and water; fixed), "date" (five levels; day 0, 7, 14, 28, 42; fixed), "system type" (two levels; RAS and AS; fixed), "replicate system" (2 RAS or 5 AS; nested in system type: random) and "tank" (five levels, tank 1, 2, 3, 4 and 5, nested in replicate system: random). Because of the highly skewed distribution of bacterial species and the large number of zeros contributed by rare species, the assumption of multivariate normal distribution was unrealistic. For that reason a permutation-based multivariate ANOVA (PERMANOVA) was used to analyze the data set (Anderson, 2001). This method allows multivariate data to be analyzed on the basis of any distance or dissimilarity measure. The distance matrix was based on Bray Curtis dissimilarity (Bray and Curtis, 1957) due to its desirable properties when compared to other distance measures for analyzing environmental data. For example, the Bray Curtis coefficient does not increase the similarity between two samples when a common species absence occurs (Clarke, 1993) which is a very useful property when analyzing biological assemblage data with many zeros. For each term in the analysis, 9999 permutations of raw data units were performed to calculate P values, and when there were not enough possible permutations a Monte Carlo sample was drawn from the theoretical asymptotic permutation distribution (Anderson and Robinson, 2003).

In addition to PERMANOVA, analysis of similarities (ANOSIM) was used to give an insight into the degree of separation between the tested groups of samples. ANOSIM tests the null hypothesis that the average rank similarity between samples within a group is the same as the average rank similarity between samples belonging to different groups. The analysis produces an R statistic that generally ranges from 0 to 1 (Chapman and Underwood, 1999). An R of 1 indicates complete separation whereas an R of 0 indicates that the null hypothesis is true. The statistical significance of R statistic is assessed by random permutations of the group membership to obtain the empirical distribution of R under the null-model and is free of any assumption of normality (Clarke, 1993).

Although neither PERMANOVA nor ANOSIM explicitly assume common variances among groups, they are both sensitive to differences in multivariate dispersion. To test the hypothesis of equal within group dispersion (for both methods) PERMDISP analysis as a multivariate non-Euclidean equivalent to traditional Levene's test was used (Anderson, 2006). The analysis was used for two reasons: i. as a complementary test to avoid any kind of misinterpretation of the outcome of the two previous methods mostly due to type II error, and ii. to give insight of within and between groups variation. Homogeneity of dispersion among groups was calculated as an average distance (\pm SE) of group members (samples) from the group's centroid. PERMDISP was used to test the null hypothesis of no difference between groups dispersion. Significant effects on group dispersion were tested for "tank", "replicate system", "system type" and "date".

Non metric multi-dimensional scaling (nMDS) was performed to represent the samples in a low dimensional space in a way that relative distances of all points are in the same rank order as the relative dissimilarities of the samples as measured by Bray Curtis index. "Stress" values in nMDS indicate how well the multidimensional relationships among the samples are represented in the low dimensional space.

Hierarchical agglomerative clustering with group average linking (based on Bray Curtis similarity) was used to identify "natural groupings" (meant as non-predefined groups) of samples, in such a way that partitioning of groups indicates differences in the microbial community between them. To verify cluster patterns even for the most clearly congregated samples, cluster analysis was used in combination with nMDS plots, as well as the results from the estimation of the components of variation in PERMANOVA.

All statistical analyses were performed by using the multivariate statistical software package Primer V6 (Primer-E Ltd, Plymouth, UK).

2.3 Results

During the experiment, the water quality was maintained within preset limits resulting in above 99% survival. Fish grew on average 11.17 ± 0.06 % g in RAS and 11.03 ± 0.05 % body weight d^{-1} in AS with a feed conversion ratio of 0.64 ± 0.01 in RAS and 0.70 ± 0.01 (\pm SD) in AS. The final weight reached was 1.24 ± 0.03 g in RAS and 1.17 ± 0.03 g in AS. No significant differences were observed between replicate systems, neither for water quality nor for fish growth ($P > 0.05$).

2.3.1 Overall contribution of factors in microbial dynamics

PERMANOVA of DGGE data revealed significant effects of all main factors ("system type", "replicate system", "date" and "origin") except for "tank" (Table 2.1). A similar picture emerged for the pyrosequencing data. The highest fraction of total variation was explained by the main factor "origin", followed by "system type", "date" and "replicate system" (Figure 2.1).

Table 2.1. Overall PERMANOVA test based on DGGE data for main experimental factors. P values are based on 9999 Monte Carlo (MC) permutations. Effects of the interaction terms are not shown in the table.

Factor	df	Pseudo-F	P(MC)
System type	1	5.9632	0.0001
Date	4	4.9623	0.0001
Origin	1	9.1458	0.0001
Replicate system	5	5.6948	0.0001
Tank	8	1.0661	0.2891

Lowest similarity was observed between gut and water microbiota. Gut samples were separated into RAS and AS systems, confirming that system is the principal factor differentiating gut microbiota. In both systems, gut samples differed significantly between day 07 and 42. Differences between gut samples from “replicate systems” were less pronounced than for “origin”, “system type” and “date”.

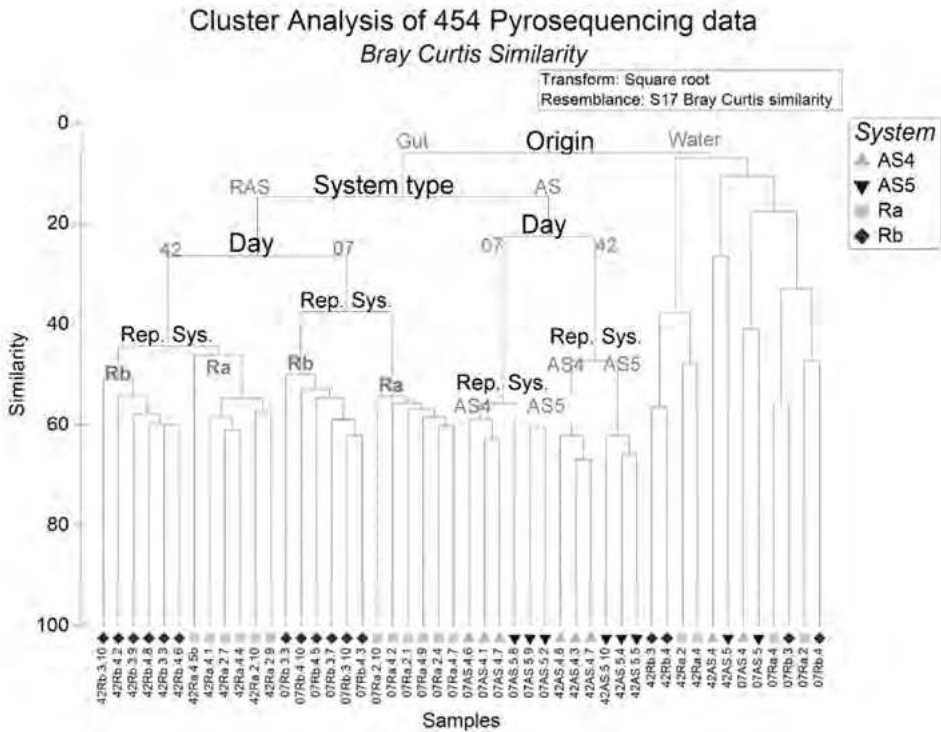


Figure 2.1. Hierarchical clustering with Unweighted Pair Group with Arithmetic Mean (UPGMA) linkage of gut and water samples based on 454 data. On y-axis: similarity percentage based on Bray Curtis similarity, on x-axis: all individual samples of gut (left) and water (right). D07 and 42: sampling day 7 & 42, AS 4 & 5: replicate active suspension systems 4 & 5, Ra and Rb: replicate recirculating system a and b. Numbers 2, 4 & 3, 4: replicate tanks from Ra and Rb respectively. Last digits following the tank number indicate the number of replicate fish in each tank. Since only one water sample was taken from each tank, the last digits were omitted from water sample's ID. (e.g. 42Rb.3.10: Gut of day 42, from recirculating system b, tank 3, fish 10 whereas 07Ra.2: Water of day 7, from recirculating system a, tank 2)

2.3.2 Variation in gut and water microbiota from replicate systems

Recirculating systems (RAS): Gut microbiota of Ra and Rb differed consistently ($P < 0.05$; Table 2.2) during the 42 days experimental period, irrespective of the choice of analytical method (DGGE and 454) or statistical test (PERMANOVA or ANOSIM). The nMDS ordination plots of the DGGE data showed a clear distinction between the microbiota of individuals

reared in Ra and Rb for all dates (Figure. 2.2), confirming the R statistic in ANOSIM (Table 2.2). The comparison of water microbiota between Ra and Rb also differed consistently (Figure 2.3).

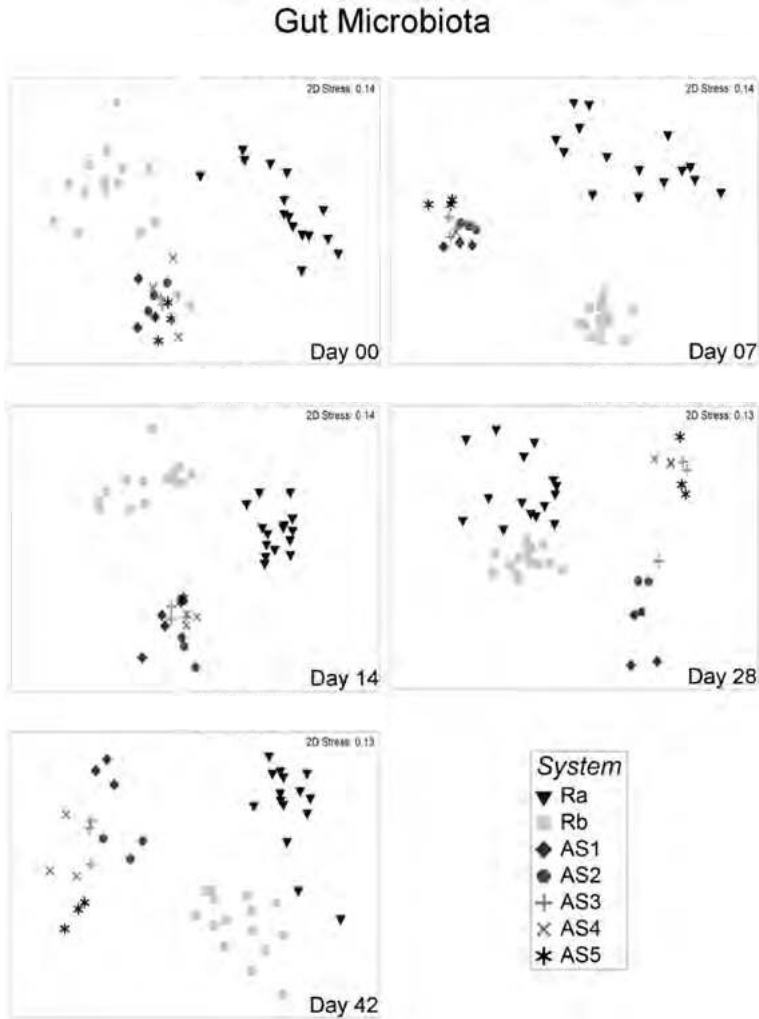


Figure 2.2. Non metric dimensional scaling (nMDS) of gut microbiota from individuals reared in different systems over time. Each point represents the gut microbiota of one individual. Plots are based on Bray Curtis distance after square root transformation of relative abundance DGGE data. D00, 07, 14, 28, 42: sampling days 0, 7, 14, 28 & 42 respectively, AS1-5: replicate active suspension system 1 to 5, Ra & Rb: replicate recirculating system a & b. Stress values are reported for the two dimension and are indicative of the goodness of fit of data into the plot.

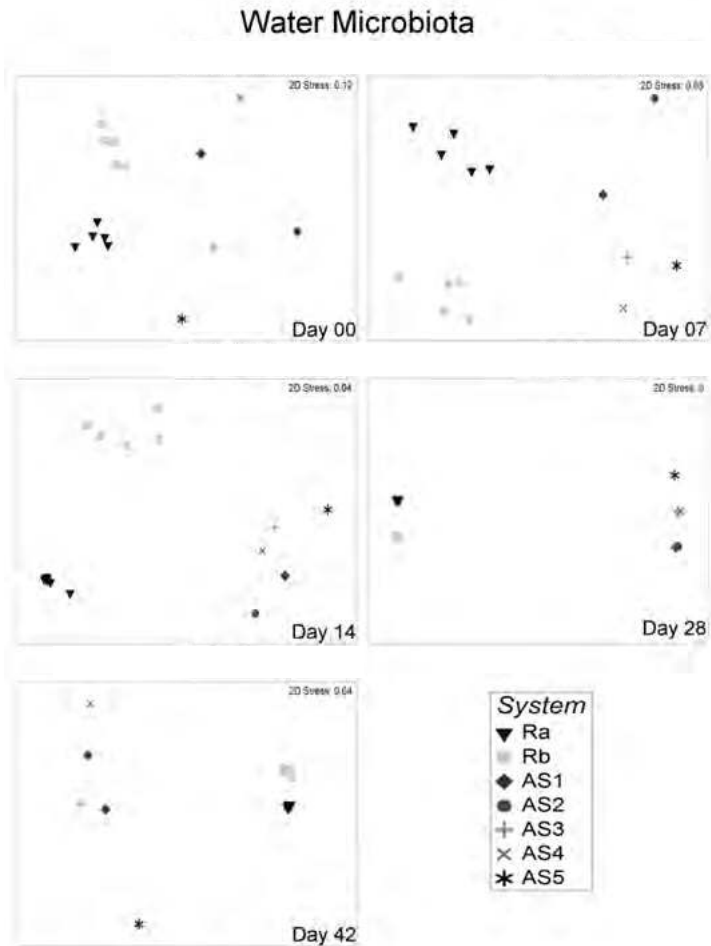


Figure 2.3. Non metric dimensional scaling (nMDS) of water microbiota from different systems over time. Each point represents the water microbiota from each tank. Plots are based on Bray Curtis distance after square root transformation of relative abundance DGGE data. D00, 07, 14, 28, 42: sampling days 0, 7, 14, 28 & 42 respectively, AS1-5: replicate active suspension system 1 to 5, Ra & Rb: replicate recirculating system a & b. Stress values are reported for the two dimensions and are indicative of the goodness of fit of data into the plot.

Active suspension systems (AS): Gut microbiota was different between individuals reared in different AS systems at day 07 and day 14, whereas this was not the case on day 0 (Table 2.2). On day 07, the five AS systems were not statistically different when using PERMANOVA. Nevertheless, out of 10 possible pairwise comparisons, six comparisons indicated significant differences between the five AS systems (P values of each of the pairwise tests are not shown). ANOSIM's R statistic suggested a clear distinction between AS systems for both DGGE and 454 data on day 07. At day 14, the same pattern emerged (6/10 pairwise tests showed differences, and ANOSIM's R statistic was 0.542).

Table 2.2. Pairwise comparisons between replicate systems for RAS and AS separately, based on DGGE and 454 pyrosequencing data sets. Tests were performed by sample type per sampling day, with P values for each comparison from two different statistical tests (PERMANOVA and ANOSIM). ANOSIM results are complementary to the PERMANOVA to provide information on the degree of separation between groups, suggested by R statistic. The 2/10, 6/10 & 7/10 indicate the number of significant out of the total available comparisons. D00, 07, 14, 28, 42: sampling day 0,7,14,28 & 42 respectively, AS1-5: replicate active suspension system 1 till 5, Ra & Rb: replicate recirculating system a & b. P values are based on 9999 Monte Carlo permutations.

Statistical test	Analytical Method	D00		D07		D14		D28		D42		
		AS1-5	Ra-Rb	AS1-5	Ra-Rb	AS1-5	Ra-Rb	AS1-5	Ra-Rb	AS1-5	Ra-Rb	
Perm. ANOVA	GUT	DGGE	2/10	P:0.0001	6/10	P:0.0003	6/10	P:0.0001	7/10	P:0.0003	7/10	P:0.0001
		454	P:0.2079				P:0.0025				P:0.0242	
ANOSIM	GUT	DGGE	R:0.428	R:0.925	R:0.787	R:0.772	R:0.542	R:0.922	R:0.745	R:0.596	R:0.907	R:0.862
		454	P:0.0005	P:0.0001	P:0.0001	P:0.0001	P:0.0001	P:0.0001	P:0.0001	P:0.0001	P:0.0001	P:0.0001
Perm. ANOVA	WATER	DGGE	P:0.0002		P:0.0212		P:0.0002		P:0.0001		P:0.0008	
		454	R:0.996		R:0.972		R:1		R:1		R:0.988	
ANOSIM	WATER	DGGE	P:0.008		P:0.008		P:0.008		P:0.008		P:0.008	

At days 28 and 42, AS systems were different (Table 2.2). Due to lack of replicate samples pairwise comparisons between water samples of AS systems were not possible. To evaluate differences between RAS and AS systems, pooled data of Ra and Rb were compared with pooled data from AS. Pairwise comparisons (Table 2.3) for PERMANOVA and ANOSIM showed that microbiota in gut or water were different between RAS and AS from day 07 onwards ($P < 0.001$).

Table 2.3. Pairwise comparisons between RAS and AS systems based on DGGE and 454 pyrosequencing data sets. Tests were performed by sample type per sampling day, with P values for each comparison from two different statistical tests (PERMANOVA and ANOSIM). ANOSIM analysis is complementary to PERMANOVA as it provides information on the degree of separation between groups, suggested by R statistic. N.A.: No pyrosequencing data available for that day. D00,07,14,28,42: sampling day 0,7,14,28 & 42 respectively, AS1-5: replicate active suspension system 1 till 5, Ra & Rb: replicate recirculating system a & b. P values are based on 9999 Monte Carlo permutations.

Statistical test	Analytical Method	RAS vs. AS								
		D00	D07	D14	D28	D42				
Perm. ANOVA	GUT	DGGE	P:0.0710	P:0.0022	P:0.0202	P:0.0063	P:0.0099			
		454	NA	P:0.0152	NA	NA	P:0.0056			
ANOSIM	GUT	DGGE	R:0.244	R:0.731	R:0.496	R:0.881	R:0.872			
		454	P:0.0020	P:0.0001	P:0.0001	P:0.0001	P:0.0001			
Perm. ANOVA	WATER	DGGE	P:0.0983		P:0.0187		P:0.0136		P:0.0062	
		454	R:0.820		R:0.944		R:1		R:0.990	
ANOSIM	WATER	DGGE	P:0.0010		P:0.0003		P:0.0020		P:0.0020	

3.2.1 Variation in gut microbiota of larvae reared in different tanks

The extent of variation in gut microbiota of animals reared in replicate tanks of the same Recirculating system was evaluated based on pairwise comparisons of profiles obtained by either DGGE (five tanks per system) or 454 pyrosequencing (two tanks per system). On day 0, gut microbiota was similar ($P > 0.05$ for all pairwise comparisons) between all replicate tanks in either Ra or Rb. For all subsequent sampling days, the majority (72% of all possible comparisons) of the pairwise tests indicated that gut microbiota was not different between replicate tanks ($P > 0.05$, Table 2.4).

Table 2.4. Pairwise comparisons between individuals reared in replicate tanks of the same system. Analysis was performed both on DGGE and 454 pyrosequencing data sets. D00,07,14,28,42: sampling day 0,7,14,28 & 42 respectively, Ra & Rb: replicate recirculating system a & b. Tank numbers 6-10 & 11-15, refer to replicate tanks of Ra and Rb, respectively. P values are based on 9999 Monte Carlo permutations. P values < 0.05 are highlighted in bold.

Statistical test	Analytical Method	Ra tanks	Ra tanks					Rb tanks					
			D00	D07	D14	D28	D42	D00	D07	D14	D28	D42	
PERMANOVA	DGGE	6-7	0.286	0.429	0.124	0.130	0.029	11-12	0.709	0.099	0.273	0.047	0.520
		6-8	0.535	0.494	0.155	0.026	0.009	11-13	0.631	0.168	0.383	0.533	0.737
		6-9	0.217	0.042	0.026	0.081	0.011	11-14	0.330	0.052	0.068	0.044	0.493
		6-10	0.365	0.017	0.043	0.298	0.009	11-15	0.269	0.014	0.091	0.122	0.024
		7-8	0.338	0.260	0.295	0.177	0.298	12-13	0.272	0.266	0.325	0.547	0.768
		7-9	0.150	0.015	0.027	0.302	0.294	12-14	0.212	0.071	0.013	0.049	0.341
		7-10	0.126	0.008	0.083	0.233	0.105	12-15	0.114	0.017	0.028	0.228	0.024
		8-9	0.453	0.055	0.037	0.081	0.243	13-14	0.604	0.149	0.151	0.557	0.596
		8-10	0.458	0.025	0.092	0.068	0.147	13-15	0.383	0.055	0.187	0.653	0.074
		9-10	0.542	0.067	0.665	0.432	0.230	14-15	0.319	0.144	0.408	0.104	0.079
	454	7-9	NA	0.432	NA	NA	0.411	13-14	NA	0.452	NA	NA	0.432

2.3.3 Temporal dynamics in gut and water microbiota

The temporal dynamics in gut microbiota was tested separately for Ra and Rb and for AS (AS1 to AS5) systems. PERMANOVA on DGGE data revealed a significant impact of time on gut microbiota (Table 2.5). Pairwise comparisons of DGGE patterns obtained for consecutive sampling dates were performed, and for all systems there was a significant time effect for every pairwise comparison (Table 2.5, $P < 0.05$). Also for water samples, the temporal variation of microbiota was tested separately for Ra and Rb. At "system type" level, pairwise comparisons of samples taken at consecutive dates indicated that the water-associated microbiota was different between dates ($P < 0.05$) (Table 2.5).

Table 2.5. Pairwise comparisons between consecutive days for RAS and AS replicate systems separately. Analysis is based on DGGE data sets. Tests were performed by sample type, with P values for each comparison from two different statistical tests (PERMANOVA and ANOSIM). ANOSIM results are complementary to the PERMANOVA as they provide information on the degree of separation between groups, suggested by R statistic. D00, 07, 14, 28, 42: sampling day 0, 7, 14, 28 & 42 respectively, AS1-5: replicate active suspension system 1 till 5, Ra & Rb: replicate recirculating system a & b. P values are based on 9999 Monte Carlo permutations.

Statistical test	Groups (dates)	AS					RAS		
		AS1	AS2	AS3	AS4	AS5	Ra	Rb	
		P (MC)	P (MC)	P (MC)	P (MC)	P (MC)	(P)	(P)	
PERMANOVA	GUT	00, 07	0.010	0.001	0.005	0.010	0.003	0.002	0.006
		07, 14	0.009	0.013	0.002	0.001	0.001	0.001	0.010
		14, 28	0.008	0.006	0.015	0.001	0.003	0.002	0.002
		28, 42	0.010	0.005	0.033	0.008	0.005	0.005	0.004
ANOSIM	Global R (all groups)	R:1	R:0.997	R:0.833	R:1	R:1	R:0.906 P:0.001	R:0.938 P:0.001	
PERMANOVA	WATER	Groups (dates)	AS					Ra	Rb
		00, 07	0.0079					0.0003	0.0017
		07, 14	0.0041					0.0006	0.0009
		14, 28	0.0028					0.0001	0.0001
	28, 42	0.0091					0.0003	0.0004	
ANOSIM	Global R (all groups)	R:0.788 / P: 0.0001					R:0.999 P:0.0001	R:0.989 P:0.0001	

2.3.4 Within group dispersion as a measure of dissimilarity between individuals

One way ANOVA on Bray Curtis similarity indicated a clear tank or system effect, with individuals being more similar within than between tanks. Between replicate systems the similarity was even lower (Figure 2.4 A & B). When comparing similarity between individuals of replicate systems, AS replicate systems were more similar than RAS systems (Figure 2.4 C). These differences in Bray Curtis similarity concurred with differences in group dispersion (Figure 2.4 D). Mean group dispersion indicated that microbiota of individuals reared in AS systems were less dispersed, thus more similar, than for individuals reared in RAS until culture day 28 (Figure 2.4 C & D). By day 42 the differences in dispersion became non-significant (P (perm) = 0.095). There was a significant overall time effect on the group dispersion within system. In AS systems dispersion increased (individuals became less similar) over time (AS: $F = 26.205$ ($df_{\text{system}}: 4, df_{\text{time} \times \text{samples}}: 70$), P (perm) = 0.001), whereas in RAS systems dispersion decreased (RAS: $F = 11.683$ ($df_{\text{system}}: 4, df_{\text{time} \times \text{samples}}: 145$), P (perm) = 0.0001), until the dispersion within the two systems converged on day 28 and 42 (Figure 2.4 D). These trends were confirmed by the Bray Curtis similarity means over time for AS and RAS (Figure 2.4 C).

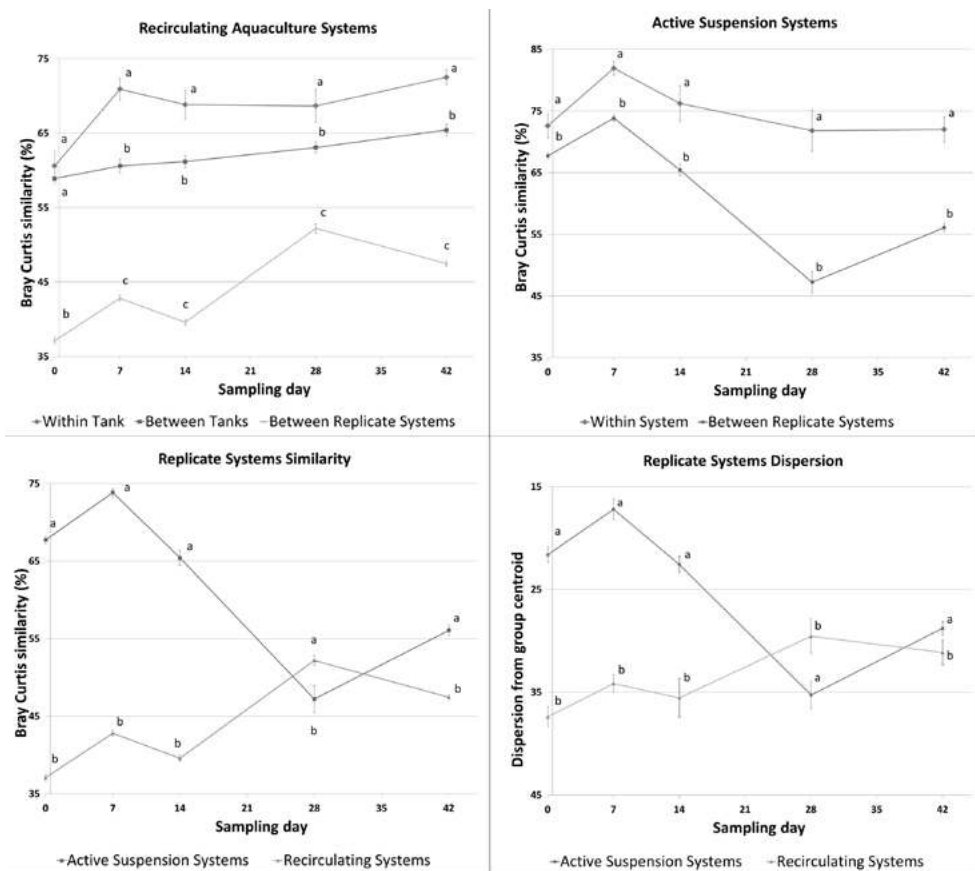


Figure 2.4. Bray-Curtis similarity (%) (A, B & C) and dispersion from centroid based on Euclidian distance (D) for each sampling day. Points represent mean values of gut microbiota between individuals reared either in the same tank (Within tank), replicate tanks (Between tank) or between systems (Between System). (A): Comparison for Recirculating Aquaculture Systems (RAS). (B) Comparison for Active Suspension (AS). (C) Comparison between RAS and AS Systems & (D) Dispersion of samples from group centroid in RAS and AS systems. Error bars show standard error. Different data labels (a, b and c) per sampling day indicated significant difference ($P < 0.05$) based on one-way ANOVA and Bonferroni ranking test for A, B & C and permutation dispersion test for D, ($P(\text{perm}) < 0.05$).

2.4 Discussion

In this study we characterized the spatio-temporal variation in the gut microbiota of tilapia larvae, reared in two different types of aquaculture systems. As was mentioned in the introduction, a subset of samples was analyzed using two different analytical methods, namely DGGE and pyrosequencing of PCR-amplified 16S rRNA gene fragments. The comparison revealed that data obtained by both methods were not contradicting each other. DGGE as a method has some specific limitations, for instance, the separation of relatively small DNA fragments, the co-migration of DNA fragments with different

sequences, the detection of hetero-duplex molecules and the limited sensitivity of detection of rare community members (Muyzer, 1999). In addition, multiple comparisons among different DGGE gels might lead to a false positive conclusion due to high gel to gel variation (Muyzer et al., 1993; Ferris et al., 1996). However, in this study these errors were small and did not jeopardize the broader picture, as it was also confirmed by the pyrosequencing data analysis.

2.4.1 Differences in gut and water microbiota in different tanks

The results showed that variation in individual gut microbiota within tanks was similar to the variation between tanks. This can probably be explained by the fact that larvae shared the water source and feed. To this end, it is interesting to note that pairwise comparisons indicated that the microbiota in larvae reared in replicate tanks in the same RAS were mostly similar, but not in all cases (Table 2.4). Bakke et al. (2013), pointed out that when sampling a few fish in only two replicate tanks, on one occasion gut microbiota differed between two tanks, whereas on another occasion gut microbiota was similar between two other tanks. When sampling only a few individuals in only two tanks the power of the analysis is low. We sampled only three individuals per tank resulting in 10 possible unique permutations. This allows only for a maximum significance level of 10% and in such cases Monte Carlo permutation was used. Nevertheless, unique permutation based P values are preferred when the minimum significance level drops below 1%; this will be realized when sampling a minimum of individuals per tank. Anderson et al. (2006) suggested that examining average within/between group dissimilarities and dispersion, as well as using unconstrained ordination plots, helps to reveal the nature of differences among groups detected by PERMANOVA. In our case, nMDS plots of the DGGE data and the cluster analysis of the pyrosequencing data did not show a clear separation of gut microbiota between larvae reared in replicate tanks. Moreover, ANOSIM's R statistic of tank pairwise comparisons was very low or even negative, also suggesting there is no tank effect on gut microbial communities. Bakke et al. (2013), in contrast to our findings, reported differences in gut microbiota between replicate tanks. This might be due to cumulative differences in water microbiota between replicate tanks and variation in microbiota of daily fed live feeds as opposed to the pelleted commercial diet used in our study. Another reason might be that Bakke et al. (2013), extracted DNA from whole cod larvae after homogenization. Although larvae were disinfected externally, the possibility of contamination cannot be excluded. In our study, fish guts from comparatively much larger tilapia larvae were dissected aseptically after sterilizing body surfaces, with lower risk for contamination. High within tank variation in gut bacteria of cod larvae was also reported by Fjellheim et al. (2011). Here too, larvae were fed live feeds, and whole larvae were used for DNA extraction. In addition, larvae were sampled only from one tank per treatment, and conclusions were drawn based on a combination of culture dependent

and independent techniques. These results should be considered with caution, because the cultivability of microbiota varies with species composition.

2.4.2 Differences in gut and water microbiota between replicate systems

Gut microbiota between replicate AS systems became different within one week ($P < 0.05$), whereas gut microbiota of the individuals reared in Ra and Rb was different already from day 0 ($P < 0.05$; $43.8\% \pm 0.26$ SE Bray Curtis similarity). Microbiota in water was also different ($P < 0.05$) between Ra and Rb. Verschuere et al. (1997), monitored the water microbial communities in three identical *Artemia* culture series, showing distinct microbial communities developing in each of them, suggesting differentiation is stochastic. This concurs with the observed differences of microbiota in gut and water between replicated RAS or AS systems in this study (Figure 2.1). On each sampling day, based on their gut microbiota, larvae reared in Ra differed from those reared in Rb. Similarly, larvae reared in AS4 and AS5 differed (Figure 2.1; $P < 0.05$). This difficulty to replicate systems when studying individual gut microbiota makes experimental design challenging.

In our study, water quality parameters and fish growth were not significantly different between replicate systems (data not shown), yet their microbial communities differed. The observed differences in microbial composition do not necessarily imply differences in functionality (Mouchet et al., 2012). Functional redundancy suggests that functional diversity of an ecosystem is additive when species are complementary, or decreases, when species share functions (Bell et al., 2005). Our results suggest that different treatments (for example, testing dietary effects on gut microbiota) should preferably be tested in tanks within the same system, to reduce variation due to system replication.

2.4.3 Differences in gut and water microbiota between different types of rearing systems

Except for day 0, water and gut microbiota differed between RAS and AS, suggesting a clear system effect. Larval growth, feed conversion and survival between RAS and AS were similar (data not shown), and it is thus safe to assume that observed differences in gut microbiota were not caused by growth related factors or health status of the larvae. Regarding water, rearing system type also affected microbial communities. Possible underlying mechanisms will be explored in a separate paper focusing on differences in bacterial community species composition based on pyrosequencing data.

One question is whether differences in gut microbiota can be explained by differences in water microbiota. Water microbiota, together with feed microbiota, have a large impact on gut microbiota in early life stages (Hansen and Olafsen, 1999). Bakke et al. (2013), suggested that relatively small differences in water microbiota may impose significant

differences in larval microbiota, and this might also be the case in our study. Cluster analysis of Bray Curtis similarity of relative abundance data showed that only 10% of the gut and water microbiota was overlapping (Figure 2.1). Nevertheless, species sub-dominant or even below the detection threshold in the water might be dominant in the gut, or vice versa.

The lack of significant differences in gut microbiota between RAS and AS on day 0 might be due to two reasons; i. high similarity between the microbial communities of the two systems or ii. high within system variation (dispersion). Anderson (2006) suggested that PERMANOVA test should be used combined with a test of homogeneity of multivariate dispersion (PERMDISP). Our results showed that dispersion in RAS was significantly higher at day 0, (compared to the rest of the days), and this was most likely the reason that gut microbiota from larvae reared in RAS did not differ significantly from the ones reared in AS. This might as well explain why water microbial communities between the two systems did not differ on day 0, as PERMDISP confirms that water microbiota among the five different AS systems was more dispersed on day 0 than on any other day of the experiment.

2.4.4 Temporal variation in gut and water microbiota

It is interesting to observe, that in spite of the enormous changes during early development, the effect of “date” was not the most pronounced among factors. The “date” effect to a large extent is linked to structural and functional changes of the gut during early development, including changes in the gut microbiota (Wilson and Castro, 2010). Changes could be induced by fluctuations in pH, gastric secretions and digestive enzymes activity, presence of bile salts, nutrients availability (from endogenous to exogenous feeding), as well as some stochastic events (Hansen and Olafsen, 1999; Verschueren et al., 1997).

When plotting the temporal trajectories of gut microbiota of larvae reared in RAS and AS, both systems clearly differed from day 07 onwards. While the trajectories of the five AS systems were very different, changing almost stochastically (plots not shown), the two replicate RAS trajectories were similar even if the two replicate RAS did not share the same water source (Figure 2.5).

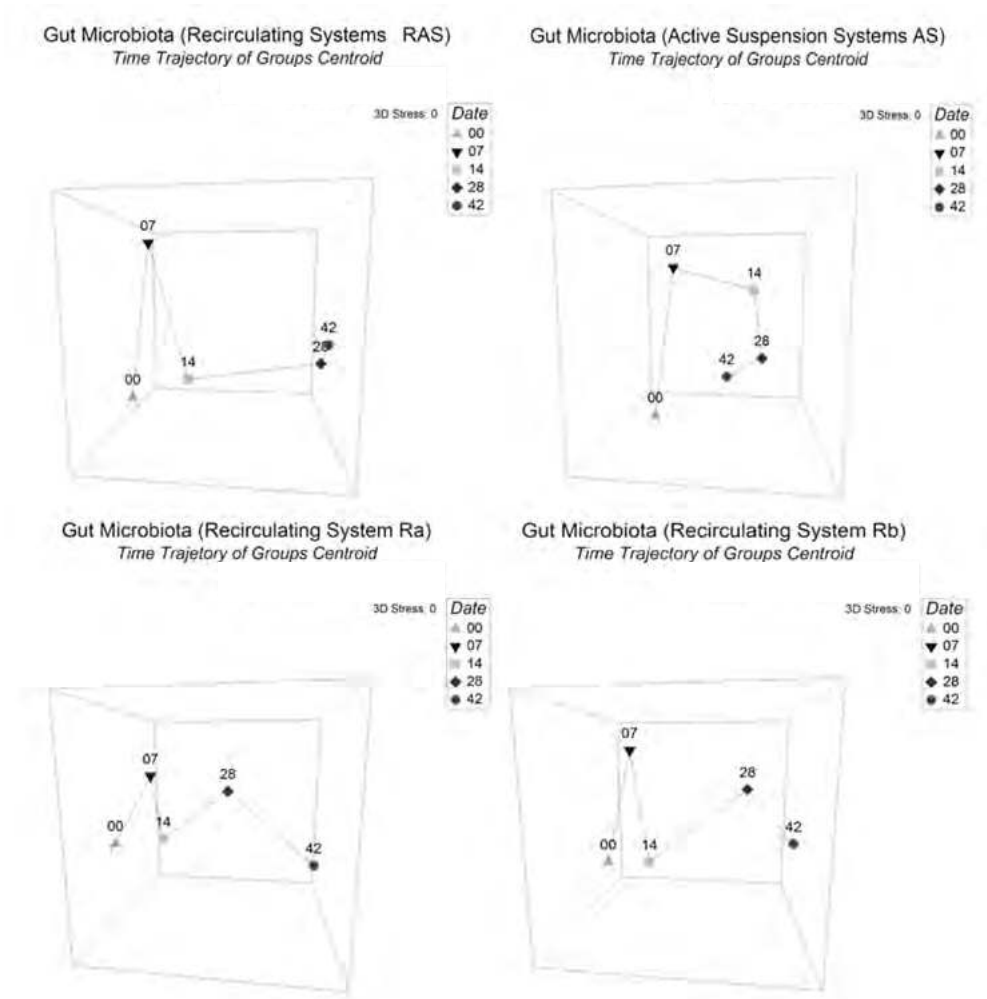


Figure 2.5. Three dimensional nMDS plots of gut microbiota from different systems over time (trajectory). Plots are based on Bray Curtis distance after square root transformation of relative abundance DGGE data. D00, 07, 14, 28, 42: sampling days 0, 7, 14, 28 & 42 respectively. Each point in the plots represents the group centroid and the shift of group average microbiota in time. Zero stress values for each plot are indicative of the fit due to the representation of the centroids. AS and RAS: active suspension and recirculating system, Ra & Rb: replicate recirculating system a & b.

There was a clear distinction of day 0, 7 & 14 from days 28 and 42 for the RAS systems, whereas for the AS systems such a separation was not evident. This agrees with the observed overall (all five points) lower dispersion of AS gut samples compared to dispersion of RAS gut samples. This might be due to two reasons; i. Gut microbiota changed less over time in AS than in RAS; ii. Microbiota of individuals was more similar on each sampling day in AS than in RAS. A possible explanation is that in AS systems

solids remain in the fish tank and most of the organic carbon and nitrogen is available for heterotrophic bacteria. These bacteria are abundant in high concentrations in the water reaching densities of 10^7 CFU mL⁻¹ (Burford et al., 2003). Bacteria, protozoa, algae and zooplankton form bioflocs, are directly available to fish (2007). Grazing on bioflocs might have caused gut microbiota in AS to be more uniform than in RAS.

2.5 Conclusions

Microbiota in water and in larval guts between replicate systems was very different. When individuals share the same water, the rearing tank had a minor effect on gut microbiota. Compositional replication of the microbial communities at system level was not successful. Apparently, our understanding and control of underlying deterministic and stochastic factors is insufficient. This poses many challenges when researching treatment effects on gut or water microbiota. We recommend to investigate treatment effects on gut microbiota within the same system (fish share the same water source), rather than between replicate systems, unless systems can be replicated within treatment. Our results showed that gut microbiota of individuals between tanks of the same system did not differ, whereas between replicate systems they did. The observed rapid and stochastic changes of microbiota in gut and water over time, suggests that long term studies should be interpreted carefully. Observations of start and endpoint do not provide information about the temporal variation in between.

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We would like to thank Hans Heilig, Menno Ter Veld and Muhamad Amin for their contribution on samples collection and laboratory analysis. We also thank all participants of the EU-project Promicrobe, whose enthusiasm and expertise helped to shape ideas expressed in this manuscript.

Supplementary material

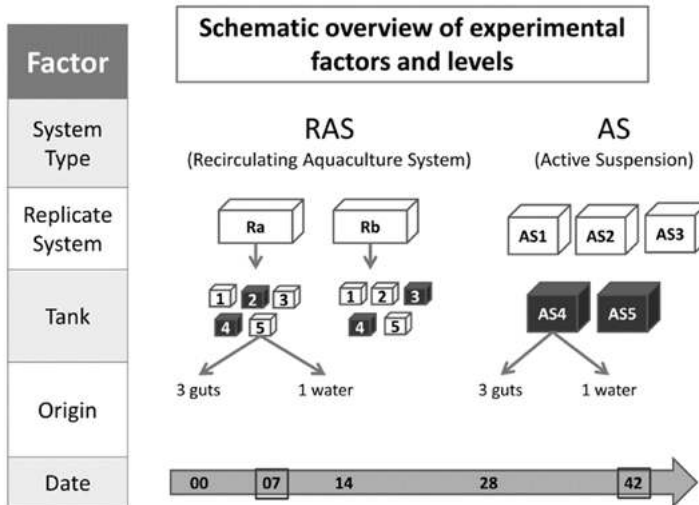


Figure S2.1. Schematic overview of experimental factors and levels. Five active suspension (AS) and 2 recirculating aquaculture systems (RAS) were used. The replicate RAS are named Ra and Rb; the replicate AS systems are named AS1 through AS5. Each RAS contained five tanks which shared the same water source. AS systems did not have sub-divisions. For DGGE analysis, three guts and water were sampled from each tank in RAS (10 tanks total) and each AS (5 systems) on sampling day 00, 07, 14, 28 & 42. Sub-sets of samples for DGGE of Ra2, Ra4, Rb3 and Rb4 (dark shaded tanks), and active suspension systems AS4 and AS5 (also dark shaded), taken on days 07 and 42, were used for pyrosequencing.

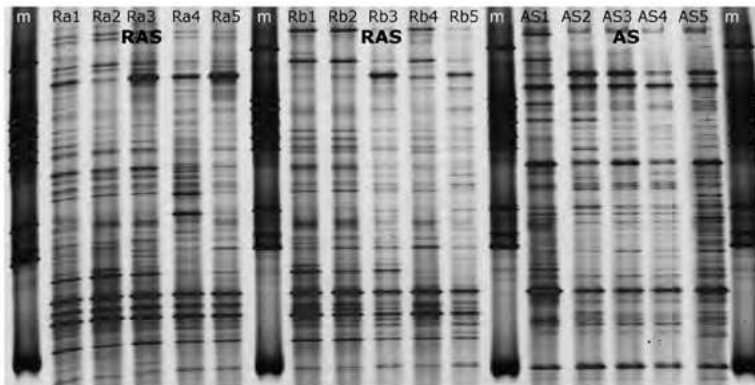


Figure S2.2. Denaturing gradient gel electrophoresis (DGGE) of gut microbiota on day 28. Each lane displays the banding pattern (fingerprint) of gut microbiota from an individual fish. Samples were taken from different systems on experimental day 28. Ra and Rb: Recirculating aquaculture systems a and b, from tank 1 to 5, AS1-5: Active suspension systems 1 through 5, m: standard reference marker consisting of an amplicon mix of 10 different cloned bacterial 16S rRNA genes used for digital gel normalization.

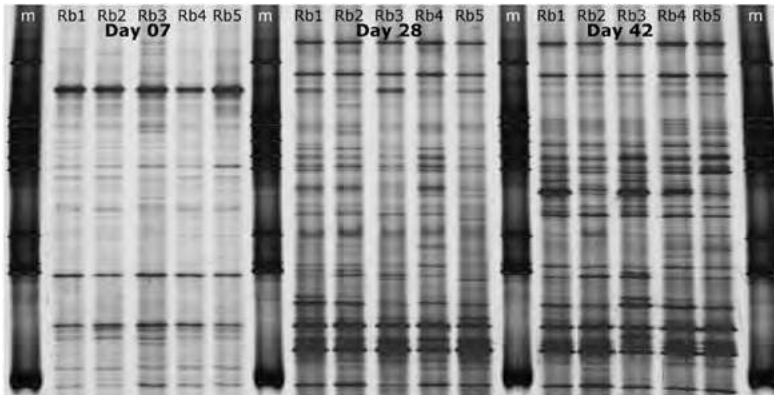


Figure S2.3. Denaturing gradient gel electrophoresis (DGGE) of gut microbiota of system Rb over time. Each lane displays the banding pattern (fingerprint) of gut microbiota from an individual fish. Samples were taken from the same system on experimental day 07, 28 and 42. Rb: Recirculating aquaculture systems b, from tank 1 to 5, m: standard reference marker consisting of an amplicon mix of 10 different cloned bacterial 16S rRNA genes used for digital gel normalization.

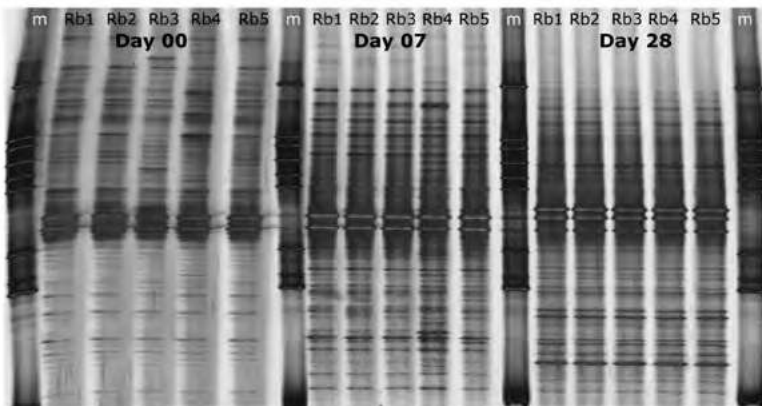


Figure S2.4. Denaturing gradient gel electrophoresis (DGGE) of water microbiota from system Rb over time. Each lane displays the banding pattern (fingerprint) of water microbiota from each tank. Samples were taken from the same system on experimental day 00, 07 and 28. Rb: Recirculating aquaculture systems b, from tank 1 through 5, m: standard reference marker consisting of an amplicon mix of 10 different cloned bacterial 16S rRNA genes used for digital gel normalization.

Chapter 3

The impact of rearing environment
on the development of
gut microbiota in tilapia larvae



This chapter has been published as:

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srep18206

This study explores the effect of rearing environment on water bacterial communities (BC) and the association with those present in the gut of Nile tilapia larvae (*Oreochromis niloticus*, Linnaeus) grown in either recirculating or active suspension systems. 454 pyrosequencing of PCR-amplified 16S rRNA gene fragments was applied to characterize the composition of water, feed and gut bacteria communities. Observed changes in water BC over time and differences in water BCs between systems were highly correlated with corresponding water physico-chemical properties. Differences in gut bacterial communities during larval development were correlated with differences in water communities between systems. The correlation of feed BC with those in the gut was minor compared to that between gut and water, reflected by the fact that 4 to 43 times more OTUs were shared between water and gut than between gut and feed BC. Shared OTUs between water and gut suggest a successful transfer of microorganisms from water into the gut, and give insight about the niche and ecological adaptability of water microorganisms inside the gut. These findings suggest that steering of gut microbial communities could be possible through water microbial management derived by the design and functionality of the rearing system.

3.1 Introduction

Gut microbiota influences a wide range of biological processes of their host, including digestion, innate immunity, proliferation of epithelial cells and structural and functional maturation of the gut in humans (Rawls, 2007; Sekirov et al., 2010), domesticated terrestrial animals (Chaucheyras-Durand and Durand, 2010; McFall-Ngai et al., 2013) and fish (Nayak, 2010b; Sullam et al., 2012). However, our understanding of the roles and drivers for community diversity and species dominance within the gut is presently limited (Walker et al., 2011).

Fish are exposed to higher microbial loads in the aquatic environment than terrestrial domesticated animals are in air or soil (Verschuere et al., 2000). Thus, this closer contact with the surrounding water likely affects early gut colonization. Whilst water seems to affect the fish gut microbiota already from mouth opening onwards (Reitan et al., 1998), feed microbiota becomes important at a later development stage (Hansen and Olafsen, 1999; Ringo and Birkbeck, 1999). However, although water and feed are the two main sources of microorganisms available to fish, the factors underlying the successful colonization of ingested microbes and the community assembly inside the gut are still poorly understood (De Schryver and Vadstein, 2014). For this reason, predictability and repeatability of gut microbiota manipulations is currently limited.

The nutrient rich environment of high density aquaculture is conducive to the proliferation of microbes. To this end, aquaculture management aims to minimize the amount of nutrients in the rearing tank. For example, in recirculating aquaculture systems (RAS) the first step of water purification is solids removal (Crab et al., 2007; Gutierrez-Wing and Malone, 2006). In contrast, in active suspension (AS) systems, fish excreta and feed left overs are mineralized inside the rearing tank. AS systems are characterized by the formation of bioflocs (aggregates of microorganisms, colloids, organic polymers and dead cells), which contribute to the maintenance of good water quality while providing additional nutrients to the fish (De Schryver et al., 2008).

In the present study, we hypothesized that water bacterial communities would differ between the two rearing environments (i.e. RAS vs. AS systems) due to differences in systems' design. Assuming that gut colonization is strongly affected by the composition of the microbiota in the water, we expected that composition of gut communities would differ between systems as well. Compared to RAS, bacteria in the water of AS systems have a higher chance of being successfully transferred in the gut, due to the constant grazing of larvae on bioflocs. Thus, a greater influence of AS water microbiota on the gut is expected. We used 454 pyrosequencing of PCR-amplified bacterial 16S ribosomal RNA (rRNA) gene fragments to characterize for the first time the composition of gut BCs of Nile tilapia *Oreochromis niloticus* (Linnaeus) reared for 42 days in RAS or AS tanks from

the moment of first feeding. The progressive gut colonization in fish reared in the two systems and the similarity of water and feed BCs with those in the gut were evaluated at different time points. Furthermore, the potential role of the most predominant bacteria found in this study is discussed in detail based on a comprehensive literature review.

3.2 Materials and methods

3.2.1 Experimental animals and set up

The experiment was performed in accordance with relevant guidelines and approved by the Ethical Committee of Wageningen University for animal experiments (Registration code: 2009055d). One batch of three days post-fertilization (3 dpf) eggs were washed out from the mouth of a female Nile tilapia and incubated for four days at 27°C. Eggs were incubated in a tank until swim-up stage and received water from the same source. For rearing the larvae, two different culture systems were used: a recirculating aquaculture system (RAS) and an active suspension (AS) system with two replicates each (Ra&Rb and AS1&AS2), which were initially filled with the same water coming from a well. Replicate systems did not share the same water. Only for RAS systems, each replicate also contained 2 replicate tanks (Ra1, Ra2 & Rb1, Rb2) which were connected to the same water treatment unit (biofilter). In each tank, 100 randomly selected swim-up larvae (7 dpf) were introduced before first feeding and before mouth opening. Mouth opening occurred within the experimental rearing systems. Feeding started 9 dpf (referred to as day 0) and was continued for 42 days. Fish were fed three times a day to apparent satiation with a starter feed (F-0.5 GR Pro Aqua Brut – Trouw Nutrition, France; 57% crude protein, 15% crude fat, 8.5% carbohydrates, 11% crude ash, 0.6% crude fiber, 1.7% phosphorus). A 42 day culture period was considered sufficiently long for all major stages of the gut development (Fujimura and Okada, 2007). Water, feed and gut samples were collected on day 7 and 42. Further details about the experimental setup, animals and description of the rearing systems can be found elsewhere (Giatsis et al., 2014).

3.2.2 Sampling of guts, water and feed

On sampling days, three tilapia larvae were randomly collected from Ra1, Ra2, Rb1, Rb2, AS1 and AS2 for gut BC analysis. After euthanization, the larvae were rinsed with 70% ethanol and sterile water, before dissecting the gut. Gut samples were flash-frozen in liquid nitrogen and stored individually at -80 °C until further processing. Furthermore, 250 mL of water was collected from each tank. Water was simultaneously filtered through a 0.45 µm (type HAWP) over a 0.22 µm (type GTTP) membrane filter (Millipore - Isopore™) using a vacuum apparatus. Filters were stored at -80°C until processing. The 0.45 µm filters were

used in order to avoid clogging of the 0.22 μm with large size particles during filtration. However, both filters were used for DNA extraction from water. Finally, also 2 grams of feed were collected and stored at -80°C until processing. All gut, water and feed samples were stored and analyzed individually. Samples were not pooled neither were the corresponding DNA extracts.

On a weekly basis, total ammonia nitrogen (TAN-N), nitrite (NO_2^- -N), nitrate (NO_3^- -N), orthophosphate (PO_4^{3-}), carbon dioxide (CO_2), urea, dissolved oxygen (DO), pH, conductivity and temperature ($^{\circ}\text{C}$) were measured in each tank according to Giatsis et al. (2014).

3.2.3 Isolation of DNA

DNA was extracted from larval gut samples using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands), whereas the FastDNA SPIN kit for soil (MP Biomedicals, Ohio, USA) was used for water and feed samples. The use of different physical, mechanical, chemical and enzymatic lysis protocols, different purification and precipitation techniques and different reagents, have shown to affect genomic DNA yield and quality. This was confirmed by Lever et al. (2015), however, the authors furthermore concluded that the observed effects are almost always sample dependent. This being said, before our study, genomic DNA was extracted from all sample types by using two extraction protocols. Since the DNeasy Blood & Tissue Kit resulted in a very low DNA yield from the water and feed samples and a high DNA yield was obtained with the FastDNA SPIN kit for soil, and hence the latter was used for all water and feed samples in this study. In contrast, the FastDNA SPIN kit for soil showed a low yield for DNA extraction from gut samples, while a high yield was obtained with the DNeasy Blood & Tissue Kit. Therefore, the DNeasy Blood & Tissue Kit was used for all gut samples in this study.

Modifications of the manufacturer's protocol with respect to enhancement of cell lysis (i.e. bead beating), DNA purification and elution processes, were made as previously described by Giatsis et al. (2014).

3.2.4 PCR and 454 Pyrosequencing

Barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated by PCR using the 27F-DegS primer (Van den Bogert et al., 2011) appended with titanium sequencing adaptor A and an 8 nt sample-specific barcode (Hamady et al., 2008) at the 5' end. A detailed protocol for the PCR is given by Giatsis et al. (2014). Nucleotide sequences were generated by pyrosequencing using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany). These sequence data were submitted to the European Bioinformatics Institute under study accession No PRJEB4462 and sample accession No ERS343984 – ERS344037.

3.2.5 Data handling

Pyrosequencing data were analyzed using the QIIME 1.5.0 pipeline (Caporaso et al., 2010) and quality filtering was performed as follows: low quality sequences were removed using default parameters. Removed reads included (i) reads with less than 200 or more than 1000 nucleotides; (ii) reads with more than 6 ambiguous nucleotides, homopolymer runs exceeding 6 bases, reads with missing quality scores and reads with a mean quality score lower than 25; and (iii) reads with mismatches in the primer sequence. Operational taxonomic units (OTUs) were identified at the 97% identity level. Representative sequences from the OTUs were aligned using PyNAST (DeSantis et al., 2006). The taxonomic affiliation of each OTU was determined using the RDP classifier at a confidence threshold of 80% against the 12_10 Greengenes core set (Wang et al., 2007). Possible chimeric OTUs were identified using QIIME's ChimeraSlayer and removed from the initially generated OTU list, producing a final set of non-chimeric OTUs.

To visualize possible commonalities and anti-correlations between gut, water and feed BCs, physical samples and OTUs were plotted as nodes in a bipartite network. In the network analysis, the average relative abundance of OTUs in replicate samples was represented. To cluster the OTUs and hosts in this network, the Edge-weighted Spring Embedded algorithm as implemented in Cytoscape 3.0.2 was used (Saito et al., 2012).

3.2.6 Statistical analysis

A repeated measure ANOVA was applied for water quality measurements on day 7 and 42 with "system" (RAS or AS) as main factor and "day" (7 or 42) as repeated measure. Based on Draftsman plots (variable pair-wise scatter plots) of water quality data, the TAN values were log-transformed (Figure S3.1). Subsequently, all environmental variables were normalized. The Euclidean distance of normalized environmental variables was used as dissimilarity measure. Gut, water and feed BC data were expressed as relative abundance of all OTUs in each sample, and Bray Curtis similarity was calculated based on square root transformed data.

A distance-based linear model (distLM) was used for analyzing and modelling the relationship between the bacterial community composition, as described by a resemblance matrix, and the predictor variables (i.e. water parameters). When not enough possible permutations were available, P-values for testing the null hypothesis of no relationship between the two datasets were obtained by Monte Carlo permutations (a sample was drawn from the theoretical asymptotic permutation distribution) (Anderson and Robinson, 2003). Forward selection of the environmental variables was applied and distance-based redundancy analysis (dbRDA) was used for visualization (Legendre and Anderson, 1999; Anderson et al., 2006).

Based on Bray Curtis similarity of relative abundance data, RELATE analysis evaluated the relatedness between water and gut BC by calculating Spearman's ρ correlation coefficient between all elements. Similarity percentages (SIMPER) analysis was used to determine the OTUs which contributed most to the discrimination or relatedness of samples in each group. To measure alpha-diversity of the bacterial communities: the total number of observed species (S), Pielou's evenness ($J' = H' / \log(S)$) and Shannon ($H' = -\sum(P_i \cdot \ln(P_i))$) were calculated (P_i is the proportion of the total count (abundance) arising from the i th species). As species richness and evenness can only be compared between samples when sample sizes are equal (Hurlbert, 1971), resulting reads were randomly selected so as to standardize to the sample with the least number of obtained reads ($n = 1719$). To visualize community evenness, dominance plots were created based on relative abundance data. OTU relative abundance (y-axis) was averaged per sample type (gut and water), for each system (AS and RAS) per day (7 and 42). Cumulative relative abundance was plotted against the increasing species rank (x-axis).

Statistical analyses were performed using Primer 6 (version 6.1.11) (Primer-ELtd., Plymouth, United Kingdom) and its PERMANOVA add-on package (Gorley and Clarke, 2006). Venn diagrams were produced in *Venny* online freeware (Oliveros, 2007).

3.2.7 Phylogenetic analysis

The first 150 OTUs contributing most to differences between groups were considered for a more thorough phylogenetic analysis. Representative sequences of these OTUs were aligned using the online SINA alignment service of the ARB-Silva database (Pruesse et al., 2012). Aligned sequences were imported into ARB in the Silva release 115 SSU NR 99 tree (Quast et al., 2013) using the ARB parsimony method without changing the tree topology. Three near neighbors of all 150 OTUs were selected according to the following criteria: (i) they were at least 800 nucleotides long and included the entire sequenced amplicon, and (ii) neighbors per OTU were picked from different published studies. An alignment containing only the neighbors was exported before constructing a Bayesian tree. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). Default parameters were used, except allowing a minimum block length of 5 and gaps in 50% of positions. Phylogenetic trees were calculated by Bayesian analysis, using a locally installed version of MrBayes 3.2 (Ronquist et al., 2012). All parameters were treated as unknown variables with uniform prior-probability densities at the beginning of each run, and their values were estimated from the data during the analysis. All Bayesian analyses were initiated with random starting trees and were run for 10^7 generations. The number of chains was set to four and Markov chains were sampled every 1000 iterations. Points prior to convergence were determined graphically and discarded. Calculated trees were imported into ARB, and short sequences obtained in this study were subsequently added by use of the ARB parsimony method without changing the tree topology.

3.3 Results

3.3.1 The effect of rearing system and time on water quality

Analysis of environmental data showed significant differences in water quality between RAS and AS. Conductivity, NO₃-N and PO₄-P were higher, whereas CO₂, DO and pH were lower in RAS (P<0.05) than in AS (Table 3.1).

Table 3.1. ANOVA results with system as main factor and day as repeated measure. Average water quality parameters calculated for recirculating aquaculture system (RAS) or active suspension (AS) and for day 7 and 42 (n =4) are given. The right column gives the "System * Day" interaction P values (bold for P < 0.05). Within system or day, bold values indicate significant difference (P < 0.05). TAN: Total ammonia nitrogen; NO₂-N: Nitrite nitrogen; NO₃-N: Nitrate nitrogen; PO₄-P: Phosphate phosphorous; CO₂: Carbon dioxide; T: Temperature; DO: Dissolved oxygen; Cond.: Conductivity.

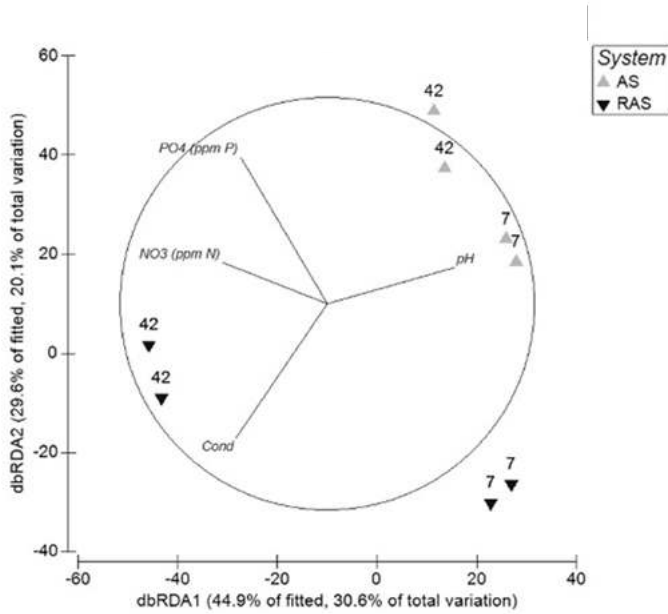
Parameter	System				Day				Syst*Day
	RAS		AS		7		42		
	7	42	7	42	RAS	AS	RAS	AS	
TAN (mg L ⁻¹)	0.03	0.05	0.06	0.04	0.03	0.06	0.05	0.04	0.047
NO ₂ (mg L ⁻¹)		0.03		0.11		0.04		0.10	0.303
NO ₃ (mg L ⁻¹)	12.4	54.3	4.5	28.2	12.4	4.5	54.3	28.2	0.000
PO ₄ (mg L ⁻¹)		1.46		1.04		0.37		2.13	0.098
CO ₂ (mg L ⁻¹)	59.1	9.4	72.9	39.0	59.1	72.9	9.4	39.0	0.003
Urea (mg L ⁻¹)		0.02		0.07		0.03		0.06	0.465
T (°C)		27.3		27.5		27.2		27.6	0.884
pH	8.06	7.28	8.15	8.10	8.06	8.15	7.28	8.10	0.002
DO (mg L ⁻¹)		7.72		8.00		8.18		7.55	0.150
Cond (µS cm ⁻¹)		402		820		260		820	0.002

Conductivity, NO₃-N and PO₄-P increased, and CO₂, DO and pH dropped between day 7 and 42 (P<0.05). In contrast, temperature and concentrations of NO₂-N, urea and TAN were neither significantly different (P>0.05) between RAS and AS, nor between day 7 and 42.

3.3.2 Correlation of environmental parameters with water bacterial communities

Distance-based linear model (DistLM) analysis revealed that pH, conductivity, NO₃-N and PO₄³⁻-P together explained 68% (R² sequential) of the observed total variation in the composition of the bacterial community in the water. Conductivity and pH strongly correlated with differences in BCs between RAS and AS, while PO₄-P and NO₃-N correlated with differences in the communities between day 7 and 42 within system (Figure 3.1). The same parameters showed a highly significant system * day interaction with repeated measures ANOVA of water quality data (Table 3.1).

Distance based Redundancy Analysis (dbRDA) of water microbiota and environmental parameters



Distance based Linear Model (DistLM) - MARGINAL TESTS

Variable	SS(trace)	Pseudo-F	P	Proportion of explained variation
NO ₃ -N (ppm N)	11842	4.2141	0.0002	0.29647
PO ₄ -P (ppm P)	11195	3.8941	0.0002	0.28027
pH	12081	4.3359	0.0011	0.30245
Cond	11895	4.2408	0.0001	0.29779
res.df: 10				

DistLM - SEQUENTIAL TESTS (Specified solution)

R ²	RSS	No. Variables	Selection of variables
0.68073	12753	4	NO ₃ -N, PO ₄ -P, pH, Cond

Figure 3.1. Distance based Redundancy Analysis (dbRDA) of water microbiota. Relative position of water samples in the biplot is based on Bray Curtis similarity of square root transformed relative abundance at the OTU level. Vectors indicate the weight and direction of those water quality parameters that were best predictors of water bacterial composition as suggested by the results of the distance-based linear model (distLM). The dbRDA axes describe the percentage of the fitted or total variation explained by each axis while being constrained to account for group differences. Sample IDs indicate the sampling day (7 and 42) and the rearing system (AS & RAS: Active suspension and recirculating aquaculture system with two replicates each). NO₃⁻-N: Nitrate nitrogen; PO₄³⁻-P: Phosphate phosphorous; Cond.: Conductivity.

3.3.3 Similarity of water bacterial communities between replicate systems over time

Overall, 11,658 OTUs were identified in all samples. At OTU level, the average similarity of water BC within AS on day 7 was 64% (Table 3.2). The 5 OTUs (SIMPER analysis), in order of relative abundance, explaining most of the average similarity were OTU 9828 (order Sphingomonadales), 4552 (genus *Limnohabitans*), 8066 (genus *Sediminibacterium*), 2756 (genus *Rhodobacter*) and 11945 (genus *Nitrospira*). Combined, these 5 OTUs accounted for 49% of the bacterial community in AS water on day 7 (Table S3.1).

Table 3.2. Percentage average Bray Curtis similarity between gut, water and feed bacterial communities. Values are based on SIMPER analysis of square root transformed relative abundance data at the OTU level. Values within brackets indicate the standard deviation of the average gut Bray Curtis similarity within group, as a measure of multivariate within group dispersion. AS & RAS: Active suspension and recirculating aquaculture systems respectively; AS1 & AS2: Replicate active suspension system 1 & 2; Ra & Rb: Replicate recirculating aquaculture system a & b; 7 and 42: Sampling day 7 and 42.

% Average Bray Curtis similarity						
	Gut			Water		
	Between replicate AS (AS1, AS2)	Between replicate RAS (Ra, Rb)	Between AS & RAS	Between replicate AS (AS1, AS2)	Between replicate RAS (Ra, Rb)	Between AS & RAS
Day 7	68.9 (±7.6)	55.1 (±15.9)	21.6	63.8	59.0	23.9
Day 42	59.9 (±18.1)	56.4 (±11.0)	10.8	25.8	60.8	1.8
	Between day 7 & 42 of gut			Between day 7 & 42 of water		
AS	21.6			17.3		
RAS	27.2			3.5		
	Day 7			Day 42		
	Between gut & water	Between gut & feed	Between water & feed	Between gut & water	Between gut & feed	Between water & feed
AS	4.1	0.1	0.1	8.5	0.1	0.1
RAS	3.9	0.1	0.1	8.1	0.1	0.1

On day 42, the water BC average similarity within AS decreased to 26% (Table 3.2). Almost 60% of this similarity was attributed to OTU 8066 (genus *Sediminibacterium*), 14254 (family Comamonadaceae), and 4686 (order Sphingobacteriales) (Table S3.1). Other predominant OTUs of AS water BC on day 42 included: OTU 5688 (genus *Fimbriimonas*) and 7261 (genus *Armatimonas*).

Overall, water bacterial communities in AS systems on day 7 was 17% similar with BC present on day 42 since most of the predominant OTUs on one day were absent or rare on the other (Table 3.2). Despite the low similarity, 85 OTUs were shared between the two dates, with a cumulative relative abundance of 63% on day 7 and 52% on day 42 (Table

3.3). OTU 8066 contributed most to the similarity, whereas OTU 9828 contributed most to the dissimilarity between the two days (Table S3.1).

Table 3.3. Number of unique and shared OTUs and relative abundance of shared OTUs between days. Shaded abundance values correspond to shaded sampling group in the first column. Sampling groups are compared between sampling days for each system. OTU: Operational taxonomic unit; AS & RAS: Active suspension and recirculating aquaculture systems; 07 & 42: Sampling days 7 and 42 respectively.

Shared OTUs between days per system				
Group	Number of shared OTUs	% abundance of shared OTUs	OTUs shared among gut or water samples	OTUs shared between all gut & water samples
Gut_07_AS vs. Gut_42_AS	131	76.4	52	
		86.4		
Gut_07_RAS vs. Gut_42_RAS	302	91.0		2
		51.2		
Water_07_AS vs. Water_42_AS	85	62.9	18	
		51.9		
Water_07_RAS vs. Water_42_RAS	98	76.1		
		56.8		

In RAS, the average similarity of water BC on day 7 was 59% (Table 3.2). More than half of this similarity was attributed to only two predominant OTUs: OTU 7333 (genus *Polynucleobacter*) and 9828 (family Sphingomonadales), which together had a cumulative relative abundance of 46.8% on that day (Table S3.1).

On day 42, similarity of water BC within RAS remained at levels similar to day 7. This similarity was mostly attributed to OTU 7090 (family Rhodocyclaceae), 14286 (genus *Limnohabitans*), and 4377 (genus *Rhodobacter*). Other predominant OTUs of RAS water BC on day 42 included: OTU 5688 (genus *Fimbriimonas*) and 7261 (genus *Armatimonas*). The aforementioned five OTUs accounted for 55.2% of the total bacterial community on that day (Table S3.1).

Bacterial communities in the RAS water differed considerably between day 7 and 42 (3.5% similarity) (Table 3.2). Predominant OTUs in water on day 7 were almost absent on day 42 and vice versa. For example, OTU 7090 was absent on day 7 whilst accounted for 35% of the community on day 42, thus contributing about 20% to the difference between the two days (Table S3.1). Despite the low similarity, water BC in RAS shared 98 OTUs between day 7 and 42, with a cumulative relative abundance of 76% and 57%, respectively (Table 3.3).

3.3.4 Similarity of water bacterial communities between systems

Already on day 7, the average similarity between AS and RAS was only 24% (Table 3.2). The two systems shared 113 OTUs with a cumulative relative abundance of 62.5% and 73% in AS and RAS systems, respectively (Table 3.4). On day 42, the similarity of water BC between the two systems decreased to 2%, since almost all predominant OTUs in AS water, were absent in RAS and vice versa. Overall, water from the two systems shared 40 OTUs with a cumulative relative abundance of 30% in AS and only 4% in RAS (Table 3.4).

In terms of evenness, an overall system (AS & RAS) * day (7 & 42) interaction was observed. On day 7, 17 OTUs had a cumulative relative abundance of 60% in AS water, whereas on day 42, the same percentage was attributed to six OTUs. In RAS water, 60% of the relative abundance was attributed to 6 OTUs on day 7 and three OTUs on day 42 (Table S3.1). Additionally, over time species richness decreased from 683 to 394 OTUs in AS water and from 815 to 552 in RAS. This observation is also in agreement with the results of the dominance plots and the biodiversity indices which showed that the richness and evenness of water bacterial communities in AS decreased between day 7 and 42, whereas this difference was less pronounced in RAS (Figure S3.2 & Table S3.2).

Table 3.4. Number of unique and shared OTUs within and between systems. Percentages indicate the relative abundance of shared OTUs within each system. Operational taxonomic unit; AS & RAS: Active suspension and recirculating aquaculture systems; 07 & 42: Sampling days 7 and 42 respectively.

Shared OTUs between systems per day			
Group	Total number of OTUs	Number of shared OTUs	Within group % abundance of shared OTUs
Gut_07_AS	732		70.7
Gut_07_RAS	1003	247	71.4
Gut_42_AS	279		43.9
Gut_42_RAS	879	90	50.7
Water_07_AS	683		62.5
Water_07_RAS	815	113	73.0
Water_42_AS	394		30.3
Water_42_RAS	552	40	3.6

3.3.5 Similarity of gut bacterial communities between replicate systems over time

On day 7, average similarity of gut bacterial communities within AS was 69% (Table 3.2). Half of this similarity was attributed to OTUs 4790 (species *Mycobacterium llutzerense*), 1651 (genus *Rhodobacter*), 10562 (genus *Gordonia*), 10599 (family Bradyrhizobiaceae) and 8299 (genus *Sporocytophaga*). Those five OTUs accounted for a cumulative relative abundance of 42.2% in AS (Table S3.1). On day 42, the similarity of gut BC between samples

dropped to 60%. More than 80% of this similarity was attributed to OTUs 4563 (family Isosphaeraceae), OTU 12537 (genus *Arthrobacter*) and OTU 4790 (species *Mycobacterium llatzerense*). Those predominant OTUs were encountered on both days, albeit at different relative abundances (Table 3.5). *M. llatzerense* (OTU 4790) was the only OTU with a high relative abundance on both sampling days. Despite the 131 shared OTUs between the two days, gut BC was 21.6% similar between days (Table 3.2, Table 3.3).

Within RAS, average similarity of gut BC between samples was 55% on day 7 (Table 3.2). More than 60% of this similarity was due to OTUs 10599 (family Bradyrhizobiaceae), 1005 (genus *Rhodococcus*), 4790 (*Mycobacterium llatzerense*) and 12555 (genus *Agrococcus*) (Table S3.1). On day 42, average similarity between gut samples remained similar. This similarity was mostly attributed to OTUs 7966 (family Peptostreptococcaceae), 4790 (*Mycobacterium llatzerense*), 7955 (family Mogibacteriaceae), 1005 (genus *Rhodococcus*) and 2787 (*Cetobacterium somerae*). These five OTUs accounted for a cumulative relative abundance of 59.5% in RAS. All predominant gut OTUs of day 7 were also present on day 42, albeit at lower relative abundance, whereas the OTUs most predominant on day 42 were absent on day 7 (Table S3.1). Similar as for AS, *M. llatzerense* was the shared OTU with a high relative abundance on both sampling days. In RAS, 302 gut OTUs with a cumulative relative abundance of 91% and 51% respectively, were shared between days (Table 3.3).

3.3.6 Similarity of gut bacterial communities between systems

On day 7, similarity of gut BC between systems was 22% (Table 3.2). Most of the predominant OTUs were present in both systems, although at different relative abundance (Table 3.5). OTUs 1651 and 8299 were predominant in AS but absent in RAS and OTU 3934 was predominant in RAS though absent in AS. Despite the low similarity in gut BC, the two systems shared 247 OTUs with a cumulative relative abundance of about 71% in both systems (Table 3.4).

On day 42, similarity between systems decreased to 11% (Table 3.2). This was partly due to the difference in the abundance of OTU 4563 (family Isosphaeraceae) between the two systems (40.8% in AS and absent in RAS) (Table 3.5). Among the shared OTUs on that day, *M. llatzerense* was the OTU with high relative abundance in both systems. Overall, the number of shared OTUs between systems decreased over time from 247 to 90 (Table 3.4).

In terms of biodiversity of the BCs, a system (AS & RAS) * day (7 & 42) interaction was observed. On day 7, 60% of cumulative relative abundance in AS was attributed to 13 OTUs whereas on day 42 to only two; this is clearly depicted also in the large decrease of the Shannon index over time (Table S3.2). On the contrary, in RAS, the 60% of the abundance was attributed to five and six OTUs on day 7 and 42, respectively (Table S3.1) while the biodiversity indices remained on similar levels. Over time, in AS the total

number of observed gut species decreased from 732 to 279, whereas this decrease was not so evident in RAS. This observation is in agreement with the results of the dominance plots for day 7 (Figure S3.2).

Table 3.5. Heatmap of the most predominant gut, water and feed OTUs on day 7 and 42. Values represent the % average relative abundance of each OTU within each sample group and colour codes are proportional to increasing OTU abundance (from green: lower, to red: higher relative abundance). 07 and D42: sampling day 7 & 42; AS & RAS: Active suspension and recirculating aquaculture systems respectively.

Taxonomy		OTU Number	Gut 07_AS	Gut 42_AS	Gut 07_RAS	Gut 42_RAS	Water 07_AS	Water 42_AS	Water 07_RAS	Water 42_RAS	FEED	
Actinobacteria	<i>M. flazzerense</i>	4790	19.37	16.17	11.42	12.06	1.23	0.16	3.39	0.23	0.00	
	<i>Gordonia</i>	10562	5.94	0.00	2.36	0.09	0.01	0.00	0.00	0.01	0.00	
	<i>Rhodococcus</i>	1005	0.44	0.00	15.17	9.21	0.01	0.00	0.56	0.06	0.00	
	<i>Agrococcus</i>	12555	0.01	0.00	6.80	0.13	0.00	0.00	0.03	0.00	0.00	
	<i>Corynebacterium</i>	5872	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.34	
	<i>Arthrobacter</i>	12537	1.17	19.29	0.00	0.01	0.07	0.36	0.00	0.00	0.00	
Bacteroidetes	<i>Sporocytophaga</i>	8299	3.80	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	
	<i>Sediminibacterium</i>	8066	0.00	0.00	0.00	0.00	4.29	20.30	0.01	0.07	0.00	
Firmicutes	<i>Peptostreptococcaceae</i>	7966	0.44	0.04	0.00	18.13	0.01	0.00	0.00	1.70	0.00	
	<i>Kestanolensis</i>	1521	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	4.12	
	<i>Mogibacteriaceae</i>	7955	0.02	0.01	0.00	8.40	0.00	0.00	0.00	0.26	0.00	
	<i>Facklamia</i>	3532	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.94	
Fusobacteria	<i>Psychrilyobacter</i>	2650	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.11	
Nitrospirae	<i>Nitrospira</i>	11945	0.00	0.00	0.01	0.01	4.10	0.77	0.18	0.01	0.00	
Planctomycetes	<i>Isophaeraceae</i>	4563	3.27	40.75	0.00	0.00	0.03	1.95	0.00	0.00	0.00	
Proteobacteria	Alpha	<i>Bradyrhizobiaceae</i>	10599	3.78	0.27	18.92	0.63	0.08	0.02	0.66	0.02	0.00
		<i>Sphingomonadales</i>	9828	0.00	0.00	0.00	0.00	18.30	0.09	15.66	0.16	0.00
		<i>Rhodospirillaceae</i>	4701	0.00	0.01	0.00	0.00	0.05	1.40	0.03	10.73	0.00
		<i>Rhodobacter</i>	1651	9.35	0.05	0.00	0.05	1.10	0.01	0.00	0.00	0.00
		<i>Rhodobacter</i>	4377	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.55	0.00
	Beta	<i>Limnohabitans</i>	4552	0.01	0.00	0.00	0.00	7.03	3.00	0.04	0.01	0.00
		<i>Polynucleobacter</i>	7333	0.00	0.00	0.00	0.00	0.33	0.40	31.16	0.11	0.00
		<i>Comamonadaceae</i>	14254	0.00	0.03	0.00	0.00	0.07	5.25	0.00	0.00	0.00
		<i>Rhodocyclaceae</i>	7090	0.00	0.00	0.00	0.00	0.00	0.01	0.00	35.63	0.00
		<i>Limnohabitans</i>	14286	0.00	0.00	0.00	0.00	0.04	0.01	0.03	10.73	0.00
	Gamma	<i>Photobacterium</i>	14212	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.90

3.3.7 Predominant OTUs of feed bacterial communities

Since only one feed sample was analyzed per sampling day, no statistical comparison between sampling days was performed. OTUs 5872 (genus *Corynebacterium*), 14212 (genus *Photobacterium*), 3747 (genus *Sporosarcina*), 2650 (genus *Psychrilyobacter*), 3532 (genus *Facklamia*) and 1521 (species *Anoxybacillus kestanbolensis*), were among the most abundant OTUs on both days (Table S3.1, Table 3.5).

3.3.8 Relationship between gut, water and feed bacterial communities

RELATE analysis revealed a strong correlation (Spearman rank correlation Rho 0.807, $P < 0.0001$) between bacterial communities in gut and water (Table S3.3). In other words, differences among water and gut samples followed similar patterns, even though the bacterial community composition in gut and water was different ($P < 0.001$). On day 7, Proteobacteria dominated the water of both systems. In RAS, Proteobacteria was also the dominant phylum on day 42, but Fusobacteria and Firmicutes, which were absent on day 7, appeared. In AS systems, Bacteroidetes and Armatimonadetes replaced a large fraction of the Proteobacteria by day 42. Feed-associated BC was dominated by Firmicutes and Proteobacteria, followed by Actinobacteria and Fusobacteria (Figure 3.2).

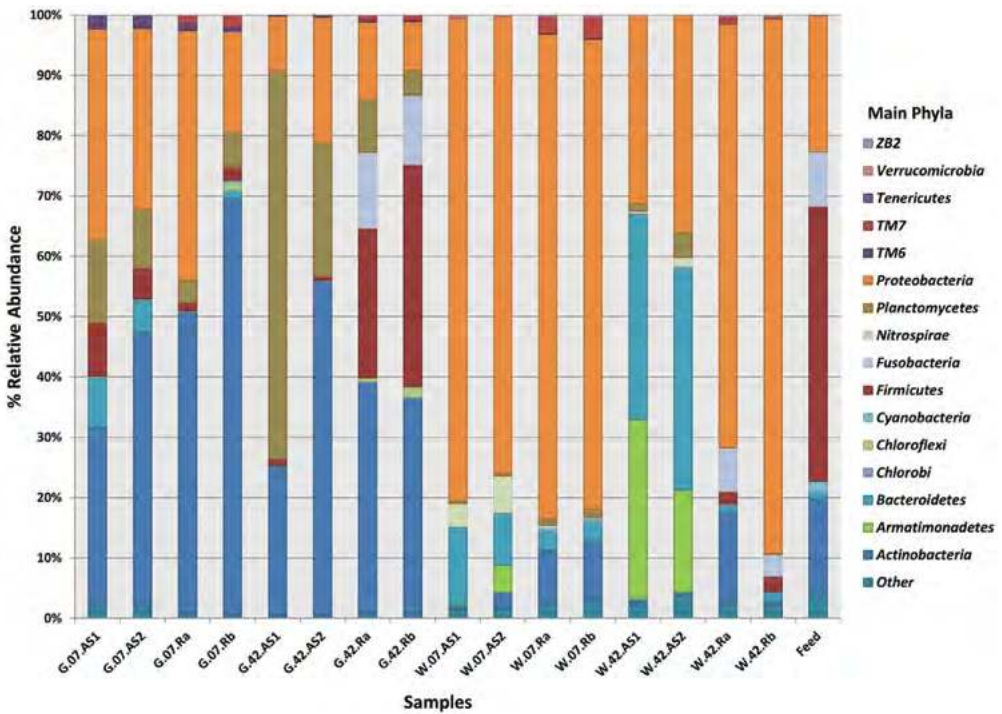


Figure 3.2. Cumulative bar charts of the main phyla present in either gut or water samples. Percentages show the relative abundance of each phylum in the gut or water of each replicate system on either day 7 or 42. Phyla in the feed represent the average values of feed samples from both day 7 and 42. AS1 & AS2: Replicate active suspension system 1 and 2, Ra & Rb: Recirculating aquaculture system a and b.

In spite of a high inter-sample correlation, average similarity between gut and water bacterial communities in AS was between 4% and 8% for both days (Table 3.2). This was mostly due to the low number of subdominant OTUs shared between gut and water (Figure 3.3). This low similarity between gut and water on day 7 was attributed to 41

shared OTUs which represented 50% of the cumulative relative abundance in gut and 29% in water. On day 42, 40 shared OTUs between gut and water had a cumulative relative abundance of 90% and 40%, respectively (Table 3.6).

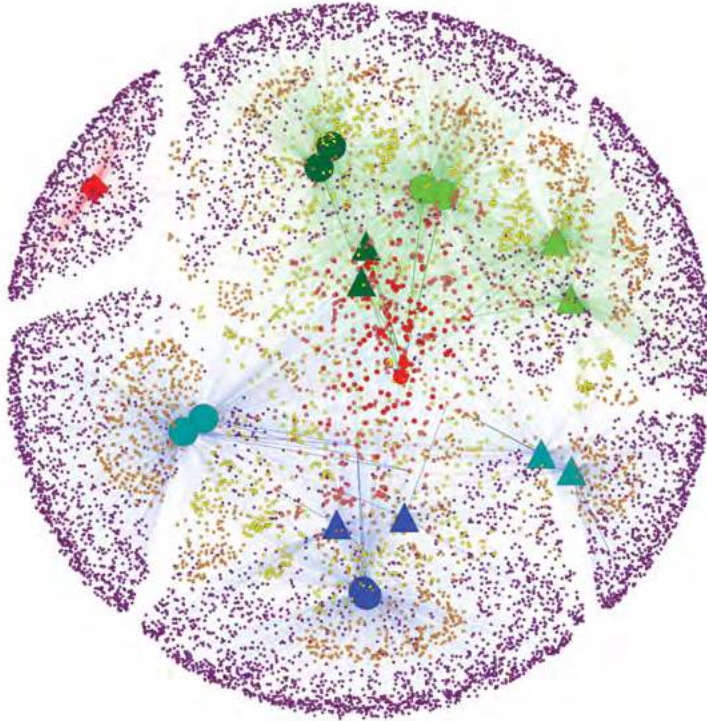


Figure 3.3. Network-based analyses of gut, water and feed bacterial communities. The network diagram is color-coded by: sample type (gut: green, water: blue and feed: red); day 07 (circles), day 42 (triangles) and feed which represents a pooled sample both from day 7 and 42 (square); system (gut AS: Active suspension system with two replicates: dark green; gut RAS: Recirculating aquaculture system with two replicates: light green; water AS: Active suspension system with two replicates: dark blue; water RAS: Recirculating aquaculture system with two replicates: light blue). Sample nodes are oversized to be distinguished from OTU nodes. Colour and size of OTU nodes represent assignments of OTUs to sample nodes based on the amount of samples that were shared with (small nodes are shared with less samples compared to larger ones); purple OTU nodes: OTUs present in only one sample; orange OTU nodes: OTUs shared between two samples; yellow OTU nodes: shared between 3 or 4 samples; pink OTU nodes: shared between 5 to 7 samples; red OTU nodes: shared between 8 to 16 samples). Thickness/brightness of edges connecting two nodes is proportional to the relative abundance of the specific OTU in the physical sample to which it connects.

In RAS, similarity between gut and water BC was 4 and 8% in the two days respectively (Table 3.2). Similarity on day 7 was attributed to 155 shared OTUs with a cumulative relative abundance of 83.5% and 20%, in gut and water respectively. On day 42, the 86 shared OTUs had a cumulative relative abundance of 81% and 12% (Table 3.6).

On the two days, gut shared with feed ten and five OTUs in AS and eight and three in RAS (Table 3.6). For both systems and days, the similarity between gut and feed was below 1% (Table 3.2). In AS, OTU 5872 was the only dominant OTU in feed encountered in the gut on day 7. Overall, only two OTUs were shared among gut, water and feed bacterial communities in AS or RAS on day 7, and only one OTU on day 42 (Table 3.6).

Table 3.6 Total number and percentage relative abundance of shared OTUs per system on each sampling day. Shaded relative abundance values correspond to shaded sampling group in the first column. Sample types are compared within system for each days. OTU: Operational taxonomic unit; AS & RAS: Active suspension and recirculating aquaculture systems; 07 & 42: Sampling days 7 and 42 respectively.

Group	Number and % abundance of shared OTUs										
	07_AS		07_RAS		42_AS		42_RAS				
Gut vs. Water	41		155		40		86				
	50.0	28.6	83.5	20.2	90.1	39.7	81.3	12.1			
Gut vs. Feed	10		8		5		3				
	0.2	18.6	1.1	2.5	0.1	1.1	0.0	0.8			
Water vs. Feed	3		3		3		3				
	3.2	0.1	2.4	0.1	0.3	0.1	0.2	0.1			
Gut vs. Water vs. Feed	2		2		1		1				
	0.1	2.2	0.1	0.9	0.2	0.1	0.0	0.2	0.1	0.0	0.1

3.4 Discussion

The higher levels of nitrate observed in RAS compared to AS could be due to the higher nitrification rates. In RAS, through the water re-use loop, settleable solids consisting mainly of organic matter, are removed before aerobic chemoautotrophic nitrifying bacteria convert ammonia via nitrite into nitrate in the biofilter (Crab et al., 2007; Gutierrez-Wing and Malone, 2006; Schreier et al., 2010). In AS, solids were not removed from the fish rearing unit, but maintained in suspension. The accumulating organic matter in AS was used as substrate for biofloc formation (Avnimelech, 2007; Azim and Little, 2008; De Schryver et al., 2008; Hargreaves, 2006). Heterotrophic bacteria in bioflocs immobilize $\text{NH}_3\text{-N}$, reducing the need for nitrification. Nitrification concurs with reduction of alkalinity. This explains why in AS the pH was higher than in RAS. However, the lower pH in RAS did not result in a higher CO_2 level, because the amount of free CO_2 in a system depends on the total amount of carbon dioxide/bicarbonate/carbonate (i.e. the total alkalinity) present (Ebeling et al., 2006).

In AS systems, a nitrification-denitrification coupling might have contributed to the observed lower $\text{NO}_3\text{-N}$ concentrations (da Silva et al., 2013; Ray et al., 2011). Nitrification occurs in the aerobic surface layer of bioflocs, whereas denitrification can take place in deeper anoxic layers (de Kreuk et al., 2005; Satoh et al., 2003; Third et al., 2003). The

high relative abundance of *Nitrospira* in water bacterial communities of AS on both days, suggests the occurrence of nitrification in those systems. *Nitrospira* is an aerobic, lithoautotrophic, nitrite-oxidizing bacterium that is commonly encountered in aquaculture systems or in freshwater activated sludge particles (Mußmann et al., 2013). However, this genus exhibits high substrate affinity and has been mainly reported in filter materials, bio-filters of freshwater, brackish and marine systems (Sugita et al., 2005; Kruse et al., 2013; Schreier et al., 2010; Itoi et al., 2007; Brown et al., 2013).

The second most abundant OTU (14254) in AS water of day 42 belonged to the Comamonadaceae family. Members of this family play a role in nitrate removal as well as in poly-hydroxy-alkanoate (PHA) metabolism. This indicates the metabolic and regulatory relationships between PHA degradation and denitrification by PHA-degrading denitrifiers in anoxic environments (Khan et al., 2002; Ginige et al., 2005; Sadaie et al., 2007; Adav et al., 2010a).

The $\text{PO}_4\text{-P}$ concentration in RAS has been higher than in AS. Microbial phosphate assimilation was suggested as the main cause of the lower phosphate levels in biofloc systems when compared to those in RAS (Brito et al., 2013; da Silva et al., 2013; Luo et al., 2014). The most predominant OTU in RAS water on day 42 was a member of family Rhodocyclaceae. This family contains mainly aerobic or denitrifying bacteria which live in aquatic habitats and exhibit very versatile metabolic capabilities. Rhodocyclaceae species are commonly reported in waste water where they play an important role in bioremediation (e.g. phosphate removal) (Quan et al., 2006; Fernández et al., 2009; Tsai et al., 2005; Ebrahimi et al., 2010; Adav et al., 2010b; Guo et al., 2011). Information at genus level on its role in phosphorus cycling would be needed to explain differences in abundance between AS and RAS.

DistLM analysis revealed a high correlation between water quality parameters and the bacterial communities present in the water. A high fraction of the total variation (68%) in water BC was explained by pH, $\text{NO}_3\text{-N}$, $\text{PO}_4\text{-P}$ and conductivity. Ebeling et al. 2006, described under which conditions autotrophic, heterotrophic, nitrifying or denitrifying processes dominate in aquatic systems (Ebeling et al., 2006). In fact, differences in water quality between RAS and AS were caused by differences in system design and management, which induced differences in composition and functionality of water BC and vice versa. Although the percentage of explained variation in water BC by environmental factors was high, it remains challenging to control bacterial communities composition, species dynamics and functionality because: (a) possibly other factors, presently not measured, are better “predictors” of the bacterial communities and (b) there is a high degree of stochasticity involved in how bacterial communities structures change over time, even in replicated systems (Giatsis et al., 2014). In addition, interpretation of the DISTLM results should be done with care, especially when sample size is low. Despite the fact that permutation

methods allowed us to reach the desirable significance level to test our hypothesis (i.e. correlation between water quality and water microbial communities), the high percentage of explained variation does not directly imply causality between the two datasets, but correlation. Future research, including more systems and data points should be performed in order to verify if the observed correlations are maintained.

The time* system interaction for water BC revealed very interesting trends. First, the similarity of water BC between replicate AS tanks decreased over time, whereas in RAS it remained stable. This suggests that sustainable “replication of water bacterial communities” is more feasible in RAS than in AS. Secondly, the average similarity of water BC between day 7 and 42 in AS was higher than in RAS. Although in RAS more OTUs were shared between days, water bacterial communities in AS remained five times more similar over time compared to that in RAS. This observation could be partly explained by some predominant OTUs in the water, which over time, were more robust in AS than in RAS. For example, *Sediminibacterium* (OTU 8066) in AS water was among the most predominant OTUs on both days. This genus contains two species exhibiting high versatility in types of used substrates including non-starch polysaccharides (NSPs), oligosaccharides and others. These species were found in lakes, freshwater reservoirs, activated sludge reactors, stream biofilms and nutrient rich environments (Kim et al., 2013; Kahlisch et al., 2012; Torrentó et al., 2011; Wang et al., 2012; Singleton et al., 2011; Khemkhao et al., 2011; Besemer et al., 2012).

Water BCs similarity between AS and RAS systems decreased considerably over time, as shown by the noticeable (from 247 to 90) decrease of the number of shared OTUs (Table 3.4). On day 7, the predominant water OTUs of either AS or RAS were found in both systems though in different relative abundances. On day 42, the predominant OTUs in water of AS systems were not encountered in RAS, and vice versa. Both systems were initially inoculated with water from the same source, and thus with a similar BC, which shows that the system design and function induced the development of a system-specific specialized bacterial community.

It is important at this point to remember that regarding water BCs in particular, the observations and patterns discovered between the two systems, or over time, are just a useful first starting point. This is firstly because in some cases sample size was low (especially for within system comparisons), though high enough to allow statistical power. Secondly, the environments in this study, although relevant to aquaculture are not natural since they operate under controlled conditions. This means that it is uncertain whether the observed patterns would occur in natural habitats or for example in an extensive outdoor aquaculture system (e.g. a fish pond). Nevertheless, this work can be the basis of future work addressing the implications that rearing systems have on water and fish gut BCs and provide awareness on system microbial management in aquaculture.

The described differences in composition of water BC between the two systems give rise to the question whether these differences had an impact on gut communities, and of course to which extent. The assumption has been that water provides the first microbial colonizers from the moment of mouth opening until first feeding, whereas feed microbiota would dominate during later development (Reitan et al., 1998; Ringo and Birkbeck, 1999; Hansen and Olafsen, 1999). Bakke et al. (2013), suggested that relatively small differences in water BC might impose significant differences in gut BC of fish larvae (Bakke et al., 2013). In line with this suggestion, our results showed a high and consistent correlation between the bacterial community composition in water and gut (Spearman's ρ : 0.807).

Although the similarity of water BC between replicate AS decreased over time, the average similarity of gut BC decreased little. Similarly in RAS, a significant decrease in average similarity of water BC over time was not observed in gut which remained eight times higher in gut than in water (Table 3.2). A considerable number of gut OTUs was shared between day 7 and 42. For example in RAS, OTU 1005 (genus *Rhodococcus*) was a predominant OTU in the gut on both days. The genus *Rhodococcus* has been reported in gut microbiota of sole, red rock fish, Norwegian mackerel, USA smelt, rainbow trout and shrimp (Kim et al., 2007; Sanchez et al., 2012; Spanggaard et al., 2000; Huber et al., 2004).

The relatively larger effect of time on water communities suggests that water BC were more sensitive to environmental changes than bacterial communities residing in the gut of fish larvae. To a certain degree, microbial community stability highly depends upon potential disturbances of the two ecosystems i.e. the gut and the water, as well as the ability of a community to respond resiliently. Preservation of homeostasis at the intestine should be in the gut microbiota's best interest, in order to provide a convenient long-term habitat. In favor of both, host also contributes to a fairly stable environment leading to a homeostatic interaction between host and gut microbiota. However, during early fish development, rapid anatomical, physiological and immunological changes occur in the gut, which makes it plausible to think that equally substantial changes should have also occurred in the gut communities. Fernandez et. al (2000), evaluated the behavior of different microbial communities under perturbed conditions and suggested that stability of the functionality does not necessarily imply stability of community structures. This means that in our study, observed changes in the gut community structure over time, could have occurred side-by-side with changes in community functionality. In the same line of thought, observed changes of water physicochemical properties might have been responsible for the relative change of water BCs over time, but whether the latter compromised functional stability of the community, is not known. Further research is required to elucidate the relationship between microbial community structure (i.e. composition, diversity) and its ability to organize in a way that assures the ability of counteracting the stress effects, by maintaining functionality.

Regarding predominant species, an interesting system * day interaction was observed in the gut communities. On day 7, the number of observed gut species in the AS systems was higher than in RAS. This higher richness coincided with higher gut community evenness i.e. higher biodiversity. On day 42 a considerable decrease both in the community richness and evenness was observed in AS. The latter means that a smaller fraction of the different species was present in dominant numbers whereas this was not so evident for fish reared in the RAS systems. Based on our data, it is not possible to clarify with certainty the reason for the observed differences between the two systems. Nevertheless, the fact that similar temporal changes in community biodiversity were also observed in water BCs might point out the role of water as a microbial reservoir available to the fish gut. However, more studies would be necessary to test how changes in community structure of a habitat impact gut microbial biodiversity of the organisms living therein and whether such changes affect the functionality of the gut community.

Similarity of gut bacterial communities between AS and RAS was overall low and further decreased over time. On day 7, OTU 1651 (genus *Rhodobacter*) was a predominant OTUs in the gut of AS, but absent in RAS. The genus *Rhodobacter* contains Gram-negative bacteria widely distributed in fresh water as well as marine and hypersaline habitats (Table S3.4, Figure S3.3). *Rhodobacter* species have been reported in the gut of grass carp, rainbow trout, Siberian sturgeon, catfish, Atlantic cod and Pacific white shrimp (Wu et al., 2012b; Wong et al., 2013; Mansfield et al., 2010; Geraylou et al., 2013; Di Maiuta et al., 2013; Fjellheim et al., 2011; Zhang et al., 2014), as well as in the water of aquaculture systems (Tang et al., 2014; Rurangwa and Verdegem, 2014).

A similar pattern was observed for the gut BC in RAS. OTU 7966, (family Peptostreptococcaceae) was predominant in the gut on day 42, absent on day 7, and present at very low relative abundance levels in AS. This suggests that both systems and developmental stage of the fish influence the presence and abundance of this OTU. Peptostreptococcaceae is a family of obligate anaerobic bacteria (Ringø et al., 1995). In salmon, Peptostreptococcaceae was detected as a member of both the allochthonous and autochthonous microbiota regardless of the fish diet, indicating that Peptostreptococcaceae might be a natural part of the intestinal microbiota of this species (Hartviksen et al., 2014). The family has been also reported in the gut microbiota of yellow catfish (Wu et al., 2012a, 2010) and common carp (van Kessel et al., 2011).

In this study, out of 11,658 OTUs, two OTUs were detected in all gut and water samples. Of these, *Mycobacterium llatzerense*, a member of the phylum Actinobacteria, was present at high relative abundance in all gut samples, and less abundant in water samples. The second OTU, a member of the Bradyrhizobiaceae, was among the predominant gut OTUs on day 7 for both systems, less abundant on day 42, and also present in all water samples. Whether its presence in the water was due to fish defecation or whether it originated from water and managed to proliferate in the fish gut is presently not known.

M. llatzerense, has not been previously reported in aquaculture systems but has been reported in hemodialysis water, and drinking water production and distribution systems (Dubrou et al., 2013). The association of *M. llatzerense* with groundwater (Table S3.4, Figure S3.3) may reflect a more psychrophilic profile of this species compared to other Mycobacteria, and might justify its presence in our systems which rely on well water. Although *M. llatzerense* has not been previously reported in fish gut microbiota, the high and consistent abundance in all guts suggests the preference of these bacteria for the gut environment of tilapia larvae. *M. llatzerense* is facultative autotrophic, aerobic, non-fermentative, and involved in hydrogen oxidation and alkaline dephosphorylation (Gomila et al., 2008).

Members of the Bradyrhizobiaceae family have been found in sewage treatment plants (Zhang et al., 2012), municipal wastewater treatment plants (Ye and Zhang, 2013), active granular activated carbon filters and drinking water distribution systems (Table S3.4, Figure S3.3) (Liu et al., 2012b). This is in agreement with the presence of Bradyrhizobiaceae in all water samples of this study. In fish, the Bradyrhizobiaceae family was overrepresented in the gut microbiota of sea bass (Carda-Diéguez et al., 2014), and was also reported in the stomach of yellow catfish (Wu et al., 2012a). Since the family contains more than ten different genera, it is difficult to speculate about the role and functionality of these microorganisms in the fish gut.

More information and relevant literature regarding major functions and processes as well as environment preference of the encountered OTUs are provided in Table S3.4 of the supplementary material.

Interestingly, the OTUs shared between gut and water had a higher relative abundance in the gut than in the water. In turn, almost none of the dominant OTUs in water were detected in the gut. This could suggest that host-specificity for particular microbial species is modulated by selective pressures within the host gut attributed to gut habitat (i.e. physiology, anatomy) and host's genotype (Yan et al., 2012; Rawls et al., 2006; Navarrete et al., 2012). On the other hand in water, low abundance of the predominant gut OTUs can be partly explained by fish defecation. Apparently, conditions in water are suboptimal for the growth of defecated bacteria mostly due to the ecological preference of the latter for the gut habitat (i.e. pH, anoxic conditions, etc.), adhesion sites and nutrients availability therein. Despite the significant effort in defining the forces which impact the assembly of gut microbial communities, the underlying mechanisms attributed to the impact of the environment over the host selectivity are still poorly understood.

Feed predominant bacteria were mostly absent in water and gut. Only 1% of gut OTUs was shared with feed at very low relative abundances. It is important at this point to mention that these results do not underestimate the importance of feed per se on gut microbial communities. Feed composition, nutrients, production technology, etc. have been all found to affect gut microbiota in mammals (Scott et al., 2011; Flint et al., 2007; Walker et al.,

2011; Turnbaugh et al., 2009b; Muegge et al., 2011; Delsuc et al., 2014) and fish (Merrifield et al., 2011; Ingerslev et al., 2014; Meziti et al., 2012; Hartviksen et al., 2014). This is not the case in our study where the same feed was used in all treatments. We put forward that the low number of subdominant bacteria shared between the feed and the gut indicates the low influence that feed communities had in the gut compared with those present in the water. Bakke et al. 2013, working with cod larvae, suggested that microbiota in water had a stronger influence than microbiota in the feed on the microbial community composition in the gut (Bakke et al., 2013). This was clearly pictured in our OTU network (Figure 3.3) where only a few OTUs present in feed were also present in water or gut samples. *Corynebacterium* (OTU 5873) was an exception since it was the predominant OTU in feed but also present in the gut of fish larvae in AS on day 7. *Corynebacteria* are found in a broad variety of habitats such as soil, plants and food products (Yassin et al., 2003).

Shared and unique OTUs between gut, water & feed microbiota

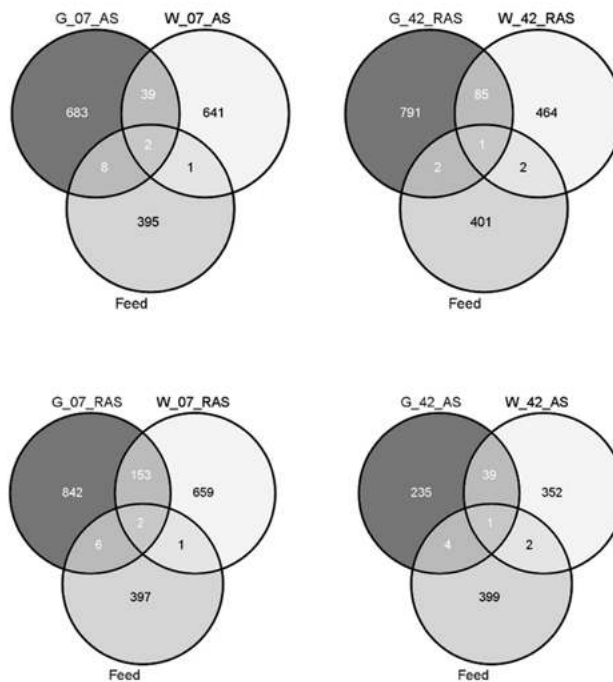


Figure 3.4. Venn diagrams of bacterial communities between sample types. Number of reads in all samples was normalized to the minimum number of reads found in one of the samples (1719 reads). The Venn diagram illustrates overlap of bacterial OTUs between gut, water and feed samples for two different systems over time. Numbers inside circles indicate the number of unique OTUs in one of the subsets based on absence/presence data from replicate systems. Numbers of unique OTUs indicate those OTUs that were present in both replicate systems and in at least one of the samples within each replicate system. Numbers in the overlapping regions indicate the number of shared OTUs between subsets. AS & RAS: active suspension and recirculating aquaculture systems; 07 and 42: Sampling day 7 and 42 respectively.

The low average similarity and the small number of OTUs shared between gut, water and feed (Table 3.2, Figure 3.4) suggest that the microbial community in the gut is not a simple reflection of the microbes from the environment. In our study, bacterial communities in rearing water influenced those present in the gut; nevertheless they only had a limited effect on the predominant gut species. Factors affecting community development include: suitability of adhesion sites and substrates availability, host physiology, gut anatomy and abiotic components in different sections of the gut, as well as the host's non-specific and specific immunity (Clements et al., 2014; Hansen and Olafsen, 1999). Thus, the resulting gut microbial community can be very different from the microbiota in water or feed. Sullam et al. (2012) stated that fish hindgut microbial communities resemble much more those of mammals, than those present in water or feed. Our study confirms that fish, like other vertebrates, harbor specialized gastrointestinal communities which are a skewed selection of microbes present in water and feed.

3.5 Conclusion

The culture system strongly affected the composition and development of bacterial communities in water. Despite low similarities between gut and water, observed changes in water bacterial communities were highly correlated with changes in the gut. However, there is no clear evidence that bacteria in the water from one system had a larger impact on gut communities than the other. The potential role of the few shared or discriminant species explaining a fraction of the observed similarity was also evaluated. Most of the predominant or shared OTUs in the fish gut have also been found in other fish species, which suggests that these microorganisms fulfil similar roles in the gut of different species. By studying the roles and functionalities of the shared species, novel insights on their niche and ecological adaptability of microbiota in water and gut, and to a lesser extent feed, will be generated. A better understanding of how microbiota in water, feed and gut interact will help improving the design, microbial management and nutrient cycling of fish rearing systems. Future research should focus on disentangling the key processes governing selection and propagation of water and feed microbiota, which ultimately become important species of the microbial community in the adult fish gut.

Acknowledgements

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Supplementary material

Table S3.1. Similarity percentages (SIMPER) indicating the most abundant OTUs contributing to the discrimination between groups, or to the relatedness of samples within a group. Average similarity: Overall Bray Curtis similarity among samples within the same group; Average dissimilarity: Overall Bray Curtis dissimilarity between samples of the two groups in comparison; Av. Abundance: Average relative abundance of an OTU within a group; Av. Sim.: Contribution of an OTU to the average group similarity; Av. Diss.: Contribution of an OTU to the average dissimilarity between the two groups in comparison; Sim/SD: Ratio of the average contribution of an OTU to the average similarity within the group, divided by the SD (in the case of AS water microbiota, # indicates that no value was calculated due to the lack of replication and estimation of the SD) ; Diss./SD: Ratio of the average contribution of an OTU to the average dissimilarity within the group, divided by the SD (low SD suggests consistency of a discriminant OTU); Contrib%: Percentage contribution of an OTU to the similarity within a group or to the dissimilarity between groups in comparison; Cum.%: Cumulative percentage contribution of OTU's based on column Contrib%; OTU: Operational taxonomic unit No; AS & RAS: Active suspension and recirculating aquaculture systems respectively; 7 and 42: Sampling day 7 and 42.

Type: Gut, Day: 7, System: AS						
Average similarity: 68.91						
Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Illatzerense	19.37	13.68	1.83	19.85	19.85	4790
Bacteria Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter	9.35	7.01	3.97	10.17	30.02	1651
Bacteria Actinobacteria Actinobacteria Actinomycetales Gordoniaceae Gordonia	5.94	4.68	6.67	6.79	36.81	10562
Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	3.78	3.22	4.68	4.68	41.49	10599
Bacteria Bacteroidetes Cytophagia Cytophagales Cytophagaceae Sporocytophaga	3.8	2.97	3.87	4.3	45.79	8299
Bacteria Planctomycetes Planctomycetia Pirellulales Pirellulaceae A17	3.3	2.79	4.85	4.04	49.84	8792
Bacteria Planctomycetes Planctomycetia Gemmatales Isosphaeraceae	3.27	2.46	2.81	3.57	53.41	4563
Bacteria Proteobacteria Alphaproteobacteria Rhizobiales	2.54	2.11	5.9	3.06	56.47	14302

Type: Gut, Day: 7, System: RAS

Average similarity: 55.06

Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	18.92	11.59	1.29	21.06	21.06	10599
Bacteria Actinobacteria Actinobacteria Actinomycetales Nocardiaceae Rhodococcus	15.17	9.28	1.37	16.86	37.91	1005
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilatzerense	11.43	9.02	3.02	16.38	54.3	4790
Bacteria Actinobacteria Actinobacteria Actinomycetales Microbacteriaceae Agrococcus	6.8	4.66	2	8.47	62.77	12555

Type: Gut, Day: 7, System: AS vs. RAS

Average dissimilarity = 78.38

Taxonomic ID	Group AS	Group RAS	Av.Diss	Diss/SD	Contrib%	Cum.%	OTU
	Av.Abund	Av.Abund					
Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	3.78	18.92	7.58	1.23	9.67	9.67	10599
Bacteria Actinobacteria Actinobacteria Actinomycetales Nocardiaceae Rhodococcus	0.44	15.17	7.36	1.52	9.39	19.07	1005
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilatzerense	19.37	11.43	5.14	1.35	6.56	25.63	4790
Bacteria Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter	9.35	0	4.68	2.51	5.96	31.59	1651
Bacteria Actinobacteria Actinobacteria Actinomycetales Microbacteriaceae Agrococcus	0.01	6.8	3.4	1.9	4.33	35.93	12555
Bacteria Actinobacteria Actinobacteria Actinomycetales Gordoniaceae Gordonia	5.94	2.36	3.27	1.59	4.17	40.1	10562
Bacteria Actinobacteria Thermoleophilia Solirubrobacterales	0	5.79	2.89	2.72	3.69	43.79	3934

Type: Water, Day: 7, System: AS

Average similarity: 63.75

Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales	18.3	14.75	#####	23.13	23.13	9828
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Limnohabitans	7.03	5.81	#####	9.12	32.25	4552
Bacteria Bacteroidetes Saprospirae Saprospirales Chitinophagaceae Sediminibacterium	4.29	4.1	#####	6.43	38.67	8066
Bacteria Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter	5	3.81	#####	5.98	44.66	2756
Bacteria Nitrospirae Nitrospira Nitrospirales Nitrospiraceae Nitrospira	4.1	2.96	#####	4.64	49.3	11945

Type: Water, Day: 7, System: RAS

Average similarity: 58.96

Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Oxalobacteraceae Polynucleobacter	31.16	24.13	3.1	40.92	40.92	7333
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales	15.66	9.97	1.67	16.91	57.83	9828

Type: Water, Day: 7, System: AS vs. RAS

Average dissimilarity = 76.04

Taxonomic ID	Group AS		Group RAS		Contrib%	Cum.%	OTU
	Av.Abund	Av.Abund	Av.Diss	Diss/SD			
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Oxalobacteraceae Polynucleobacter	0.33	31.16	15.42	2.94	20.28	20.28	7333
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales	18.3	15.66	3.96	1.61	5.21	25.49	9828
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales	7.03	0.04	3.49	5.37	4.6	30.08	4552
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales	1	5.25	2.49	1.09	3.28	33.36	10813
Bacteria Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter	5	0.11	2.44	3.86	3.21	36.57	2756

Bacteria Bacteroidetes Saprospirae Saprospirales Chitinophagaceae Sediminibacterium	4.29	0.01	2.14	20.93	2.81	39.38	8066
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales	4.22	0	2.11	2.55	2.77	42.15	4582
Bacteria Nitrospirae Nitrospira Nitrospirales Nitrospiraceae Nitrospira	4.1	0.18	1.96	3.19	2.58	44.73	11945
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilutzerense	1.23	3.39	1.4	1.31	1.85	46.58	4790
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilutzerense	0.01	2.72	1.36	0.94	1.79	48.36	9738
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae	2.4	0.29	1.17	1.03	1.54	49.9	12945
Bacteria Armatimonadetes Armatimonadia Armatimonadales Armatimonadaceae Armatimonas	2.09	0	1.05	1.02	1.38	51.28	7261
Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae Novosphingobium	0.78	2.6	0.92	0.99	1.21	52.49	6299

Type: Gut, Day: 42, System: AS

Average similarity: 59.85

Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Planctomycetes Planctomycetia Gemmatales Isosphaeraceae	40.76	27.08	1.56	45.25	45.25	4563
Bacteria Actinobacteria Actinobacteria Actinomycetales Micrococcaceae Arthrobacter	19.29	14.57	3.77	24.34	69.59	12537
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilutzerense	16.17	8.74	0.97	14.6	84.19	4790

Type: Gut, Day: 42, System: RAS

Average similarity: 56.35

Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Firmicutes Clostridia Clostridiales Peptostreptococcaceae	18.13	13.02	2.8	23.11	23.11	7966
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilutzerense	12.06	9.44	2.99	16.75	39.86	4790
Bacteria Firmicutes Clostridia Clostridiales [Mogibacteriaceae]	8.4	6.16	2.21	10.93	50.79	7955
Bacteria Actinobacteria Actinobacteria Actinomycetales Nocardiaceae Rhodococcus	9.21	5.74	1.73	10.18	60.96	1005
Bacteria Fusobacteria Fusobacteria Fusobacteriales Fusobacteriaceae Cetobacterium somerae	11.73	4.31	0.75	7.64	68.61	2787

Type: Gut, Day: 42, System: AS vs. RAS							
Average dissimilarity = 89.22							
Taxonomic ID	Group AS	Group RAS	Av.Diss	Diss/SD	Contrib%	Cum. %	OTU
	Av.Abund	Av.Abund					
Bacteria Planctomycetes Planctomycetia Gemmatales Isosphaeraceae	40.76	0	20.38	1.91	22.84	22.84	4563
Bacteria Actinobacteria Actinobacteria Actinomycetales Micrococcaceae Arthrobacter	19.29	0.01	9.64	2.57	10.8	33.64	12537
Bacteria Firmicutes Clostridia Clostridiales Peptostreptococcaceae	0.04	18.13	9.04	2.01	10.14	43.78	7966
Bacteria Fusobacteria Fusobacteria Fusobacteriales Fusobacteriaceae Cetobacterium somerae	0	11.73	5.86	0.85	6.57	50.35	2787
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium Ilutzerense	16.17	12.06	5.78	1.87	6.48	56.83	4790
Bacteria Actinobacteria Actinobacteria Actinomycetales Nocardiaceae Rhodococcus	0	9.21	4.6	1.56	5.16	61.99	1005
Bacteria Firmicutes Clostridia Clostridiales Mogibacteriaceae	0.01	8.4	4.2	2.3	4.7	66.69	7955
Bacteria Proteobacteria Gammaproteobacteria Legionellales Legionellaceae Tatlockia	6.02	0	3.01	0.78	3.37	70.07	2401
Bacteria Proteobacteria Gammaproteobacteria Enterobacteriales Enterobacteriaceae Plesiomonas shigelloides	1.77	2.63	1.53	1.07	1.72	71.79	12852
Bacteria Actinobacteria Actinobacteria Actinomycetales Mycobacteriaceae Mycobacterium celatum	0.01	3.04	1.51	0.99	1.7	73.48	12238
Bacteria Actinobacteria Thermoleophilia Solirubrobacteriales	0	2.73	1.37	1.15	1.53	75.01	3934
Type: Water, Day: 42, System: AS							
Average similarity: 25.82							
Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum. %	OTU	
Bacteria Bacteroidetes Saprospirae Saprospirales Chitinophagaceae Sediminibacterium	20.31	11.43	#####	44.28	44.28	8066	
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae	5.25	2.9	#####	11.24	55.52	14254	
Bacteria Proteobacteria Alphaproteobacteria Rhodospirillales Rhodospirillaceae	1.4	1.19	#####	4.6	60.12	4701	
Bacteria Bacteroidetes Sphingobacteria Sphingobacteriales	7.02	0.93	#####	3.58	63.7	4686	

Type: Water, Day: 42, System: RAS

Average similarity: 60.79

Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Proteobacteria Betaproteobacteria Rhodocyclales Rhodocyclaceae	35.64	30.69	3.97	50.48	50.48	7090
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Limnohabitans	10.73	6.8	4.31	11.18	61.66	14286
Bacteria Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter	12.56	3.32	0.41	5.47	67.13	4377

Type: Water, Day: 42, System: AS vs. RAS

Average dissimilarity = 98.24

Taxonomic ID	Group AS	Group RAS	Av.Diss	Diss/SD	Contrib%	Cum.%	OTU
	Av.Abund	Av.Abund					
Bacteria Proteobacteria Betaproteobacteria Rhodocyclales Rhodocyclaceae	0.01	35.64	17.82	4.63	18.14	18.14	7090
Bacteria Bacteroidetes Saprospirae Saprospirales Chitinophagaceae Sediminibacterium	20.31	0.07	10.12	2.13	10.3	28.43	8066
Bacteria Armatimonadetes Fimbriimonadia Fimbriimonadales Fimbriimonadaceae Fimbriimonas	14.54	0	7.27	0.94	7.4	35.84	5688
Bacteria Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter	0	12.56	6.28	0.9	6.39	42.23	4377
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Limnohabitans	0.01	10.73	5.36	1.71	5.46	47.68	14286
Bacteria Armatimonadetes Armatimonadia Armatimonadales Armatimonadaceae Armatimonas	8.11	0	4.05	0.94	4.13	51.81	7261
Bacteria Bacteroidetes Sphingobacteria Sphingobacteriales	7.02	0	3.51	1.08	3.57	55.39	4686
Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae	5.25	0	2.63	2.09	2.67	58.06	14254
Bacteria Fusobacteria Fusobacteria Fusobacteriales Fusobacteriaceae Cetobacterium somerae	0	5.02	2.51	1.58	2.55	60.61	2787
Bacteria Actinobacteria Actinobacteria Actinomycetales ACK-M1	0	4.96	2.48	0.9	2.52	63.14	5270

Type: Feed						
Average similarity: 59.36						
Taxonomic ID	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%	OTU
Bacteria Proteobacteria Gammaproteobacteria Vibrionales Vibrionaceae Photobacterium	10.9	9.94	#####	16.75	16.75	14212
Bacteria Fusobacteria Fusobacteria Fusobacteriales Fusobacteriaceae Psychrilyobacter	7.11	6.78	#####	11.42	28.16	2650
Bacteria Firmicutes Bacilli Lactobacillales Aerococcaceae Facklamia	3.94	3.83	#####	6.46	34.62	3532
Bacteria Firmicutes Bacilli Bacillales Bacillaceae Anoxybacillus kestanbolensis	4.12	3.78	#####	6.37	40.99	1521
Bacteria Firmicutes Bacilli Bacillales Planococcaceae Sporosarcina	8.06	3.2	#####	5.39	46.38	3747
Bacteria Actinobacteria Actinobacteria Actinomycetales Corynebacteriaceae Corynebacterium	12.34	2.94	#####	4.95	51.32	5872

Table S3.2. Average alpha-diversity indices of gut, water and feed bacterial communities. Number of obtained sequences was standardized among samples to the minimum number of reads obtained in one of the samples ($n = 1719$). S: Richness (observed OTUs); J': Pielou's evenness ($J' = H' / \log(S)$); H': Shannon index ($H' = -\sum (P_i * \ln(P_i))$), based on the OTU level. AS & RAS: Active suspension and recirculating aquaculture systems; 07 & 42: Sampling days 7 and 42 respectively.

Sampling Group	S	J'	H'(ln)
Gut_07_AS	732	0.626	4.132
Gut_07_RAS	1003	0.532	3.674
Gut_42_AS	279	0.395	2.225
Gut_42_RAS	879	0.519	3.521
Water_07_AS	683	0.690	4.504
Water_07_RAS	815	0.540	3.618
Water_42_AS	394	0.601	3.594
Water_42_RAS	552	0.494	3.118
Feed	406	0.666	3.997

Table S3.3. RELATE analysis testing for matched resemblance matrices based on Spearman's rank correlation coefficient. Water and gut microbiota data sets were square root transformed and resemblance matrices were based on Bray Curtis similarity among samples. Rho statistic indicates the degree of "relatedness" between the two matrices with highly related matrices having Rho statistic closer to one. BCs: Bacterial communities.

Resemblance matrices	Water BCs Vs. Gut BCs
Rank correlation method	Spearman's ρ
Sample statistic (Rho)	0.807
Significance level of sample statistic	0.0001
Number of permutations	9999
Number of permuted statistics greater than or equal to Rho	0

Table S3.4. List of the most predominant gut, water and feed OTUs on day 7 and 42 as presented on the heatmap Table 3. Column "Processes" and "Environment" provide information about the major functions and processes as well as the environments in which the most predominant OTUs were encountered as predicted based on a literature review (column "References").

Taxonomy	OTU number	Processes	Environment	References
Actinobacteria Actinomycetales Mycobacteriaceae <i>Mycobacterium</i> <i>llatzerense</i>	4790	Facultative autotrophic, aerobic, hydrogen oxidation	Haemodialysis water; tap water production plant and distribution system	(Dubrou et al., 2013; Gomila et al., 2008)
Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae <i>Rhodobacter</i>	1651	Non-sulphur purple bacteria, photosynthesis, lithotrophs, aerobic and anaerobic respiration, N ₂ fixation	Hypersaline, saline and freshwater	(Bräuer et al., 2011; Glöckner et al., 1999; Hiraishi et al., 1995; Kawasaki et al., 1993; Stapleton et al., 2000; Yang et al., 2012)
Actinobacteria Actinobacteria Actinomycetales Gordoniaceae <i>Gordonia</i>	10562	High GC-content, Gram+ bacteria, degrade many xenobiotics, potentially pathogenic.	Soil and mangrove rhizospheres, waste water treatment plants, diseased humans, hydrocarbon polluted soil, oil wells	(Arenskötter et al., 2004)
Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	10599	Contains 10 genera, rhizobium associated and animal associated (e.g. cat scratch disease). Many free living in marine or fresh water, some with N ₂ -fixation.	Sewage treatment plants, municipal waste waters, drinking water distribution. In gut of sea bass and stomach of yellow catfish.	(Liu et al., 2012b; Wang et al., 2013; Yang et al., 2012; Ye and Zhang, 2013; Zhang et al., 2012)

Taxonomy	OTU number	Processes	Environment	References
Actinobacteria Actinobacteria Actinomycetales Nocardiaceae <i>Rhodococcus</i>	1005	Chemoheterotrophic, some members are facultative chemolithoautotrophs. Many processes. Some species used as immunostimulants, others for production of digestive enzymes.	Wide distribution, including soil, water and in cells. Reported in sole, red rock fish, Norwegian mackerel, USA smelt, rainbow trout and shrimp gut.	(Boutin et al., 2013a; Khan et al., 2011; Kim et al., 2007; Sanchez et al., 2012; Sharifuzzaman et al., 2014; Spanggaard et al., 2000; Tinh et al., 2007)
Proteobacteria Alphaproteobacteria Sphingomonadales	9828	No info about functional role. Sphingolipids in outer membrane of cell wall.	Drinking water systems, groundwater treatment biofilms.	(Holinger et al., 2014; Liu et al., 2012a, 2012b; Ultee et al., 2004; Vílchez et al., 2011)
Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae <i>Limnohabitans</i>	4552	Aerobic, facultative anaerobic, chemo-organotrophic. Affinity for substrates with organic acids, monosaccharides and amino acids.	Bacteria found in water column of freshwaters.	(Hahn et al., 2010; Jezbera et al., 2013; Kasalický et al., 2010; Šimek et al., 2013; Zeng et al., 2012; Kasalický et al., 2013)
Bacteroidetes Saprosirae Saprosirales Chitinophagaceae <i>Sediminibacterium</i>	8066	Versatile in types of substrates that are degraded. Aerobic or facultative anaerobic.	Lakes, freshwater reservoirs, activated sludge reactors, stream biofilms, soil	(Besemer et al., 2012; Khemkhaio et al., 2011; Kim et al., 2013; Qu and Yuan, 2008; Singleton et al., 2011; Torrentó et al., 2011)



Figure S3.1. Draftsman plots for the untransformed water quality parameters. Pairwise plots provide indication of data skewness or multi-collinearity among parameters for data transformation, or exclusion of parameters from the distance-based linear models analysis. TAN: Total ammonia nitrogen; $\text{NO}_2\text{-N}$: Nitrite nitrogen; $\text{NO}_3\text{-N}$: Nitrate nitrogen; $\text{PO}_4\text{-P}$: Phosphate phosphorous; CO_2 : Carbon dioxide; T: Temperature; DO: Dissolved oxygen; Cond.: Conductivity.

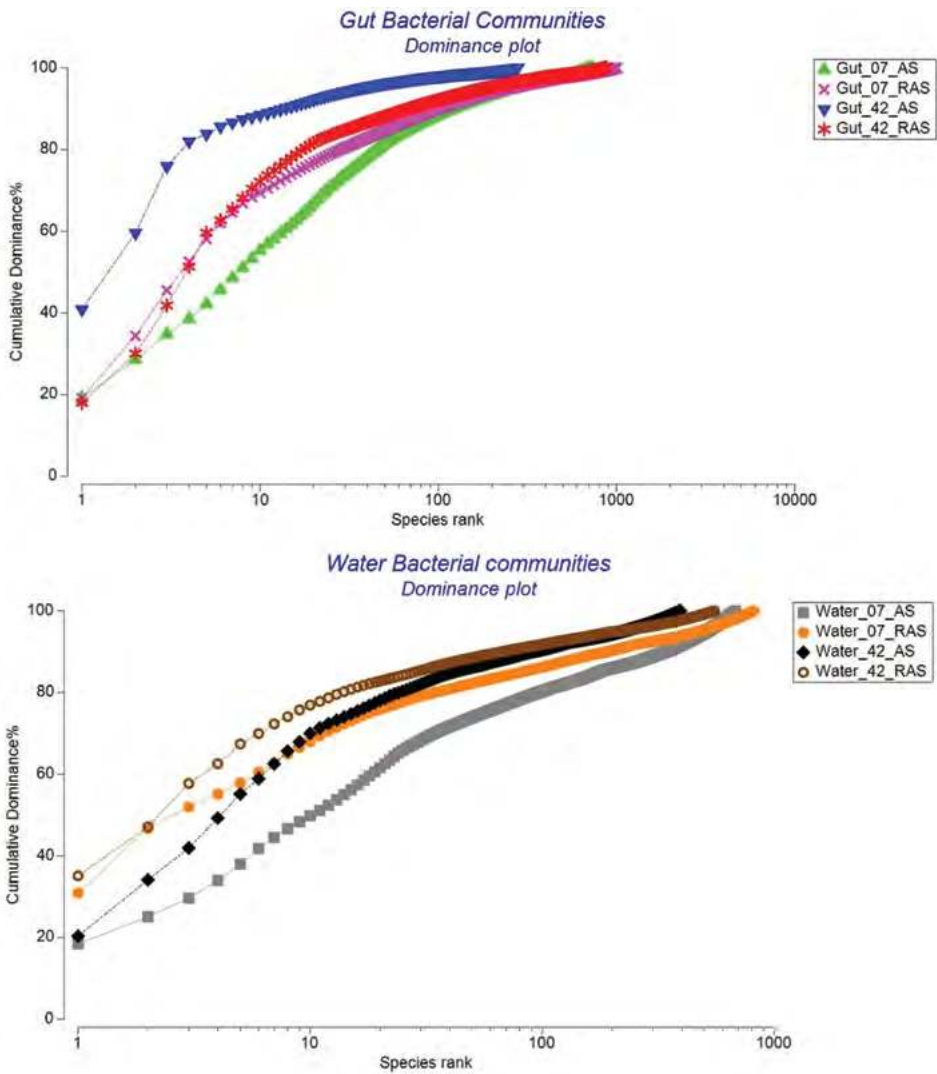


Figure S3.2. Dominance plots of gut and water OTUs from the two experimental days. Plots are based on average relative Bray Curtis OTU abundance from each system. The x-axis shows the log scale of the OTUs ranks in increasing order and the y-axis consists of the cumulative relative abundance of each OTU in each sample group. 07 and 42: sampling day 7 & 42; AS & RAS: Active suspension and recirculating aquaculture systems respectively.

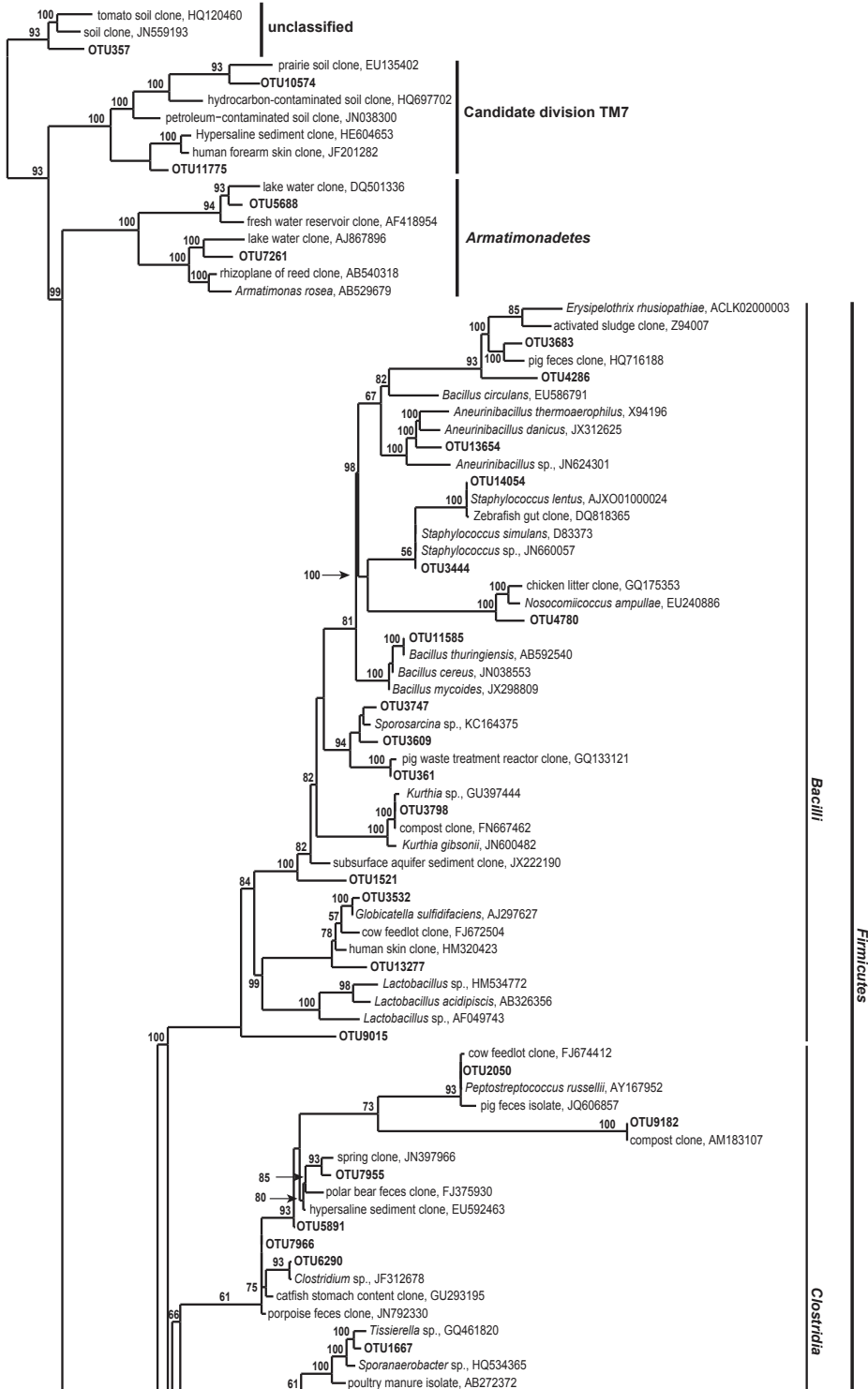


Figure S3.3.

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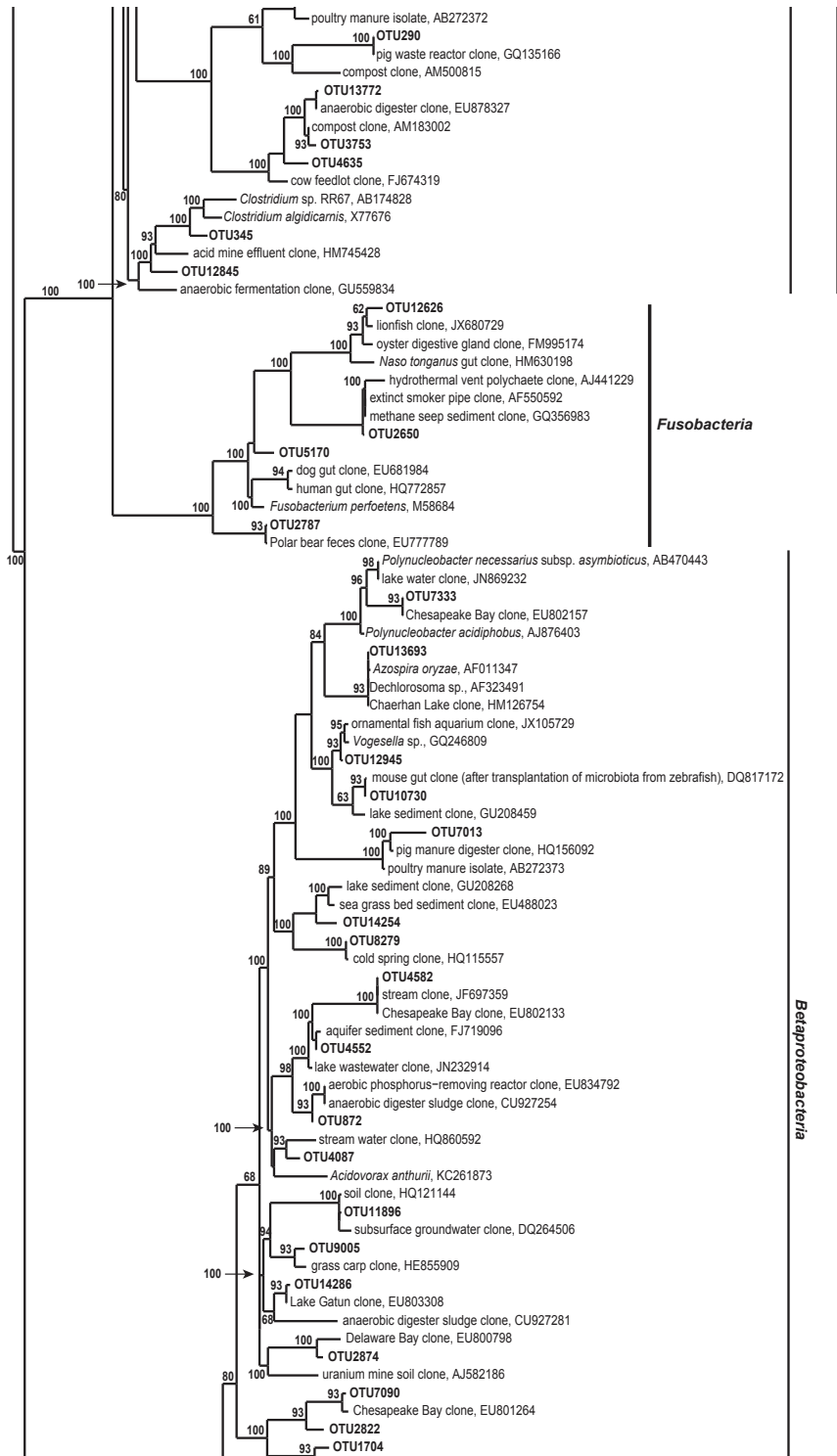


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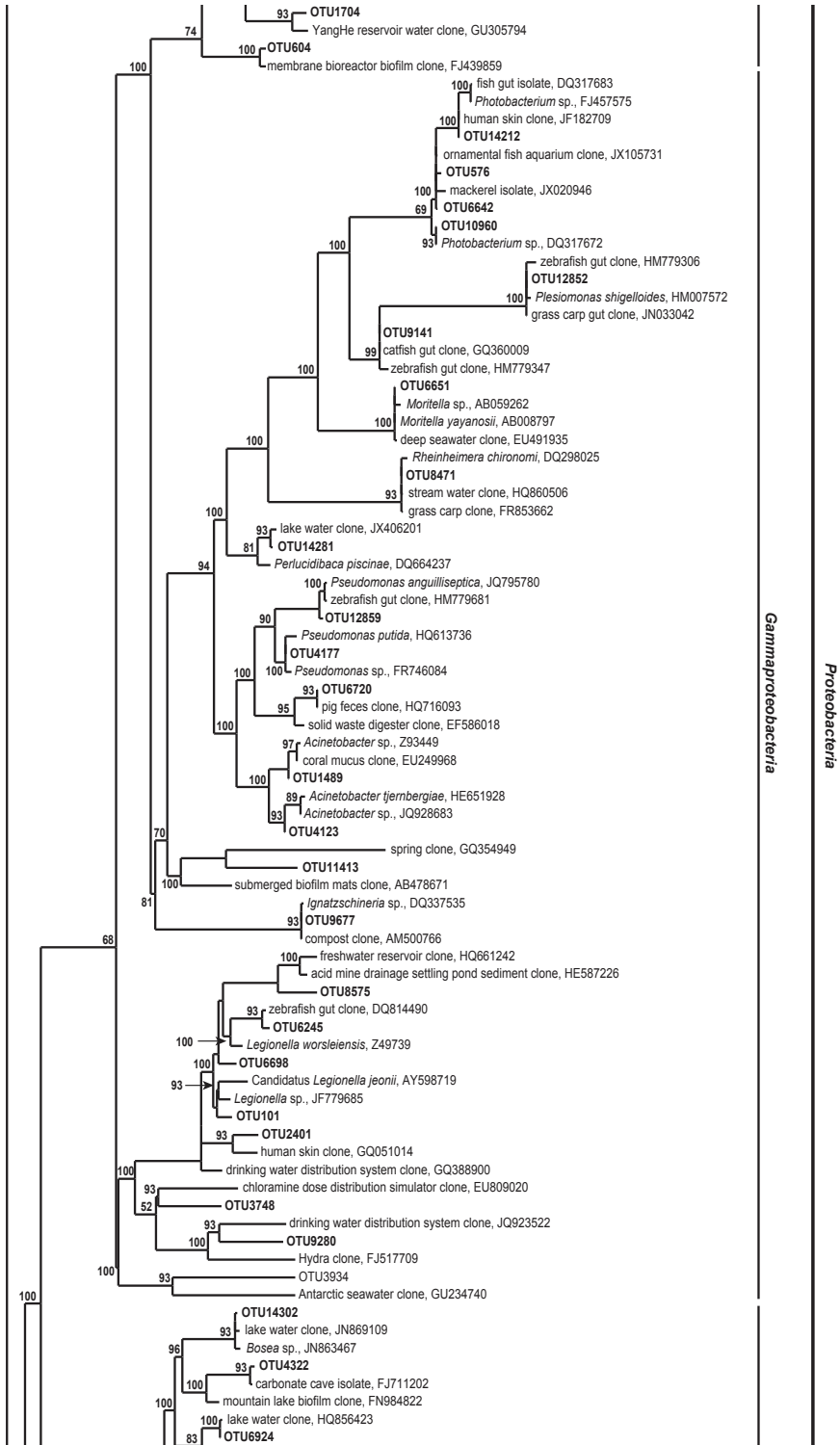


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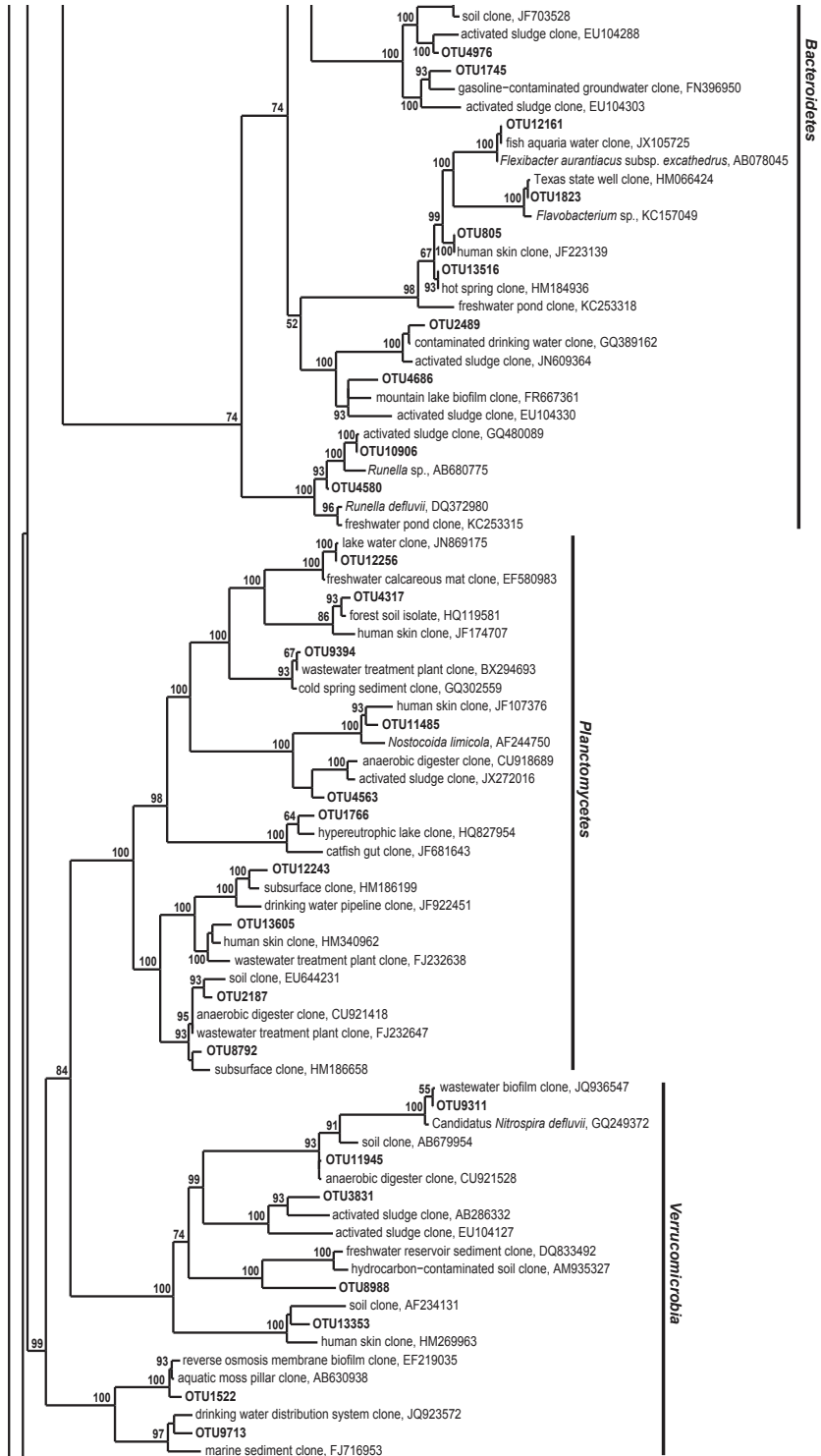


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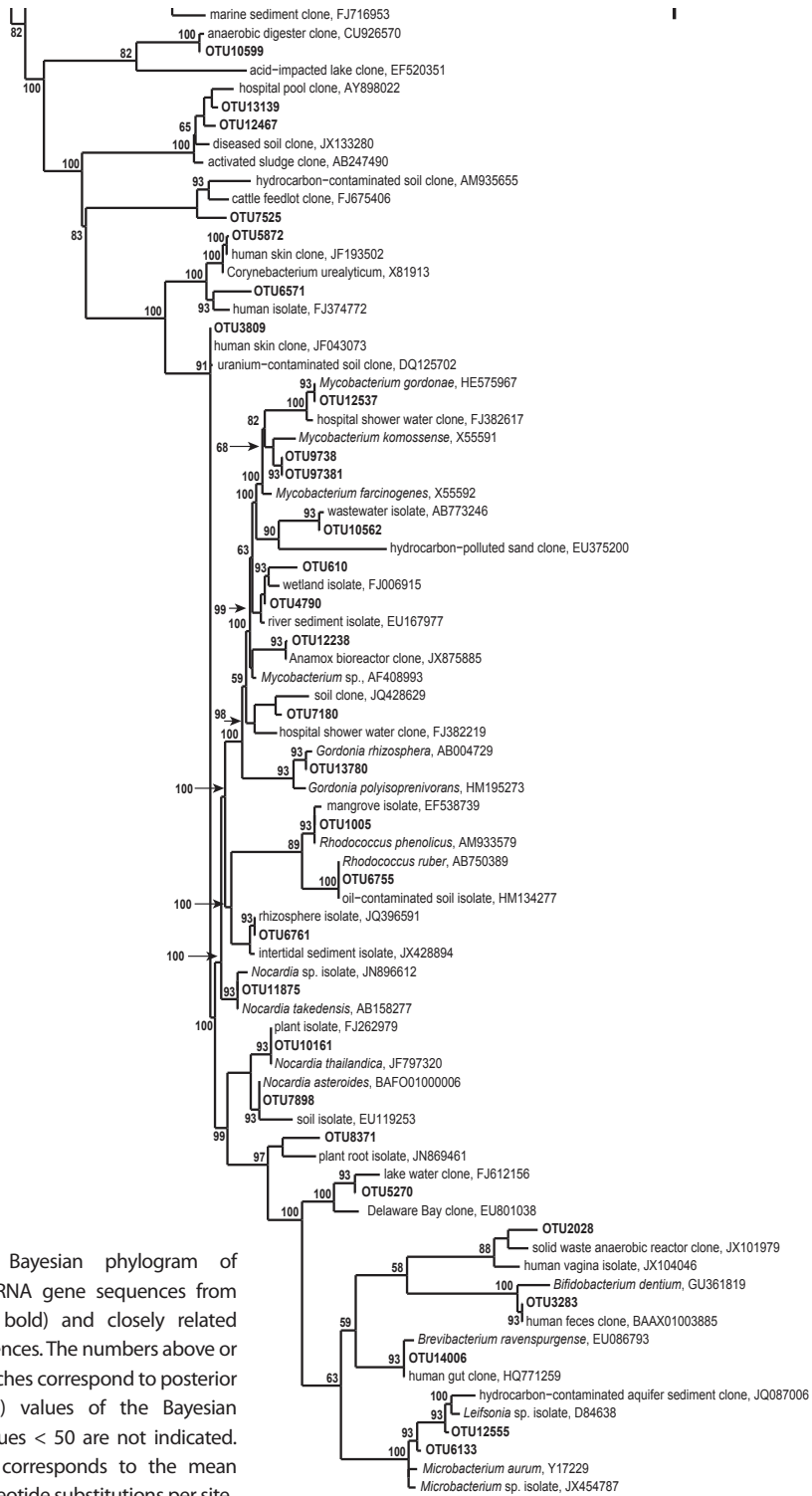


Figure S3.3. Bayesian phylogram of selected 16S rRNA gene sequences from this study (in bold) and closely related reference sequences. The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. PP values < 50 are not indicated. The scale bar corresponds to the mean number of nucleotide substitutions per site.

Chapter 4

Steering the gut microbiome of tilapia larvae with microbial diets



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Fish live and share their environment with microorganisms. The autochthonous microbiota of fish mostly originates from those present in their feed and water. However, the fish gut seems to be selective for the establishment of certain species, while at the same time being hostile to others. Despite the significant effort in defining the forces that impact the assembly of gut microbial communities, the underlying mechanisms attributed to the impact of the environment over the host selectivity are still poorly understood. In the present study the role of feed microbiota on gut communities of tilapia larvae was investigated. Fish were fed three experimental diets incorporated with sludge produced under aerobic, methanogenic or denitrifying conditions. We performed 16S rRNA gene sequence-based comparisons between gut microbial communities from different treatments and we associated them with the ones present in feed and water. Gut microbiota shared a much higher number of OTUs with microbiota in sludge-based feeds than with water, resulting in distinct gut communities between treatments. This finding implies that the tilapia gut microbiota has a certain plasticity, which makes it amenable to interventions. Nevertheless, in spite of observed changes in microbiota composition between treatments, a persistent core gut OTUs was maintained in all treatments.

4.1 Introduction

The establishment of the microbial community in the gut is an essential part of the development in humans and animals, including fish (O'Hara and Shanahan, 2006; Ley et al., 2008; Sommer and Bäckhed, 2013; Lepage et al., 2012). Fish live and share the environment with dense microbial communities. This close association in the aquatic environment causes externally exposed body surfaces of fish, including the gut, to be quickly colonized after hatching or mouth opening (Reitan et al., 1998; Verschuere et al., 2000; Hansen and Olafsen, 1999). The digestive tract provides different (micro) habitat niches, resulting in the colonization of diverse microbiota (Sommer and Bäckhed, 2013; Hansen and Olafsen, 1999).

The autochthonous microbiota of fish originates from the environment. As fish produce axenic eggs, fish larvae have their first contact with microbes right after hatching (Verschuere et al., 2000). Legacy effects attributed to environmental factors, such as maternal influences, are known to affect the establishment of the gut microbiota in fish (Keskin et al., 1994; Olafsen, 2001; Sullam et al., 2012). However, the gut environment is selective for the establishment of certain species from the environment and the forces governing host selection are poorly understood.

In fish larvae, the selection of certain microbes in the gut has been suggested to be host-dependant (Li et al., 2012b). For instance, difference in gut microbiota between fish species sharing the same environment might be related to differences in the anatomy, immunology, feeding behaviour and physiology. In fish, host-selective capabilities were revealed in axenic zebrafish (*Danio rerio*), which received a faecal transplant derived from mice. The implanted mouse community subsequently shifted towards a state resembling a native zebrafish community (Rawls et al., 2006). In addition, zebrafish originating from the wild shared a core gut microbiota with those reared in captivity, demonstrating a host-specific microbial community in the gut (Roeselers et al., 2011). Also Wong et al. (2013) found that rainbow trout (*O. mykiss*) raised at different densities and fed different diets shared a core gut microbiota (Wong et al., 2013).

In a recent study, Giatsis et al. (2015) showed that feed microbiota had a very low impact on gut microbial communities. However, in that study a commercial pelleted feed was used. This suggests that perhaps feed microbial composition and/or load was not appropriate for a successful establishment of feed bacteria in the gut. Therefore, in this study three different microbial diets were prepared based on 1% inclusion (on dry matter basis) of sludge derived from aerobic, methanogenic or denitrifying reactors. Nile tilapia larvae were fed with a regular diet (i.e. control) or with the sludge-based diets and the effect of diet microbiota on the gut communities was assessed. All larvae were reared within the same system and shared the same water source in order to avoid variation between treatments due to differences in water microbiota.

We hypothesized that if gut microbial composition is strongly shaped by selective pressures in the gut, then similar gut microbial communities should develop in all dietary treatments. Contrary, if the gut microbial community is strongly shaped by the composition of the microbial community present in the diet, this will result in a significantly different gut microbiota between treatments.

4.2 Materials and Methods

4.2.1 Ethics statement

The experiment was performed in accordance with relevant guidelines and approved by the Ethical Committee of Wageningen University for animal experiments (Project Name: Promicrobe; Registration code: 2012050b).

4.2.2 Sludge and experimental diets preparation

Three different types of sludge were produced in triplicate under aerobic, methanogenic and denitrifying conditions in customized reactors. Three sealable glass bottles (20 L volume) with integrated electric heaters (100 W) were filled with 10 L tap water for each treatment. Magnetic stir bars were placed inside the bottles (450 rpm) to ensure solids suspension. In the aerobic sludge treatment, an air diffuser (air supply 5 L h⁻¹) was placed to ensure oxygen-rich conditions. Both methanogenic and denitrifying reactors received N₂ gas (0.1 L h⁻¹). In the denitrifying reactors, NaNO₃ was added to maintain the nitrate concentration above 200 mg L⁻¹. The pH, temperature, redox potential and nitrate concentration were measured daily and concentration of total ammonia nitrogen (TAN), nitrite and dissolved oxygen (DO) were measured weekly with a pH/Oxi 340i electrode (WTW GmbH, Weilheim, Germany) and the Merck Nitrate, Ammonium and Nitrite Test, respectively.

The sludge reactors were inoculated daily with faecal matter originating from a Recirculating Aquaculture System (RAS). The faecal matter was produced in RAS stocked with adult Nile tilapia fed a commercial diet, harvested with a sedimentation cone and stored into a glass bottle kept in ice to minimize the bacterial breakdown of collected matter. Approximately 1350 mL of faeces were collected daily at 8:45 AM, left standing undisturbed for solids to settle, collected by siphoning, manually mixed for 30 s and then equally divided over the nine sludge-reactors. For the diets preparation, sludge from the nine reactors was sampled daily and sludge-based diets were freshly prepared as described below.

4.2.3 Sludge incorporated diet production

In total four experimental diets were tested. The three microbial diets consisted of three different types of sludge mixed with a commercial tilapia feed. The commercial feed was also used as the control treatment without sludge incorporation (Skretting Gemma Wean M0.5, 300-500 μm , 58% crude protein, 17% crude fat, 10% ash, 0.6% fibre, 1.3% phosphorus). The aerobic, methanogenic and denitrifying sludge was mixed with the commercial diet at 1% inclusion level on dry matter basis. The sludge suspension was first pipetted from the reactors and filtered through 0.2 μm (type GTTP) membrane filters (Millipore - Isopore™) by a vacuum pump for 1 minute. Subsequently, the retained sludge was collected and mixed with the commercial feed to create a solid paste. The control diet was the same commercial tilapia feed but instead of sludge, autoclaved water was used to form a paste of similar consistency as the sludge-based diets. After the preparation of each diet and the first feeding of the day, the remaining feed was sealed in an airtight bag (for the methanogenic and denitrifying diet, the oxic headspace was replaced by N_2 -gas) and stored at 5 °C until the next feeding of the day. The experimental diets were prepared on a daily basis.

4.2.4 Experimental animals and rearing conditions

A flow-through system (FTS) consisted of a buffer tank (200 L effective volume) and four holding tanks (i.e. four treatments) were used. In FTS, water flows continuously in the fish tanks and the outlet tank water is discarded without being recirculated. In such a way, cross transfer of feed particles, faecal matter and bacteria between treatments was avoided. No replicate FTS were used due to the focus of the experiment on the MC microbial communities composition in the gut of individual fish. Our previous research (Giatsis et al., 2014) revealed no differences in MC gut microbial communities composition between individuals reared in different tanks when the same water source was used.

One batch of full sibling Nile tilapia (*Oreochromis niloticus*) larvae was produced at the CARUS-Aquatic Research Facility (Wageningen UR). One hundred-5-days-post-fertilization (dpf) larvae were stocked in each tank. Feeding started at 9 dpf (referred as day 0) and was continued for 16 days. Larvae were fed four times a day to apparent satiation for 30 minutes at 9:00, 11:30, 14:00 and 16:30. Water temperature was set to 28°C, water exchange rate at 1 L h⁻¹ and aeration inside the tanks was supplied at 5 L h⁻¹. The photoperiod was set to 12 hours light/dark intervals.

Water quality parameters were maintained at safe levels for Nile tilapia larvae (pH 8.1-8.6, temperature 28°C, TAN<0.03 mg L⁻¹, NO₂-N<0.1 mg L⁻¹ and DO>6 mg L⁻¹). Temperature, dissolved oxygen and pH were measured daily with a pH/Oxi 340i electrode (WTW GmbH, Weilheim, Germany). The NO₃, TAN and NO₂ concentrations were determined with a Merck Nitrate, Ammonium and Nitrite Test, respectively.

The sampling scheme for microbiota analysis of gut, water, feed and sludge on sampling day 0, 8 and 16, is provided in Table S4.1. Details regarding DNA isolation from gut, water, feed and sludge samples have been previously described elsewhere (Giatsis et al., 2014) and are available as supplementary material with the online version of this paper (Appendix).

4.2.5 PCR and Illumina sequencing

For 16S rRNA gene-based microbial composition profiling, barcoded amplicons from the V4 region of 16S rRNA genes were generated by PCR using the 515F and 806R primers (Caporaso et al., 2010). Seventy different barcodes were used per library in which the forward and reverse primer of one sample always carried the same barcode (Table S4.2). Primer sequence and barcode were separated by a 2-nucleotide linker sequence (GA for 515F and CG for 806R). Extracted DNA was diluted to a concentration of 20 ng μL^{-1} based on Nanodrop readings. PCR was performed using a GS0001 thermocycler (Gene Technologies, Braintree, United Kingdom). The PCR mix (50 μL final volume) contained 10 μL of 5 \times HF buffer (Finnzymes, Vantaa, Finland), 1 μL 10 mM (each nucleotide) PCR-grade Nucleotide Mix (Roche Diagnostic GmbH, Mannheim, Germany), 0.5 μL of Phusion hot start II High-Fidelity DNA polymerase (2U/ μL) (Finnzymes), 1 μL 10 μM barcoded primer 515F (Biologio BV, Nijmegen, The Netherlands), 1 μL 10 μM barcoded primer 806R, 36.5 μL nuclease-free water and 1 μL 20 ng/ μL template DNA. Duplicate PCR reactions and a negative control, containing 1 μL nuclease-free water, were run for every sample. PCR was performed under the following conditions: 98°C for 30 s to activate the polymerase, followed by 25 cycles consisting of denaturation at 98°C for 10 s, annealing at 56°C for 10 s, and elongation at 72°C for 10 s, and a final extension at 72°C for 7 min. Five μL of the PCR products were analyzed by 1% (w/v) agarose gel electrophoresis in the presence of 1 \times SYBR[®] Safe (Invitrogen, Carlsbad, CA, USA). PCR products were purified using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) according to manufacturer's instructions and eluted in 10 μL TE buffer. The DNA concentration in the PCR products was measured with the Qubit[®] 2.0 Fluorometer (LifeTechnologies, Carlsbad, USA) and 70 barcoded PCR products were mixed per library using 200 ng DNA per sample. Four times 20 μL per library were run on a 1% agarose gel at 100 V for 45 minutes. The bands visible at approximately 300 bp were excised from the gel and purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) according to the manufacturer's instructions and eluted in 7 μL nuclease-free water. Subsequently the library DNA was precipitated. First 0.1 L volume of 3M sodium acetate (pH 5.2) was added. Second, 3 volumes (calculated including the added sodium acetate) of 95% ethanol were also added. The library DNA was incubated overnight at 4°C before centrifugation at 13,400 g for 30 min at 4°C. The supernatant was discarded and the pellet was rinsed with 400 μL 70% ethanol and centrifuged for 15 minutes at 4°C. Subsequently, the ethanol was discarded and the library DNA was dried in a vacuum centrifuge for 45 s. The pellet was re-suspended in 16.5 μL TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and left for 1 h at 4°C.

The DNA concentration was measured with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA) and 1 µL was checked on a 1% agarose gel. Nucleotide sequences were generated using an Illumina HiSeq2000 sequencer at GATC-Biotech, Konstanz, Germany. Raw sequence data were deposited in GeneBank at NCBI under accession number SRP062676.

The pipeline and the parameters described in Ramiro-Garcia et al. (2016) were employed to convert the raw DNA sequence data to taxonomically labelled operational taxonomic unities (OTUs).

4.2.6 Data handling and statistical analysis

Microbial community data were expressed as relative abundance of all OTUs in each sample. Bray Curtis similarity between samples was calculated based on square root transformed data. Non metric multi-dimensional scaling (nMDS) was performed to represent the samples in a low dimensional space in a way that relative distances of all points were in the same rank order as the relative Bray Curtis dissimilarities of the samples. All statistical analyses were performed by using the multivariate statistical software package Primer V6 (Primer-E Ltd, Plymouth, UK).

To visualize shared OTUs between gut microbiota from different treatments and days, a network analysis was performed. Physical samples and OTUs are shown as nodes where each OTU is connected to each physical sample in which it was found. The group attributed layout, as implemented in Cytoscape 3.0.2 was used to cluster the OTUs in the network based on presence/absence data (Saito et al., 2012). Shared and unique OTUs of water gut or feed microbial communities were visualized with Venn diagrams. Venn diagrams were constructed by using the VENNY program without applying area proportionality to the 4-set diagrams (Oliveros, 2007).

4.3 Results

4.3.1 Production of the different sludge types

The aim of our study was to investigate whether gut microbial composition is strongly shaped by selective pressures in the gut and to which extent different microbial diets are able to modify those communities during early life development in fish.

Physicochemical parameters of the sludge reactors were significantly different among treatments. Redox values (mean \pm SD) were highly negative in methanogenic (-434.05mV \pm 147.83), around zero in the denitrifying and positive in the aerobic reactors (64.82mV

± 32.79). Nitrate concentration was lower in the denitrifying and higher in both aerobic and methanogenic sludge reactor. Higher TAN was observed in the methanogenic and denitrifying reactors and highest nitrite was observed in the denitrifying ones (Table 4.1).

Table 4.1. Performance of reactors during sludge production under aerobic, methanogenic and denitrifying conditions. Table (ANOVA and Tukey's post-hoc test) shows mean values ($n = 3$, $\pm SD =$ standard deviation) of the measured parameters for each treatment during 23 weeks culture period (including the pre-experimental period). In each row, different superscripts indicate significant difference ($P < 0.05$). T: Temperature; Redox: Reduction oxidation; TAN: Total ammonia nitrogen; NO_2 -N: Nitrite nitrogen; NO_3 -N: Nitrate nitrogen; DO: Dissolved oxygen.

Parameters	Aerobic	Methanogenic	Denitrifying
T ($^{\circ}C$)	28.3 ± 1.2^a	29.3 ± 0.7^c	28.7 ± 0.7^b
pH	8.5 ± 0.3^b	7.8 ± 0.6^a	8.7 ± 0.7^c
Redox (mV)	64.8 ± 32.8^c	-434.1 ± 147.8^a	-2.3 ± 146.7^b
NO_3 ($mg L^{-1}$)	118.5 ± 116.6^b	6.7 ± 29.1^a	181.0 ± 87.6^c
TAN ($mg L^{-1}$)	1.9 ± 4.0^a	55.6 ± 28.2^b	63.3 ± 32.9^b
NO_2 ($mg L^{-1}$)	0.3 ± 0.4^a	0.9 ± 2.4^a	4.1 ± 3.1^b
DO ($mg L^{-1}$)	5.5 ± 2.3^b	0.1 ± 0.2^a	0.6 ± 0.7^a

Microbial communities of the sludge differed considerably between the three reactor types. The high incorporation of sludge into the tilapia diets (99% on dry matter) resulted in differences of diet microbiota among treatments. Feed along with sludge samples clustered together according to the four dietary treatments and there were no large temporal changes observed over time (Figure 4.1). However, temporal variation of the microbial communities in the control diet (commercial feed) was higher than in the sludge based diets.

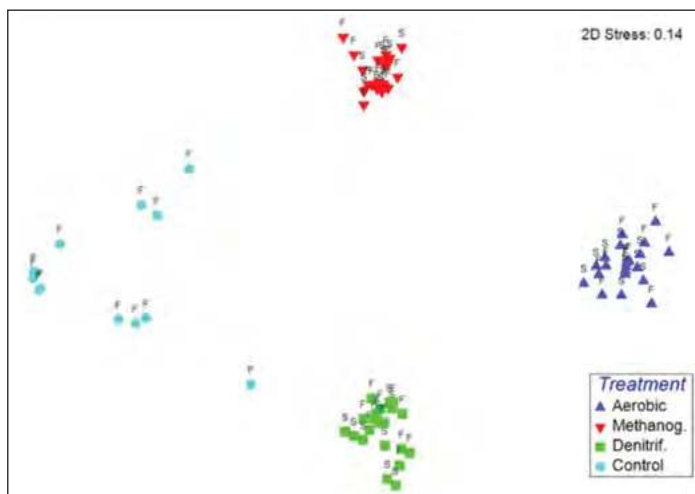


Figure 4.1. Non metric dimensional scaling (nMDS) of feed and sludge microbiota from the four dietary treatments. Each point represents a sludge (S) or feed microbiota (F) sample from days 0, 8 and 16. Beta-diversity is based on Bray Curtis after square root transformation of relative abundance data. Sludge was sampled in triplicate and feed samples in quadruplicate on each sampling day. Aerobic, Methanog., Denitrif., & Control: Aerobic, methanogenic, denitrifying and control treatment. Stress value is reported for the two dimensions and is indicative of the quality of fit of the data into the plot.

Regarding the unique and shared OTUs of the four diets, Venn diagrams showed that very few bacteria were shared between different diets and not even one OTU was present in all diets during day 16 (Figure 4.2).

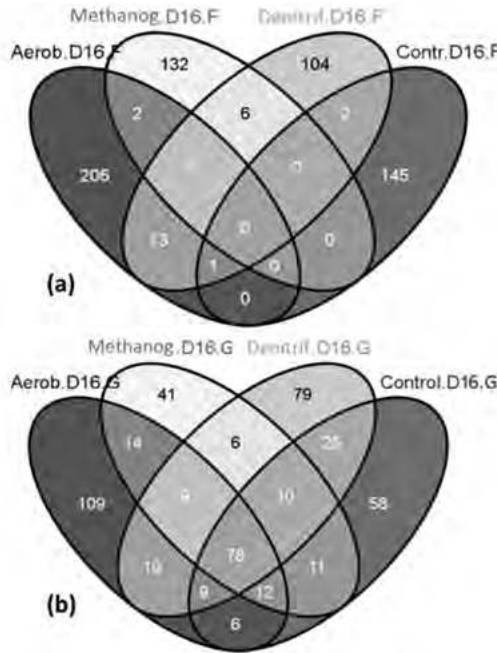


Figure 4.2. Venn diagram showing the number of shared and unique OTUs on day 16. Comparisons are made for (a) feed or (b) gut microbiota between the four treatments. Number of reads in all samples was normalized to the minimum number of reads found in one of the samples (cut off: 9719 reads) to allow a fair comparison between them. Aerob.G.16, Methanog.G.16, Denitrif.G.16 & Control.G.16: Gut microbiota OTUs in the aerobic, methanogenic, denitrifying and control treatment G. & F.: Gut and feed samples respectively; D16: Sampling day 16.

4.3.2 The effect of microbial diets on gut microbiota

Differences in the diet-associated microbiota were to a large extent observed also in the fish gut on both day 8 and 16 (Figure 4.1). Those differences were more prominent on day 16 with aerobic and methanogenic treatments clustering separately and denitrifying and control treatments overlapping (Figure 4.3). Dissimilarity of gut microbial communities due to dietary treatment was mostly attributed to the newly appeared OTUs of day 8 and 16 which instead were absent from the initial microbiota of the larvae.

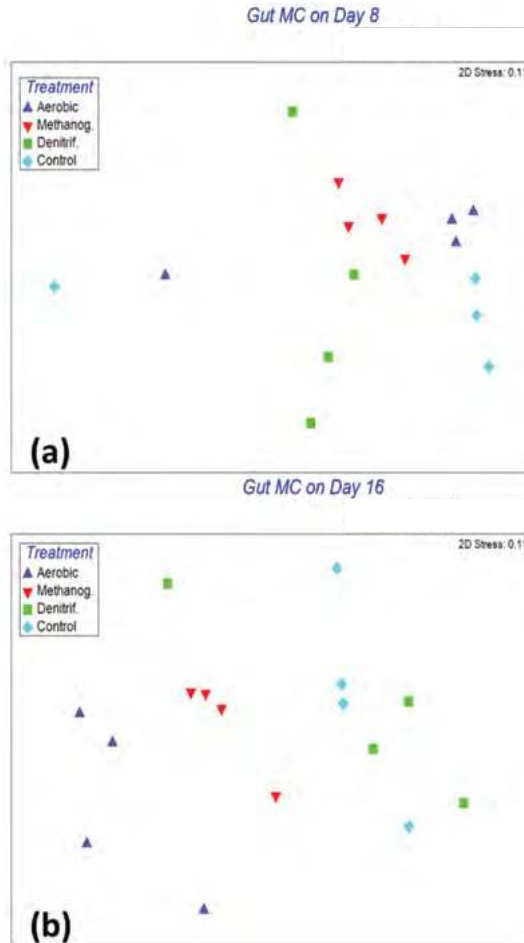


Figure 4.3. Non metric dimensional scaling (nMDS) of gut microbiota from individuals fed four different diets (complete dataset). Each point represents the gut microbiota of one individual. Plots are based on Bray Curtis distance after square root transformation of relative abundance data. Aerobic, Methanog., Denitrif., & Control: Aerobic, methanogenic, denitrifying and control treatment on day 8 (a) and day 16 (b). Stress values are reported for the two dimensions and are indicative of the quality of fit of data into the plot.

In total, 119 to 141 new OTUs appeared in the gut of larvae in the aerobic, methanogenic and denitrifying treatment on day 8, and 121 and 173 OTUs on day 16 respectively. Interestingly, in most cases, the newly appeared gut OTUs seem to have originated from the diet, whereas in the control treatment, from the water (Table 4.2).

Table 4.2. Numbers of gut OTUs appeared on either day 8 or 16 which were below threshold levels in the fish gut on day 0. Gut: Indicates the number of newly appeared OTUs in the gut; Water & Feed: Indicate the number of the newly appeared gut OTUs also present in water or feed on either day 8 or 16. Values in parenthesis show the % relative abundance of those OTUs in the gut, water or feed samples respectively on either day 8 or 16.

Treatment	Day	Newly appeared Gut OTUs	Newly appeared gut OTUs found only in	
			Water	Feed
Aerobic	8	122 (12.5%)	16 (7.2%)	30 (36.7%)
Methanogenic		141 (21.7%)	22 (11.5%)	36 (28.6%)
Denitrifying		119 (47.1%)	6 (1.3%)	44 (34.2%)
Control	16	98 (13.9%)	24 (8.9%)	4 (4.6%)
Aerobic		173 (43.7%)	9 (1.1%)	58 (27.6%)
Methanogenic		121 (34.3%)	14 (8.6%)	13 (6.1)
Denitrifying		156 (50.3%)	11 (8.6%)	19 (17.3%)
Control		140 (33.7%)	13 (10.2%)	5 (11.6%)

At the end of the experiment, not a single OTU above threshold levels was shared among all four diets. However, 78 common OTUs were present in the fish gut regardless of the dietary treatment (Figure 4.3a & b). From those, 39 and 26 highly abundant OTUs found on day 8 and 16 respectively, were already present in the gut of the initial population at day 0 (Figure S4.1). For this reason, we hypothesized that dietary effects on gut microbiota could have partly been masked by the persistent OTUs originating from the initial population (Figure 4.4).

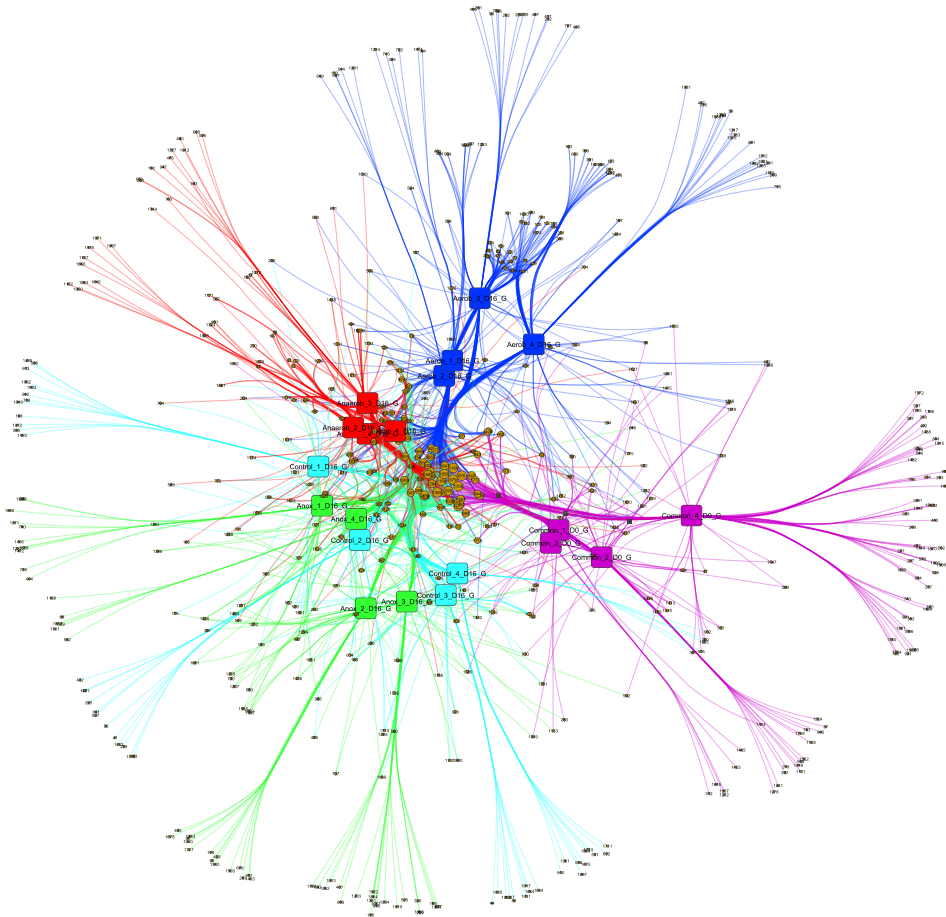


Figure 4.4. Network of shared and unique gut OTUs between all treatments on day 16 and the initial gut microbiota (Day 0). The network is color-coded by: treatment (Aerobic: blue; Methanogenic: red; Denitrifying: green; Control: cyan and initial population: purple). Sample nodes (squares) are oversized to be distinguished from OTU nodes (orange circles). Size of OTU nodes represent assignments of OTUs to sample nodes based on the amount of samples that were shared with (small nodes are shared with less samples compared to larger ones). Orange OTUs in the middle of network indicate the core OTUs found in all treatments and were also member of the initial gut microbiota.

Therefore, to obtain better insight into the changes induced by the diet, the gut OTUs, which were present in the initial population and persisted throughout the experiment, were removed from the dataset. A new dataset (subset) was plotted based on re-normalized percentage relative OTU abundance. Removal of the core OTUs resulted in more pronounced differences between gut microbiota from the four dietary treatments and particularly on day 16 (Figure 4.5a & b).

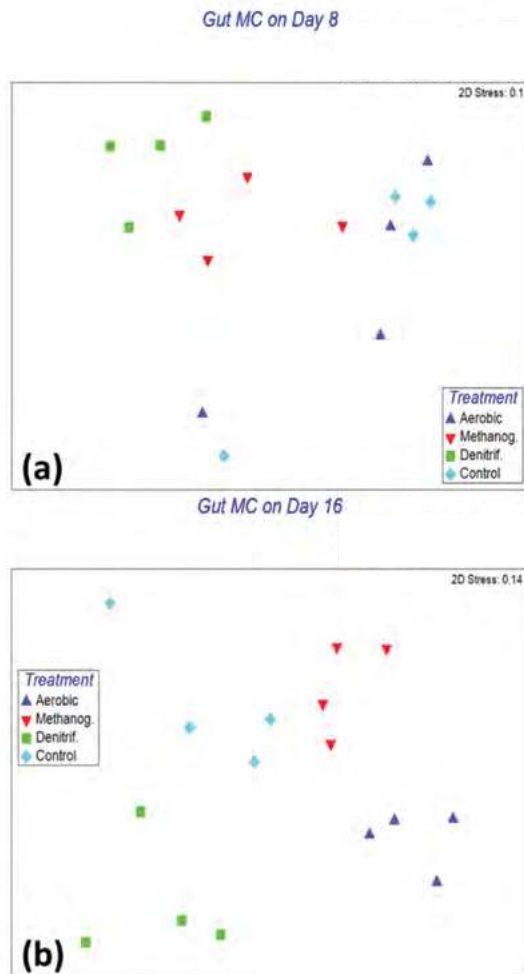


Figure 4.5. Non metric dimensional scaling (nMDS) of gut microbiota from individuals fed four different diets (reduced dataset). Each point represents the gut microbiota of one individual. Plots are based on Bray Curtis distance after square root transformation of relative abundance data. In total 26 OTUs shared between all treatments and days, and which were also present in the gut of the initial population (day 0), have been removed from the dataset. Aerobic, Methanog., Denitrif., & Control: aerobic, methanogenic, denitrifying and control treatment on day 8 (a) and day 16 (b). Stress values are reported for the two dimensions and are indicative of the goodness of fit of data into the plot.

4.3.3 The predominant and core OTUs of the gut

A heatmap of the most predominant gut OTUs (full dataset) on day 16 was created based on OTU relative abundance data (Figure 4.6). OTU 1416 (member of genus *Ralstonia*) was the most abundant OTU of the aerobic and methanogenic treatment on that day and the most abundant OTU of the initial gut microbiota (about 60%) and day 8. Another predominant gut OTU on day 16 was #676 (member of the genus *Rhodanobacter*) that was present in all treatments but surprisingly absent from both water and diet microbiota. *Rhodanobacter* was already present on day 0 and relatively highly abundant in all treatments at day 8.

The most predominant gut OTU of the denitrifying treatment at day 16 was #1850 (member of the genus *Cetobacterium*), which, although it was also found in the denitrifying sludge-diet, was absent from all samples of the other treatments and days. The next most abundant OTU of the denitrifying treatment was a member of the genus *Lactococcus* (OTU 1680), which was again absent from all other treatments on that day.

In the control treatment, #1032 (member of the genus *Aeromonas*) was the most predominant gut OTU on day 16. However, the relative abundance of this OTU differed between replicate samples indicating the high interindividual variation within the control treatment regarding predominant bacteria. *Aeromonas* was not present, neither in the water nor in the diet during that day. Other highly abundant OTUs of the control treatment were: OTU 589 (member of the genus *Rhizobium*), OTU 584 (member of the genus *Hyphomicrobium*) and OTU 676 (member of the genus *Rhodanobacter*).

In total 26 gut OTUs from the initial population were encountered in the gut of all treatments and days. Those OTUs accounted for 79% of the total OTU relative abundance on day 0 and represent a large fraction of the initial colonization of the fish gut prior to the dietary treatments (Figure S4.1). On day 16, those initial predominant OTUs accounted for a large proportion (43 to 54%) of the total OTU relative abundance in the gut of aerobic and methanogenic treatment. In the denitrifying and control treatment their abundance was lower though (37%). The lower relative abundance of shared (core) OTUs in the denitrifying and control treatment was mostly attributed to the decrease over time in the relative abundance of OTU 1416 (member of genus *Ralstonia*) in these treatments. Other predominant shared OTUs of day 8 and 16 were 676 and 679 (member of the genus *Rhodanobacter*) as well as 1056 and 1166 (members of the genus *Halomonas*) (Figure 4.6).

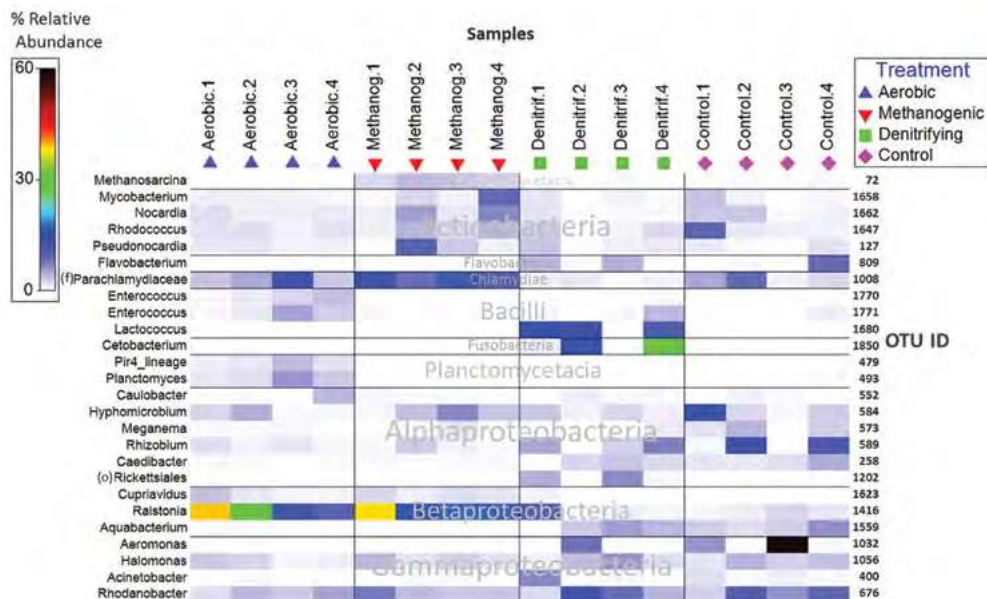


Figure 4.6. Heatmap of the five most predominant gut OTUs on day 16 in each replicate sample in the four treatments. Colours are proportional to the increasing percent relative OTU abundance (from white: lower, to red: higher) within each sample. Each column in the heatmap represents a sample, and each row represents an OTU. Columns are clustered by treatment, whereas the rows are clustered in accordance with the taxonomy of each OTU on class level. Aerobic, Methanogenic, Denitrifying & Control 1-4: Four replicate gut samples from each of the four dietary treatments on day 16. Taxonomy (left column) indicates the genus of each OTU (right column) unless otherwise stated; (f): family, (o): order. OTUs are taxonomically clustered on class level as indicated in the middle of the heatmap.

4.4 Discussion

Bacteria associated with water and feed are two of the main environmental inoculum sources for gut microbiota in fish. Whilst water starts to affect the fish gut microbiota immediately after mouth opening (Reitan et al., 1998), intrusion of microbes through feeding becomes important later (Ringo and Birkbeck, 1999; Hansen and Olafsen, 1999). Bakke et al. (2013) demonstrated that the gut microbiota of cod larvae is more strongly influenced by microbiota present in the water rather than by the microbiota associated with feed (Bakke et al., 2013). In a previous study, we confirmed a high and consistent correlation between water and gut microbiota of tilapia larvae whereas less than 1% of all OTUs was shared between gut and feed (Giatsis et al., 2015). However, our present results show that bacteria associated to the diet lead to alterations in gut microbiota and that these changes are more pronounced in the sludge-based diets rather than in the control. Contrarily to previous studies (Bakke et al., 2013; Giatsis et al., 2015), gut shared a much higher number of OTUs with the feed than with the water. However, despite marked

differences in the gut communities, the effect of the tested sludge-based diets was not sufficient to substantially modify a large core microbiota. These core gut OTUs were shared among all samples regardless the dietary treatment, and at the same time were already highly abundant in the gut of newly hatched larvae; already before the first feeding.

The concept of core gut microbiota has been proposed not only for humans (Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009a), but recently also for fish. A study conducted in rainbow trout (*Oncorhynchus mykiss*) raised under different densities and diet composition, provided evidence for a core microbiota (Wong et al., 2013). Furthermore, Roeselers et al found that a core-microbiota was shared between wild zebrafish (*Danio rerio*) shared and zebrafish reared in captivity in different geographical locations (Roeselers et al., 2011). This suggests that host-specificity for particular microbial species is modulated by selective pressures within the host gut. In the present study, it was found that gut microbiota of tilapia larvae shared a large core microbiota regardless of the dietary treatments (Figure 4.4). Moreover, the 26 core OTUs that together made up 36 to 54% of the total relative abundance in the fish gut of all treatments, accounted for almost 80% of the total relative abundance on day 0 (Table S4.1). In this regard, tilapia core microbiota of this study can be defined as the group of OTUs encountered in all fish and sampling days (regardless the diet) and at the same time were also present in the gut of the initial population prior to the dietary treatments.

Regarding the phylogenetic affiliation of the core OTUs, our analysis revealed a diverse set of Proteobacteria (especially alpha and gamma), Actinobacteria and Firmicutes. The higher the taxonomic level of comparison and the diversity within each taxon, the less meaningful the definition of core microbiota becomes in terms of functionality. For this reason, our comparative phylogenetic analysis of gut microbiota in different individuals was performed on OTU level. Among the most dominant core OTUs found in this study were members of the genera *Ralstonia*, *Halomonas* and *Rhodanobacter*. In fish, the genus *Ralstonia* (OTU 1416) has been previously found in the gut of seabass (Carda-Diéguez et al., 2014), rainbow trout (Kim et al., 2007; Navarrete et al., 2012; Pond et al., 2006), yellow catfish (Wu et al., 2010), zebrafish (Rawls et al., 2004) but also in shrimp (Durand et al., 2010). The genus *Halomonas* (OTUs 1056 & 1166) has been reported in the gut of Arctic charr (Ringo et al., 2006), Atlantic cod (Fjellheim et al., 2011), Midas cichlids (Franchini et al., 2014), queen conch (Carrascal et al., 2014), and *Artemia* brine shrimp (Tkavc et al., 2011; Riddle et al., 2013), and also in Atlantic salmon gills (Steinum et al., 2009). The “core microbiota” observed here has not been previously reported as core in tilapia. However, many of the bacterial genera found here (*Mycobacterium*, *Nocardia*, *Rhodococcus*, *Halomonas*, and *Ralstonia*) were also encountered in our previous study (Giatsis et al., 2016a) suggesting that tilapia specificity for particular microbial taxa could be modulated by selective pressures within the host.

Lack of shared predominant species across studies in tilapia does not necessarily imply difference in functionality of these communities within the gut. The concept of functional redundancy suggests that functional diversity of an ecosystem is additive when species are complementary, or decreases, when species are to some extent functionally redundant (Bell et al., 2005). Metagenomics analysis of gut communities in fish could increase our knowledge of gene content and genetic variability in this microbiome. However, metatranscriptomics analysis of the gut microbiota would be necessary to reveal whether functional patterns in the fish gut are complementary or redundant, and whether or not, these patterns match the genetic variability of the microbiome.

Mycobacteria are obligate aerobic, Gram-positive, non-spore forming, non-motile and prevalent in soil and water bacteria and have been previously reported in tilapia gut (Zhou et al., 2009; He et al., 2012, 2011, 2010), although not as core. The genus *Rhodococcus* has been reported in the gut of many fish species (Kim et al., 2007; Sanchez et al., 2012; Spanggaard et al., 2000; Huber et al., 2004). *Hyphomicrobium* was among the dominant genera in the gut of catfish (McDonald et al., 2012). Representatives of *Hyphomicrobium* are ubiquitous in nature, residing in soils, as well as fresh and wastewaters. Members of the genus have been shown to be associated with turbot and brook charr skin microbiota (Mudarris and Austin, 1988; Montes et al., 1999; Boutin et al., 2014), or water/filter microbiota (Sugita et al., 2005; Austin, 1983; Auffret et al., 2013). These findings suggest that some of the commonly found genera of this study could be involved in major metabolic functions in the fish gut. However, the observed differences in abundance of core genera across studies could to a certain degree pertain to the environmental microbiota (i.e. availability of certain bacteria), dietary interventions or other experimental treatments.

The tilapia larvae studied here were maintained under identical rearing conditions prior to the start of the experiment. The presence of the shared species is likely to be attributed to an early colonization that greatly diminished the impact of the experimental manipulations, thus reinforcing the early life imprinting hypothesis. The fact that core species of this study were not defined as core in our previous study might be related to the early exposure of larvae to different environmental microbiota (maternal or water) between the two experiments. Early microbial colonization has been suggested to have a long lasting effect on the host through early-life imprinting (Sommer and Bäckhed, 2013). The control over the composition of the gut microbiota, which is exerted by the maternal microbiota or by the exposure to the maternal environment inoculum, has been suggested in humans (Ley, 2010; Benson et al., 2010; Tannock et al., 1990) and animals (Ley et al., 2005; Zhao et al., 2013). However, the extent and the mechanisms underlying such a control, remain the major unanswered questions in gut microbiology (Flint et al., 2007). Contrary to terrestrial animals, which inherit a part of their initial microbiota through contact with the mother, fish usually spawn axenic eggs in the water (Verschuere et al., 2000). Nevertheless, the Nile tilapia is a known maternal mouth-brooder among the

cichlids. In such context, an initial contact of eggs with the maternal mouth microbiota suggests a strong influence on initial microbial colonization of the egg surfaces, before being transferred to incubators for hatching. The early life of fish larvae in intensive rearing systems takes place in incubators with both egg debris and hatching eggs. This suggests that both egg and incubator water microbial communities are important factors in establishing the indigenous larval microbiota (Olafsen, 2001; Keskin et al., 1994) and it might explain the differences in the core microbial species between our two studies.

Besides the close phylogenetic relationships of the larvae and the legacy effects during hatching (both inheritance from mother and environment), another reason for the large number of shared OTUs among fish of this study could be the common water source among treatments. A common water source might limit the variability in microbial community composition among the larvae and between treatments. In a previous study, we demonstrated that tilapia larvae shared a large number of OTUs that originated from the water when fish were fed the same diet but exposed to different water sources (Giatsis et al., 2014). However, in our current study tilapia larvae shared only a few OTUs with water, implying that the system water was not the main drive of large fraction of bacteria shared between fish from all treatments. Yet in a few cases, some of the most abundant gut OTUs (i.e. OTU 1416 on day 16 and OTU 1031 on day 8) were also encountered in the water, though in very low abundance. This finding suggests that either there is selection of some rare water bacteria in the fish gut, and/or that, predominant bacteria in the gut end up in the water through defecation. However the latter is not a valid hypothesis for bacteria present in the feed, which means that shared bacteria between gut and feed could only originate from the feed.

Although several genera predominated the gut microbiota in all treatments, analysis of the accessory microbiota (excluding the 26 core gut OTUs shared between day 0, 8 and 16), revealed that differences between gut microbial communities matched differences in dietary microbial communities. Besides the core OTUs, some of the most predominant OTUs in the gut were members of the genera *Cetobacterium*, *Lactococcus*, *Aeromonas*, *Rhizobium*, *Hyphomicrobium* and *Aquabacterium*. Both *Cetobacterium* (OTU 1850) and *Lactococcus* (OTU 1680) dominated the gut from larvae receiving the denitrifying treatment diet and were found in low abundance in the methanogenic treatment diet-fed fish. These genera were also present in the feed of these two treatments on day 8 and 16, but they were not detected in any other treatment or in the initial gut. As these two genera were not detected in the water either, it is presumed that these bacteria entered the gut through the feed. Members of the genus *Cetobacterium* (mostly *C. somerae* (Finegold et al., 2003) have been previously identified in the gut of several fish species such as catfish (Wu et al., 2012a; Di Maiuta et al., 2013), zebrafish (Roeselers et al., 2011), as well as goldfish, carp and in our previous study in tilapia larvae (Giatsis et al., 2015). The presence of this bacterium in a number of freshwater fishes suggests the importance of

these microbes as members of the fish gut community. The presence and abundance of the genus *Cetobacterium* in the methanogenic and denitrifying feed and gut, confirms the preference of these organisms for oxygen depleted habitats. Other gut OTUs which possibly originated from the feed are members of the family *Planctomycetaceae* (OTU 199 and 493), which were detected only in the gut and feed of the aerobic treatment. Most members of this family are chemo-organotrophs and obligate aerobes and tend to be slow-growers compared to other bacteria (Op den Camp et al., 2007). Both on day 8 and 16, OTU 72 (genus *Methanosarcina*) was highly abundant in the gut and among the most dominant OTUs in the feed of the methanogenic treatment. *Methanosarcina* is a genus of methane producing euryarchaeotes. These organisms are anaerobic methanogens that produce methane using all three metabolic pathways for methanogenesis (Matarazzo et al., 2012). They have been reported in diverse environments such as in groundwater, anaerobic sludge reactors, freshwater lakes but also in human digestive tract and fish faeces (Ma et al., 2013; Angelakis et al., 2012; Samuel et al., 2007; Jones et al., 2008; Van Der Maarel et al., 1999; Dridi et al., 2011).

In a study with chicken, Yin et al. (2010), suggested that bacterial succession in the gut at a very early stage of life can be influenced by exposure to proper bacterial inocula, and that host selective pressures exert an additional role in colonization process. This statement brings out three interesting points: (a) steering gut microbiota with a microbial inoculum has higher chances to succeed during early life stages of animals compared to adult; (b) the word “proper” indicates the importance of microbial composition of the inoculum for a successful colonization and (c) regardless the previous two points, host selective pressure plays a major role in determining gut colonization.

Our data showed that the microbial composition of the sludge-based diets had corresponding effects on gut microbiota. In addition, fish gut microbiota shared more OTUs with the microbial diets, whereas fish fed the control diet shared more OTUs with water. Considering the short period of this study, one could speculate that by prolonging the experimental period, shared gut OTUs between treatments would disappear; or on the other hand that, if the administration of the experimental diets was terminated, the species composition and abundance of gut microbiota would return to an equilibrium among the treatments. These assumptions raise some very interesting considerations regarding the use of probiotics for steering fish gut microbiota. The main drawbacks of probiotic use are related to low survival and proliferation rate inside the fish gut (Gatesoupe, 1999; Grześkowiak et al., 2012). Important gaps in knowledge regarding selection criteria and colonization potential of single vs. multistrain probiotics (Pérez et al., 2010; Ramos et al., 2013), as well as competitive exclusion of the probiotic strain in the gut still exist (Martínez Cruz et al., 2012). Tackling such issues will require studies of model organisms where potentially confounding variables (host genotype) can be constrained by using for example inbred fish strains or clone lines. The use of microbial fecal transplants and/

or probiotics in gnotobiotic fish could help us explore the relative contributions of legacy versus habitat (including dietary components) in selecting a microbial community. This information will facilitate the development of safe and effective methods for manipulating gut microbiota composition to promote the health of humans and animals.

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Supplementary material

Table S4.1. Sampling scheme with number of gut, water, sludge and feed samples taken for 16S rRNA gene sequencing analysis during the three experimental days. For determining the initial gut microbiota of the larvae, four gut samples were taken (not whole larvae) from the common batch before distributing larvae in the tanks and initiate feeding.

Sample	Day 0				Day 8				Day 16				Total	
	Aerob.	Methanog.	Denitrif.	Control	Aerob.	Methanog.	Denitrif.	Control	Aerob.	Methanog.	Denitrif.	Control		
Gut		4			4	4	4	4	4	4	4	4	36	
Water	Inlet	0	0	0	0	1	1	1	1	1	1	1	8	
	Tank	1	1	1	1	1	1	1	1	1	1	1	12	
	Outlet	0	0	0	0	1	1	1	1	1	1	1	8	
Sludge	3	3	3	0	3	3	3	0	3	3	3	0	27	
Feed	4	4	4	4	4	4	4	4	4	4	4	4	48	
Total		33				53				53				139

Table S4.2. Metadata of samples included in the sequencing libraries. Seventy different barcodes were used per library in which the forward and reverse primer of one sample always carried the same barcode.

#SampleID	Barcode Sequence	Library	Forward Sequence length	Reverse Sequence length	Sample Type	Barcode Number	Day	Treatment
Aerob.D0.W	AACCAGAA	1	71	70	WATER	1	0	Aerobic
Methanog.D0.W	AACCATGC	1	71	70	WATER	2	0	Methanogenic
Denitrif.D0.W	AACGGCGC	1	71	70	WATER	3	0	Denitrifying
Control.D0.W	AAGGAGCG	1	71	70	WATER	4	0	Control

#SampleID	Barcode Sequence	Library	Forward Sequence length	Reverse Sequence length	Sample Type	Barcode Number	Day	Treatment
Aerob.inlet.D8.W	ACCAATTA	1	71	70	WATER	5	8	Aerobic
Aerob.tank.D8.W	ACCGGAAT	1	71	70	WATER	6	8	Aerobic
Methanog.inlet.D8.W	ACGCTCGC	1	71	70	WATER	7	8	Methanogenic
Methanog.tank.D8.W	AGACCTGC	1	71	70	WATER	8	8	Methanogenic
Denitrif.inlet.D8.W	AGCAGATT	1	71	70	WATER	9	8	Denitrifying
Denitrif.tank.D8.W	AGTTCGCG	1	71	70	WATER	10	8	Denitrifying
Control.inlet.D8.W	AGGTGCGA	1	71	70	WATER	11	8	Control
Control.tank.D8.2.W	AGGTTATG	1	71	70	WATER	12	8	Control
Aerob.inlet.D16.W	AGTCGCGC	1	71	70	WATER	13	16	Aerobic
Aerob.tank.D16.W	AGTTATGC	1	71	70	WATER	14	16	Aerobic
Methanog.inlet.D16.W	ATATCCGC	1	71	70	WATER	15	16	Methanogenic
Methanog.tank.D16.W	ATGCCGGA	1	71	70	WATER	16	16	Methanogenic
Denitrif.inlet.D16.W	ATGCGATT	1	71	70	WATER	17	16	Denitrifying
Denitrif.tank.D16.W	CAACCTCT	1	71	70	WATER	18	16	Denitrifying
Control.inlet.D16.W	CAACGAGG	1	71	70	WATER	19	16	Control
Control.tank.D16.W	CATAAGCG	1	71	70	WATER	20	16	Control
Aerob.out.D8.W	CATGATGC	1	71	70	WATER	21	8	Aerobic
Methanog.out.D8.W	CATTGCCA	1	71	70	WATER	22	8	Methanogenic
Denitrif.out.D8.W	CCATGCGC	1	71	70	WATER	23	8	Denitrifying
Control.out.D8.W	CCATTATG	1	71	70	WATER	24	8	Control
Aerob.out.D16.W	CCGTACTA	1	71	70	WATER	25	16	Aerobic
Methanog.out.D16.W	CCGTCTGC	1	71	70	WATER	26	16	Methanogenic
Denitrif.out.D16.W	CGCAAGCT	1	71	70	WATER	27	16	Denitrifying
Control.out.D16.W	CGGAAGAG	1	71	70	SLUDGE	28	16	Control
Aerob.A.D0.S	CTAGGAGA	1	71	70	SLUDGE	29	0	Aerobic
Aerob.B.D0.S	CTCAGCGC	1	71	70	SLUDGE	30	0	Aerobic
Aerob.C.D0.S	CTCAGTCT	1	71	70	SLUDGE	31	0	Aerobic
Methanog.A.D0.S	CTCGCGTA	1	71	70	SLUDGE	32	0	Methanogenic
Methanog.B.D0.S	CTGCTGAA	1	71	70	SLUDGE	33	0	Methanogenic
Methanog.C.D0.S	CTTGCGAG	1	71	70	SLUDGE	34	0	Methanogenic
Denitrif.A.D0.S	CTTGCCCT	1	71	70	SLUDGE	35	0	Denitrifying
Denitrif.B.D0.S	GAACCGTT	1	71	70	SLUDGE	36	0	Denitrifying
Denitrif.C.D0.S	GAACGTAT	1	71	70	SLUDGE	37	0	Denitrifying
Aerob.A.D8.S	GAACTAAG	1	71	70	SLUDGE	38	8	Aerobic
Aerob.B.D8.S	GAAGCTCG	1	71	70	SLUDGE	39	8	Aerobic
Aerob.C.D8.S	GAGTTATA	1	71	70	SLUDGE	40	8	Aerobic
Methanog.A.D8.S	GATGAATG	1	71	70	SLUDGE	41	8	Methanogenic
Methanog.B.D8.S	GATGATAA	1	71	70	SLUDGE	42	8	Methanogenic
Methanog.C.D8.S	GATGCGCT	1	71	70	SLUDGE	43	8	Methanogenic
Denitrif.A.D8.S	GCCAGGTT	1	71	70	SLUDGE	44	8	Denitrifying
Denitrif.B.D8.S	GCCTTAAG	1	71	70	SLUDGE	45	8	Denitrifying
Denitrif.C.D8.S	GCTAATCT	1	71	70	SLUDGE	46	8	Denitrifying
Aerob.A.D16.S	GGAGCGCA	1	71	70	SLUDGE	47	16	Aerobic
Aerob.B.D16.S	GGAGTATG	1	71	70	SLUDGE	48	16	Aerobic
Aerob.C.D16.S	GGTACCAA	1	71	70	SLUDGE	49	16	Aerobic

#SampleID	Barcode Sequence	Library	Forward Sequence length	Reverse Sequence length	Sample Type	Barcode Number	Day	Treatment
Methanog.A.D16.S	GGTAGAAT	1	71	70	SLUDGE	50	16	Methanogenic
Methanog.B.D16.S	GTCGCAA	1	71	70	SLUDGE	51	16	Methanogenic
Methanog.C.D16.S	GTTAAGTT	1	71	70	SLUDGE	52	16	Methanogenic
Denitrif.A.D16.S	GTTCTACG	1	71	70	SLUDGE	53	16	Denitrifying
Denitrif.B.D16.S	TATTGCGC	1	71	70	SLUDGE	54	16	Denitrifying
Denitrif.C.D16.S	TCAGCGAG	1	71	70	SLUDGE	55	16	Denitrifying
Aerob.1.D0.F	TCATTCCG	1	71	70	FEED	56	0	Aerobic
Aerob.2.D0.F	TCCGTATA	1	71	70	FEED	57	0	Aerobic
Aerob.3.D0.F	TCCTCCGC	1	71	70	FEED	58	0	Aerobic
Aerob.4.D0.F	TCGAATAA	1	71	70	FEED	59	0	Aerobic
Methanog.1.D0.F	TCGATATT	1	71	70	FEED	60	0	Methanogenic
Methanog.2.D0.F	TCTATTCCG	1	71	70	FEED	61	0	Methanogenic
Methanog.3.D0.F	TGACTCAA	1	71	70	FEED	62	0	Methanogenic
Methanog.4.D0.F	TGATCTCA	1	71	70	FEED	63	0	Methanogenic
Denitrif.1.D0.F	TGCCTGCG	1	71	70	FEED	64	0	Denitrifying
Denitrif.2.D0.F	TGGTAATT	1	71	70	FEED	65	0	Denitrifying
Denitrif.3.D0.F	TGGTATGA	1	71	70	FEED	66	0	Denitrifying
Denitrif.4.D0.F	AACCAGAA	2	71	70	FEED	1	0	Denitrifying
Control.1.D0.F	AACCATGC	2	71	70	FEED	2	0	Control
Control.2.D0.F	AACGGCGC	2	71	70	FEED	3	0	Control
Control.3.D0.F	AAGGAGCG	2	71	70	FEED	4	0	Control
Control.4.D0.F	ACCAATTA	2	71	70	FEED	5	0	Control
Aerob.1.D8.F	ACCGGAAT	2	71	70	FEED	6	8	Aerobic
Aerob.2.D8.F	ACGCTCGC	2	71	70	FEED	7	8	Aerobic
Aerob.3.D8.F	AGACCTGC	2	71	70	FEED	8	8	Aerobic
Aerob.4.D8.F	AGCAGATT	2	71	70	FEED	9	8	Aerobic
Methanog.1.D8.F	AGTTCGCG	2	71	70	FEED	10	8	Methanogenic
Methanog.2.D8.F	AGGTGCAA	2	71	70	FEED	11	8	Methanogenic
Methanog.3.D8.F	AGGTTATG	2	71	70	FEED	12	8	Methanogenic
Methanog.4.D8.F	AGTCGCGC	2	71	70	FEED	13	8	Methanogenic
Denitrif.1.D8.F	AGTTATGC	2	71	70	FEED	14	8	Denitrifying
Denitrif.2.D8.F	ATATCCGC	2	71	70	FEED	15	8	Denitrifying
Denitrif.3.D8.F	ATGCCGGA	2	71	70	FEED	16	8	Denitrifying
Denitrif.4.D8.F	ATGCGATT	2	71	70	FEED	17	8	Denitrifying
Control.1.D8.F	CAACCTCT	2	71	70	FEED	18	8	Control
Control.2.D8.F	CAACGAGG	2	71	70	FEED	19	8	Control
Control.3.D8.F	CATAAGCG	2	71	70	FEED	20	8	Control
Control.4.D8.F	CATGATGC	2	71	70	FEED	21	8	Control
Aerob.1.D16.F	CATTGCCA	2	71	70	FEED	22	16	Aerobic
Aerob.2.D16.F	CCATGCGC	2	71	70	FEED	23	16	Aerobic
Aerob.3.D16.F	CCATTATG	2	71	70	FEED	24	16	Aerobic
Aerob.4.D16.F	CCGTAATA	2	71	70	FEED	25	16	Aerobic
Methanog.1.D16.F	CCGTCTGC	2	71	70	FEED	26	16	Methanogenic
Methanog.2.D16.F	CGCAAGCT	2	71	70	FEED	27	16	Methanogenic
Methanog.3.D16.F	CGGAAGAG	2	71	70	FEED	28	16	Methanogenic
Methanog.4.D16.F	CTAGGAGA	2	71	70	FEED	29	16	Methanogenic

#SampleID	Barcode Sequence	Library	Forward Sequence length	Reverse Sequence length	Sample Type	Barcode Number	Day	Treatment
Denitrif.1.D16.F	CTCAGCGC	2	71	70	FEED	30	16	Denitrifying
Denitrif.2.D16.F	CTCAGTCT	2	71	70	FEED	31	16	Denitrifying
Denitrif.3.D16.F	CTCGCGTA	2	71	70	FEED	32	16	Denitrifying
Denitrif.4.D16.F	CTGCTGAA	2	71	70	FEED	33	16	Denitrifying
Control.1.D16.F	CTTGCGAG	2	71	70	FEED	34	16	Control
Control.2.D16.F	CTTGGCCT	2	71	70	FEED	35	16	Control
Control.3.D16.F	GAACCGTT	2	71	70	FEED	36	16	Control
Control.4.D16.F	GAACGTAT	2	71	70	FEED	37	16	Control
Common.1.D0.G	GAACTAAG	2	71	70	GUT	38	0	Common
Common.2.D0.G	GAAGCTCG	2	71	70	GUT	39	0	Common
Common.3.D0.G	GAGTTATA	2	71	70	GUT	40	0	Common
Common.4.D0.G	GATGATAA	2	71	70	GUT	42	0	Common
Aerob.1.D8.G	GATGCGCT	2	71	70	GUT	43	8	Aerobic
Aerob.2.D8.G	GCCAGGTT	2	71	70	GUT	44	8	Aerobic
Aerob.3.D8.G	GCCTTAAG	2	71	70	GUT	45	8	Aerobic
Aerob.4.D8.G	GCTAATCT	2	71	70	GUT	46	8	Aerobic
Methanog.1.D8.G	GGAGCGCA	2	71	70	GUT	47	8	Methanogenic
Methanog.2.D8.G	GGAGTATG	2	71	70	GUT	48	8	Methanogenic
Methanog.3.D8.G	GGTACCAA	2	71	70	GUT	49	8	Methanogenic
Methanog.4.D8.G	GGTAGAAT	2	71	70	GUT	50	8	Methanogenic
Denitrif.1.D8.G	GTCCGCAA	2	71	70	GUT	51	8	Denitrifying
Denitrif.2.D8.G	GTTAAGTT	2	71	70	GUT	52	8	Denitrifying
Denitrif.3.D8.G	GTTCTACG	2	71	70	GUT	53	8	Denitrifying
Denitrif.4.D8.G	TATTGCGC	2	71	70	GUT	54	8	Denitrifying
Control.1.D8.G	TCAGCGAG	2	71	70	GUT	55	8	Control
Control.2.D8.G	TCATCCG	2	71	70	GUT	56	8	Control
Control.3.D8.G	TCCGTATA	2	71	70	GUT	57	8	Control
Control.4.D8.G	TCCTCCGC	2	71	70	GUT	58	8	Control
Aerob.1.D16.G	TCGAATAA	2	71	70	GUT	59	16	Aerobic
Aerob.2.D16.G	TCGATATT	2	71	70	GUT	60	16	Aerobic
Aerob.3.D16.G	TCTATTCCG	2	71	70	GUT	61	16	Aerobic
Aerob.4.D16.G	TGACTCAA	2	71	70	GUT	62	16	Aerobic
Methanog.1.D16.G	TGATCTCA	2	71	70	GUT	63	16	Methanogenic
Methanog.2.D16.G	TGCTGCG	2	71	70	GUT	64	16	Methanogenic
Methanog.3.D16.G	TGGTAATT	2	71	70	GUT	65	16	Methanogenic
Methanog.4.D16.G	TGGTATGA	2	71	70	GUT	66	16	Methanogenic
Denitrif.1.D16.G	TTAGGATG	2	71	70	GUT	67	16	Denitrifying
Denitrif.2.D16.G	AACCAGAA	3	71	70	GUT	1	16	Denitrifying
Denitrif.3.D16.G	AACCATGC	3	71	70	GUT	2	16	Denitrifying
Denitrif.4.D16.G	AACGGCGC	3	71	70	GUT	3	16	Denitrifying
Control.1.D16.G	AAGGAGCG	3	71	70	GUT	4	16	Control
Control.2.D16.G	ACCAATTA	3	71	70	GUT	5	16	Control
Control.3.D16.G	ACCGGAAT	3	71	70	GUT	6	16	Control
Control.4.D16.G	ACGCTCGC	3	71	70	GUT	7	16	Control

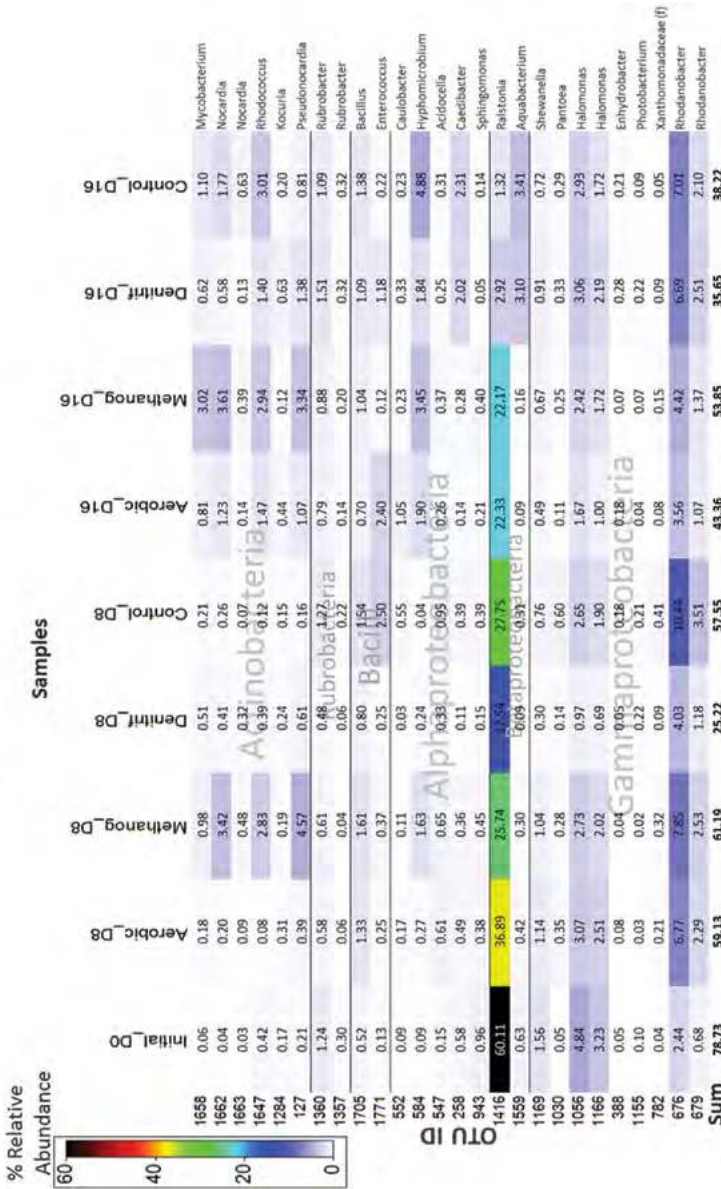
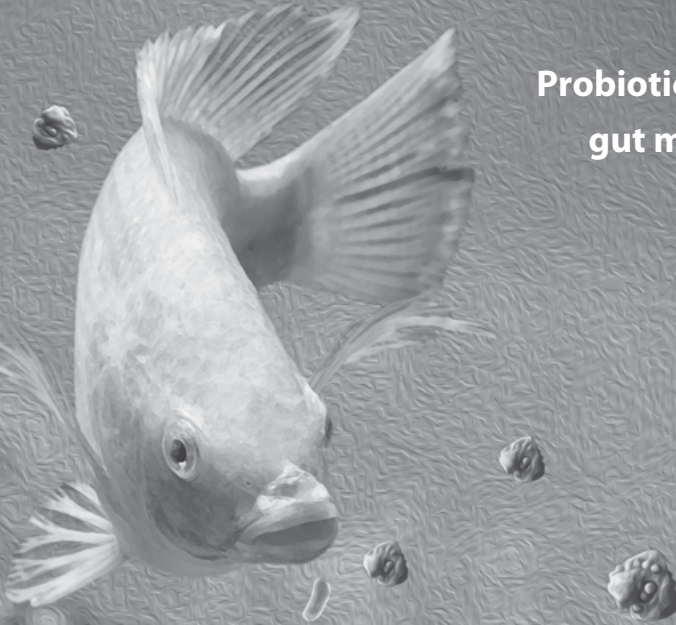


Figure S4.1. Heatmap of 26 gut OTUs shared between all treatments and days. Each column in the heatmap represents a treatment, and each row represents an OTU. Values represent the average % relative abundance of each OTU within each treatment and colour codes are proportional to the increasing OTU abundance (from green to red: higher). Initial: Initial gut microbiota from day 0; Aerobic, Methanog, Denitrif. & Control: Aerobic, methanogenic, denitrifying and control treatment; D0, D8 and D16: experimental day 0, 8 and 16 respectively. Sum: Per treatment % total relative abundance of all OTUs present in the table, #OTU ID: number of each OTU corresponding to the consensus taxonomy. Taxonomy indicates the genus of each OTU (right column) unless otherwise stated; (f): family. OTUs are taxonomically clustered on class level as indicated in the middle of the heatmap.

Chapter 5

Probiotic legacy effects on gut microbial assembly in tilapia larvae



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The exposure of fish to environmental free-living microbes and its effect on early colonization in the gut have been studied in recent years. However, little is known regarding how the host and environment interact to shape gut communities during early life. Here, we tested whether the early microbial exposure of tilapia larvae affects the gut microbiota at later life stages. The experimental period was divided into three stages: axenic, probiotic and active suspension. Axenic tilapia larvae were reared either under conventional conditions (active suspension systems) or exposed to a single strain probiotic (*Bacillus subtilis*) added to the water. Microbial characterization by Illumina HiSeq sequencing of 16S rRNA gene amplicons showed the presence of *B. subtilis* in the gut during the seven days of probiotic application. Although *B. subtilis* was no longer detected in the guts of fish exposed to the probiotic after day 7, gut microbiota of the exposed tilapia larvae remained significantly different from that of the control treatment. Compared with the control, fish gut microbiota under probiotic treatment was less affected by spatial differences resulting from tank replication, suggesting that the early probiotic contact contributed to the subsequent observation of low inter-individual variation.

5.1 Introduction

The gut microbiota influences a wide range of biological processes in humans (Rawls, 2007; Sekirov et al., 2010), domesticated terrestrial animals (Chaucheyras-Durand and Durand, 2010; McFall-Ngai et al., 2013) and fish (Nayak, 2010; Sullam et al., 2012). In fish, despite the significant contribution of several studies on gut microbiota, the current understanding of the functional significance of microbial fluctuations lags well behind that of terrestrial vertebrates. Apart from a few pathogens, host-microbe interactions in fish remain poorly understood. One reason is that the fish gut microbiota is dependent on the aquatic environment. Furthermore, compared with terrestrial animals that undergo embryonic development within an amnion, fish larvae are released into the water at an early ontogenetic stage, when their digestive tract is not yet fully developed and their immune system incomplete (Gatesoupe, 1999). Thus, the use of probiotics in aquaculture is particularly effective during early ontogenetic stages, where large mortalities are commonly observed.

Live microorganisms that confer a health benefit to the host have been demonstrated as useful in aquaculture (Pérez-Sánchez et al., 2014). Probiotics reduce infections caused by bacterial pathogens (Gatesoupe, 1999; Martínez Cruz et al., 2012) and have been successfully used as immunostimulants (Cain and Swan, 2010; Lepage et al., 2012; Song et al., 2014) and growth promoters in fish and shrimp (Pérez-Sánchez et al., 2014; Merrifield et al., 2010). However, probiotic strains often only transiently colonize the gut and quickly fall below detection limits (Gatesoupe, 1999; Grzeškowiak et al., 2012; Skjermo et al., 2015; Robertson et al., 2000; Kim and Austin, 2006; Balcázar et al., 2007). For ingested bacteria to proliferate and persist within “resident” microbiota, these microorganisms must adapt to the environmental conditions inside the gut, such as nutrient availability, pH and digestive enzymes (Hansen and Olafsen, 1999). The ability of a probiotic strain to survive and successively proliferate in the gut after the cessation of probiotics administration is both host- and probiotic strain-dependent and is highly determined by the mode and duration of administration (through water or feed) (Pérez-Sánchez et al., 2014). Based on the present level of understanding, the colonization dynamics of fish gut microbiota remain largely stochastic and are affected by gut habitat (i.e., physiology, anatomy) and host genotype (Yan et al., 2012; Rawls et al., 2006; Navarrete et al., 2012). Yin et al. (2010) suggested that in newly hatched chicks, it is possible to steer gut microbiota by feeding bacterial diets (caecal inocula), leading to the development of distinct communities. In a recent study, we observed that tilapia larvae fed with different microbial diets (sludge-based) developed distinct gut microbiota, although all larvae also shared a large number of species (Giatsis et al., 2015). It is likely that those shared species resulted from larval contact with a common water source after hatching and prior to the first feeding. However, the host-specificity for a particular microbial species modulated by selective pressures within the host gut cannot be excluded.

Bacillus subtilis is a Gram- and catalase-positive, rod-shaped, facultative, anaerobic and endospore-forming bacterium observed in air, water, soil and the gastrointestinal tract of humans and animals (Nakano and Zuber, 1998; Green et al., 1999; Hong et al., 2009; Tseng et al., 2009). Several *Bacillus* spp. (including *B. subtilis*) have been commonly used as probiotics in aquaculture, reflecting their antimicrobial activity against common fish and shrimp pathogens. The beneficial properties of these microbes primarily reflect immune system enhancement (phenoloxidase activity, phagocytic activity and clearance efficiency), competitive exclusion or antibacterial substance production (Wang et al., 2008; Liu et al., 2010; Balcázar and Rojas-Luna, 2007; Vaseeharan and Ramasamy, 2003; Aly et al., 2008; Kumar et al., 2006, 2008; Tseng et al., 2009; Salinas et al., 2005; Raida et al., 2003; Newaj-Fyzul et al., 2007). In addition, probiotic treatment with *B. subtilis* enhanced the growth and viability of beneficial lactic acid bacteria in the guts of humans and animals (Ngo Thi Hoa et al., 2000).

Prior to investigating the potential probiotic properties of *B. subtilis* in Nile tilapia, the scope of the present study was to assess the impact of the early microbial contact of tilapia larvae on the tilapia gut microbial assembly during later ontogenetic stages. We hypothesized that administration of the probiotic strain to fish larvae early in life, when the gut microbiota is still developing, enhances gut-colonization success and therefore leads to the development of distinct gut communities, even after the fish are exposed to conventional husbandry conditions in active suspension systems.

5.2 Methods

5.2.1 Ethics statement

This experiment was performed in accordance with Dutch regulations regarding the use of experimental animals and approved by the Ethical Committee of Wageningen University for animal experiments (Project Name: Promicrobe; Registration code: 2011076.c).

5.2.2 Experimental Design

The experimental period was divided into three stages: axenic, probiotic and active suspension. The first two stages were conducted under laboratory conditions, while the third stage was conducted under normal rearing conditions at the Aquatic Research Facility of CARUS, the Animal Experimental Facility of Wageningen University. The total experimental period was 28 days, which is considered sufficient for major ontogenetic changes to occur in tilapia until larvae enter the early juvenile stage (Fujimura and Okada, 2007). First, from two days post-fertilization, the eggs were reared under axenic conditions

for seven days (Days 1 - 7). Subsequently, the axenic larvae were split into two groups. Half of the larvae was divided over three replicate active suspension tanks, i.e., C1-3 (from day 8 to 28), while the other half was divided over two probiotic chambers (P-CH1 and P-CH2). Probiotic bacteria were supplied for seven consecutive days (days 8 - 14), after which the larvae from these probiotic chambers were divided over three active suspension tanks (P1-3), where these fish were further raised for another 14 days (day 15 - 28) (Figure 5.1). Throughout the text, "Control (C) treatment" refers to the axenic larvae directly transferred into xenic active suspension tanks, whereas "Probiotic (P) treatment" refers to the axenic larvae initially exposed to the probiotic strain and subsequently transferred to active suspension tanks.

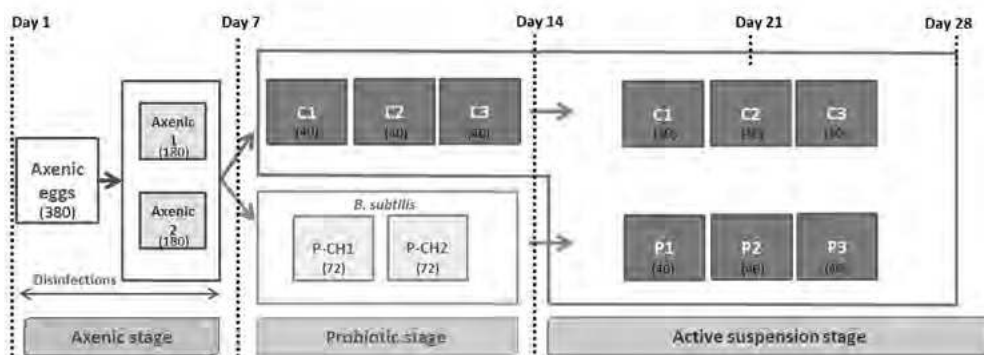


Figure 5.1. Experimental set-up during the 28-day experimental period. The period was divided into three different stages: Axenic, Probiotic and Active suspension. The numbers in parentheses indicate the initial number of eggs/larvae distributed in the tanks/chambers at each experimental stage. P-CH: probiotic chamber, C: control treatment, P: probiotic treatment. 1, 2 and 3: replicate tanks 1, 2 and 3.

5.2.3 Axenic conditions

To diminish the effects of water microbiota on early gut colonization, the larvae were initially reared under axenic conditions according to Situmorang et al., (2014). Briefly, two days post-fertilization eggs were washed from the mouth of an adult female Nile tilapia. Upon collection, the eggs were immersed in 30% hydrogen peroxide (Merck-Millipore, Amsterdam, The Netherlands) and diluted in autoclaved synthetic freshwater (ASF) with a final active peroxide concentration of 2 g L⁻¹ for 10 min at 26 ± 1°C. The synthetic freshwater contained 96 mg L⁻¹ NaHCO₃, 60 mg L⁻¹ CaSO₄·2H₂O, 60 mg L⁻¹ MgSO₄ and 4 mg L⁻¹ KCl in nanopure water (US Environmental Protection Agency, 2002). The eggs were subsequently washed four times with 250 mL of 0.2 µm-filtered ASF at 26 ± 1°C to remove loose bacteria and damaged eggs. Twenty-four hours later, a second disinfection was conducted using 100 mL of NaClO (14%) in 1 L of ASF, following the same immersion protocol as described for day 1. During the immersion, the beakers were occasionally

shaken to ensure the optimal penetration of disinfectant into the eggs. This disinfection method was applicable only prior to egg hatching, as this strategy was considered unsafe or lethal for larvae (Situmorang et al., 2014). On day 3, as soon as the eggs began to hatch, the disinfection process was conducted by the immersion of eggs/larvae into 1 L of ASF containing 100 mg L⁻¹ Bronopol (2-bromo-2-nitropropane-1,3-diol; Sigma-Aldrich 13,470-8, Zwijndrecht, The Netherlands) for 30 min. Bronopol disinfection was performed daily from day 3 to 7 during the axenic stage. All disinfection procedures were performed in a laminar flow hood, and all equipment and tools were autoclaved prior to use.

Following disinfection on day 3, the hatched eggs were aseptically distributed into 2-L sterile glass bottles (Duran GL45) containing 500 mL axenic incubation medium (Table S5.1) and incubated at a density of 300 eggs L⁻¹. Air was provided to the bottles through a filter (0.25 µm, Whatman) from a single port safety cap (Duran DG), and the bottles were placed in a moving water-bath (Julabo SW23, 110 rpm at 27°C). During the axenic period (day 1 to 7), the larvae were not fed.

On day 8, a total of 144 axenic larvae were equally distributed into the two probiotic chambers and exposed to the probiotic bacteria, whereas 120 larvae were equally distributed into the three AS aquaria and exposed to conventional (control) rearing conditions (see below for details).

5.2.4 Probiotic conditions

To rear the larvae during the probiotic stage a custom-made cabinet was used (Figure S5.1). The larvae were reared in two custom-made 2-L glass chambers installed inside the cabinet. The air in the cabinet was pressurized, and the internal surfaces were sterilized at hourly intervals through UV irradiation. The chambers were water-heated at 27 ± 1°C using the space available within the double-layered outer glass wall. Air was supplied through 0.25-µm syringe filters (Whatman) placed on single port safety caps (Duran DG). Larvae collection and water replacement were conducted through a bottom outlet valve (Figure S5.1). The larvae were sieved and washed with ASF daily and subsequently externally disinfected with Bronopol solution (100 µL L⁻¹) for 30 min (see axenic stage disinfection from day 3 onwards). The incubation chambers were replaced daily with autoclaved chambers.

The probiotic strain, *B. subtilis* (Microbiologics 0269P, Mijdrecht, The Netherlands), was grown for 24 h on E-type agar containing 15 g L⁻¹ bacteriological agar type E, 10 g L⁻¹ Tryptone, 5 g L⁻¹ NaCl, and 5 g L⁻¹ yeast extract powder in 1 L of demineralized water. The bacterial colonies were further cultured in 100 mL of liquid medium containing 7 g L⁻¹ K₂HPO₄, 2.0 g L⁻¹ KH₂PO₄, 1.0 g L⁻¹ (NH₄)₂SO₄, 1.0 g L⁻¹ glucose, 0.5 g L⁻¹ sodium citrate, and 0.1 g L⁻¹ MgSO₄·7H₂O in demineralized water for another 24 h. Prior to use, the cultures were centrifuged for 7 min (8000 × *g*) to pellet the *B. subtilis*. The supernatant was

discarded, and the cell density was adjusted to 1×10^7 cfu mL⁻¹ with 1.5 L ASF water as spectrophotometrically determined by optical density (OD₆₀₀) (Bio-Rad SmartSpec 3000). In the probiotic chambers, the water containing *B. subtilis* was replaced daily.

5.2.5 Active suspension tanks

Six 20-L aquaria, each connected to a separate 120-L active suspension tank (AST), were used for rearing the larvae under conventional conditions. One month prior to the experiment, 10 adult tilapias were stocked per AST to initiate microbial growth. Prior to the addition of the axenic larvae, adult tilapias were removed, and the water from all six ASTs was mixed and re-distributed over the tanks. Water and suspended solids in the ASTs were constantly mixed and aerated, while the temperature was maintained at 27°C. Total ammonia nitrogen (TAN-N), nitrite (NO₂⁻-N), nitrate (NO₃⁻-N), dissolved oxygen (DO), pH and temperature (°C) were monitored daily in each AST.

During their first week in the ASTs (day 8 to 14), the larvae from the control treatment and in the probiotic chambers were not fed (Figure 5.1). On day 14, the larvae from both probiotic chambers were mixed and redistributed over the three active suspension tanks (40 larvae/tank). Thereafter, the larvae from both treatments were fed daily to apparent satiation (30 min) at 09:00, 12:30 and 16:00 with a commercial crumble larval feed (Skretting Gemma Wean M0.5, 300-500 µm, 58% crude protein, 17% crude fat, 10% ash, 0.6% fibre and 1.3% phosphorus). The feed was divided into daily portions of 4 g in 15-mL Falcon tubes and subsequently sterilized with cobalt-60 gamma irradiation at 25 kGy (Synergy Health, Ede, The Netherlands) prior to the experiment to minimize the viable microbial load entering with the feed, and the 16S ribosomal RNA (rRNA) gene-targeted PCR using DNA extracted from irradiated feed did not yield any products.

5.2.6 Verification of axenic and probiotic conditions by cultivation

During the axenic and probiotic stages, daily samples of the culture medium, eggs and media/water were monitored for the presence of viable bacterial cells. The eggs, larvae and 1 mL of water were separately added into 10 mL of liquid medium (as previously described for *B. subtilis*) and incubated at 37°C for 24 h. The next day, the samples were streaked onto 2YT agar (Biotrading K604P090KP, Mijdrecht, The Netherlands) and incubated for 24 h at 37°C. At 48 h after inoculation, the agar plates were visually assessed for microbial growth.

5.2.7 Sampling of gut and water for bacterial community profiling

For each of the aquaria/chambers, the gut samples from four larvae were collected on days 14, 21 and 28 (Table S5.2, sample meta-data), and the water was also sampled from each aquarium/chamber after filtering 1 L of water through 0.45- and 0.2-µm membrane

filters (Millipore HAWP-04700 and Millipore GTTP-04700). All samples were frozen in liquid nitrogen and stored at -80°C until further analysis. All gut and water samples were stored and individually analysed. The detailed protocols on gut and water sampling are described in Giatsis et al. (2014).

5.2.8 Bacterial community profiling

DNA was extracted from gut samples using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol, with some modifications (Giatsis et al., 2014). For DNA extraction from the water samples, the FastDNA SPIN kit for soil (MP Biomedicals, Ohio, USA) was used. The DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE), and the DNA samples were stored at -80°C until further use. Detailed protocols on gut and water DNA extraction are described elsewhere (Giatsis et al., 2014).

For 16S rRNA gene-based microbial composition profiling, barcoded amplicons from the V4 region of 16S rRNA genes were generated by PCR using the 515F and 806R primers (Caporaso et al., 2010). Seventy different barcodes were used per library, in which the forward and reverse primer of one sample always carried the same barcode. The primer sequence and barcode were separated by a 2-nucleotide linker sequence (GA for 515F and CG for 806R). The extracted DNA was diluted to a concentration of $20\text{ ng }\mu\text{L}^{-1}$ based on NanoDrop (NanoDrop Technologies, Wilmington, DE) spectrophotometric readings. The PCR conditions, DNA purification and library preparations were performed according to Giatsis et al. (2015). The nucleotide sequences were generated using an Illumina HiSeq 2000 sequencer at GATC-Biotech, Konstanz, Germany. Raw sequence data were deposited into the Sequence Read Archive (SRA) at the NCBI under accession number SRP062681.

NG-Tax, an in-house pipeline, was used for the analysis of the 16S rRNA gene sequencing data (Ramiro-Garcia et al., 2016). Briefly, paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and these barcodes were used to separate reads according to sample. The operational taxonomic units (OTUs) were assigned and classified using an open reference approach and a customized SILVA 16S rRNA gene reference database (Quast et al., 2013).

5.2.9 Data handling and statistical analysis

The Bray Curtis dissimilarity was calculated based on square root-transformed relative abundance data. Principal coordinate analysis (PCoA) was performed to represent the samples in a low dimensional space; thus, the relative distances of all points represent the relative dissimilarities of the samples according to the Bray Curtis index. All statistical analyses were performed using the multivariate statistical software package Primer V7

(Primer-E Ltd, Plymouth, UK). BLAST searches were used to identify the closest relatives of selected OTUs (members of the genus *Bacillus*) (Altschul, 1997). Multiple sequence alignments of the sequences were performed in ClustalW2-Phylogeny using neighbour-joining as the clustering method, and the corresponding Newick tree file was visualized using a phylogram constructed in Treedyn (Chevenet et al., 2006).

5.3 Results

5.3.1 Axenic stage

In total, 380 fertilized eggs were available at the start of the experiment. By the end of the hatching period, 304 larvae (80% of all eggs) successfully hatched. On day 7, 120 of the axenic larvae were equally distributed into active suspension tanks C1, C2 and C3 (named as “control treatment”), whereas the remaining larvae were exposed to a high load of *B. subtilis* in chambers P-CH1 and P-CH2 (named “probiotic treatment”) for one week.

Medium and egg samples cultured on agar plates showed no proliferation of microbes throughout the axenic period, and 16S rRNA gene-targeted PCR using DNA extracted from washed and antibiotic-treated eggs and larvae yielded no products, confirming the axenic conditions.

5.3.2 Probiotic stage

The 16S rRNA profiling of water microbiota from chambers P-CH1 and P-CH2 on day 14 confirmed the presence of the probiotic strain in both chambers albeit at different relative abundances. P-CH2 was dominated with *Bacillus*, whereas P-CH1 was dominated with *Pseudomonas* (Figure 5.2). In both chambers, several OTUs belonging to the genus *Bacillus* were present; however, the most abundant *Bacillus* OTU (OTU 814) had 100% sequence identity with the added probiotic strain of *B. subtilis* (Figure S5.2). *Pseudomonas* OTU 338 was present in the water of both chambers at a relative abundance of >20%.

Subsequent analyses of the gut samples from larvae raised in the probiotic chambers showed that *B. subtilis* was among the most dominant species, regardless of the observed differences in the relative abundance of *B. subtilis* in the corresponding water samples. At the end of the probiotic treatment (day 14), *B. subtilis* accounted for approximately half of all bacteria in the gut (average relative abundance). A comparison between the gut samples from control (C1, C2 and C3 tanks) and probiotic treatments (P-CH1 and P-CH2 chambers) indicated a clear difference in the composition of the gut microbiota (Figure 5.3a). This difference reflected, in part, the high relative abundance of *B. subtilis* in the gut of larvae from the probiotic treatment (and the absence of these bacteria from the

control), according to the SIMPER analysis results (contribution: 25%). Other discriminant OTUs were members of the genera *Nocardia*, *Mycobacterium*, *Rhodococcus*, *Rhodanobacter* and *Halomonas* (Table S5.3).

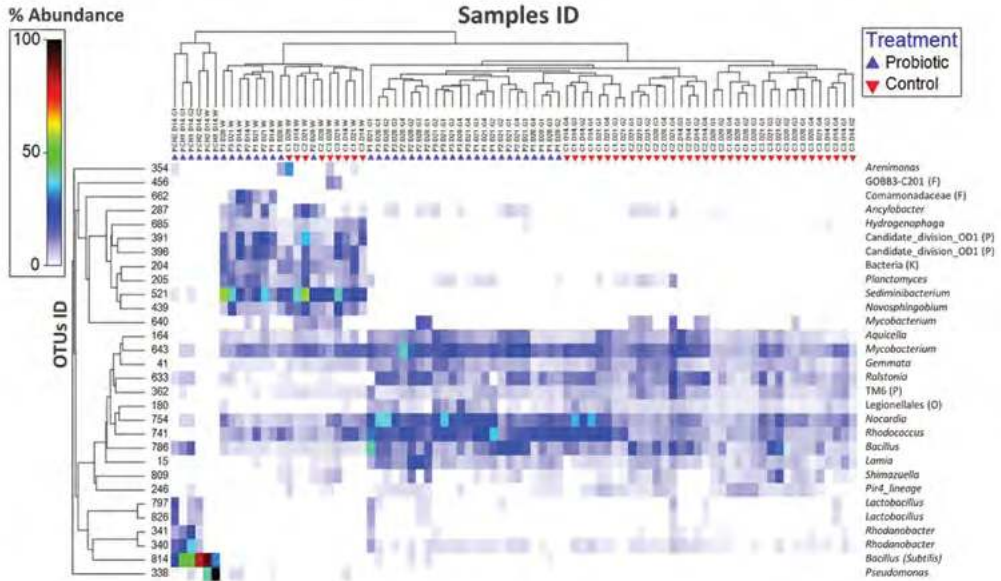


Figure 5.2. Heatmap of the 30 most predominant OTUs among all water and gut samples. Each column in the heatmap represents a sample, and each row represents an OTU. The OTUs were clustered based on group average, as groups of OTUs better define sample clusters. Only the first thirty OTUs contributing most to these clusters are displayed. The samples were clustered according to the unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering on the basis of Bray Curtis dissimilarity and based on the complete OTU dataset. The colours are proportional to the increasing percent relative OTU abundance (from white: lower, to red: higher) within each sample. P1-3 and C1-3: Replicate active suspension tanks 1-3 of the probiotic and control treatment, respectively. D14, 21 and 28: Experimental days 14, 21 and 28. G1-4: The number of replicate gut samples from each tank. G and W: Gut and water samples, respectively. P-CH1 and 2 (probiotic chamber 1 and 2) indicate that water samples were obtained from the probiotic chamber at the end of probiotic period (day 14). Taxonomy (right column) indicates the genus of each OTU ID (left column) unless otherwise stated (i.e., order (O), family (F) and phylum (P)).

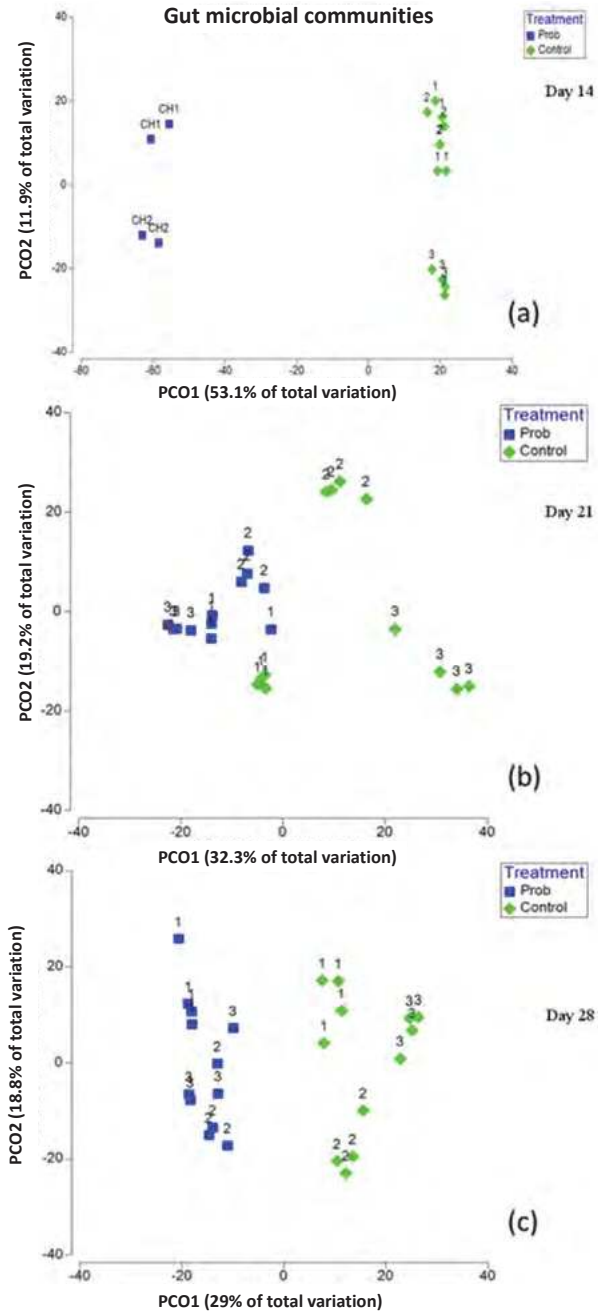


Figure 5.3. Principal coordinate analysis (PCoA) of the gut microbial communities based on the square root-transformed relative abundance data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray Curtis index. Plots (a, b and c): ordinations of all gut samples from probiotic and control treatment from days 14, 21 and 28, respectively. The percentage of total variation explained by each PCo axis is shown in the parentheses.

5.3.3 Active suspension stage

At the end of the probiotic stage (day 14), larvae from the probiotic chambers were transferred to active suspension tanks (P1, P2 and P3). One week after exposure to conventional rearing conditions (day 21), their gut microbiota was significantly different from that of the control treatment larvae, which had been acclimated to non-sterile conditions for one week longer (Figure 5.3b and Table S5.4). The replicate aquaria of the control treatment were significantly more dispersed than those of the probiotic treatment according to multivariate permutation dispersion ($P_{\text{perm}}: 0.011$) (Table S5.5). However, the observed lower dispersion within the probiotic treatment no longer reflected the presence of *B. subtilis* in the gut, as the relative abundance of these bacteria at day 21 was below detection level. The most predominant OTUs in the probiotic treatment were members of the genera *Bacillus*, *Rhodococcus*, *Nocardia*, *Mycobacterium*, *Ralstonia* and *Aquicella* (Figure 5.2). These taxa were also among the most predominant bacteria observed in the gut of the control treatment on day 21 but at different relative abundances. One *Bacillus*-affiliated OTU (OTU 786) was present in all gut samples of both treatments on day 21, with an average relative abundance of 4.1% ($SD \pm 2.3$). BLAST analysis showed that this bacterium was a different species than the administered strain, as its 16S rRNA gene sequence was only 94% identical to that of the probiotic strain of *B. subtilis* (Figure S5.2).

A comparison of the gut microbiota on day 28 showed significantly different communities in the two treatments (Figure 5.3c and Table S5.4). As on day 21, the probiotic strain remained below the detection level. The most predominant genera at day 28 in both treatments were similar to those observed at day 21, albeit at different relative abundances. The within-treatment variability of replicate tanks was no longer significantly different ($P_{\text{perm}}: 0.121$) between control and probiotic treatments (Table S5.5). Notably, the homogeneity of dispersion is a precondition to accurately interpret, but not perform, a PERMANOVA. On day 21, significant differences in the gut communities were detected in both PERMDISP (dispersion) and PERMANOVA (location). Thus, to uncover the nature of the differences among groups, the results are also discussed with respect to the average within- and between-group dissimilarities and the position of the samples from different groups in the PCoA.

5.3.4 Succession of water and gut microbiota

The effect of time on the water and gut microbiota composition was evaluated by comparing the profiles on days 14, 21 and 28. The water microbiota in the tanks was not significantly different between the two treatments on any of the sampling days, but a different pattern was revealed over time (Table S5.6), i.e., water microbial communities from the control treatment clearly clustered by tank, whereas this pattern was not observed in the probiotic treatment (Figure 5.4a and b). *Sediminibacterium* (OTU 521) was

the most abundant genus in the water of both treatments on days 21 and 28, with average relative abundances between 18% and 35%, respectively (Figure 5.2).

Regarding the gut samples, the cluster analysis revealed clearly different patterns between treatments. In the control treatment, the variability of the gut microbial communities was higher between tanks than between days (Figure 5.4c), consistent with the pattern observed in the water (Figure 5.4a). In the probiotic treatment, the tank effect was not as clear as in the case of the control (Figure 5.4d).

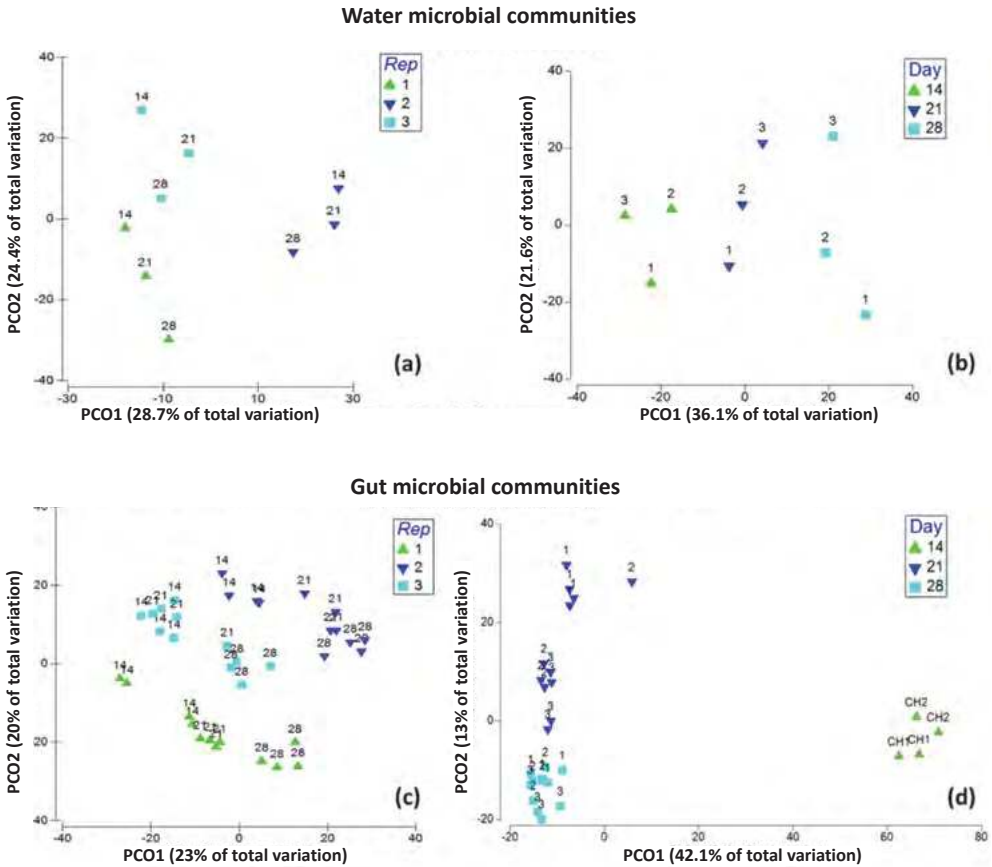


Figure 5.4. Principal coordinate analysis (PCoA) of microbial communities in the gut and water samples of the control and probiotic treatments. The plots are based on the square root-transformed relative abundance data for the OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray Curtis index. Ordination plots of water (a and b) and gut (c and d) microbial communities from the control (a and c) and probiotic treatments (b and d), respectively. The numbers 14, 21 and 28 indicate the three experimental days that the samples were collected. Rep 1-3: replicate tank 1 to 3. The percentage of total variation explained by each PCo axis is shown in the parentheses.

5.4 Discussion

Probiotics have been widely applied in aquaculture for many years. However, assessing the effectiveness of their use is problematic because the probiotic strains residing in the gut transiently and rapidly fall below detection limits. This effect most likely reflects the low survival and proliferation rate of probiotics in the fish gut (Gatesoupe, 1999; Grześkowiak et al., 2012). For prolonged gut colonization by probiotics, it is paramount to understand the principles governing microbial community assembly and the persistence of specific populations.

In the present study, we attempted to enhance the colonization success of the probiotics in a “virgin” gut ecosystem by maintaining the larvae in axenic conditions prior to exposure to a probiotic strain. The results indicated that *B. subtilis* was present in the waters of both probiotic chambers, albeit at different relative abundances. In the first chamber, *B. subtilis* was the most dominant water OTU; in the second chamber, populations belonging to the genus *Pseudomonas* were present at a higher relative abundance, although the same amount of *B. subtilis* was added daily to both chambers. Apparently, we were not successful in maintaining the gnotobiotic conditions of the water, as both chambers were contaminated with *Pseudomonas*.

However, regardless of the presence of *Pseudomonas* in the water of both P-CH, *Pseudomonas* was barely detected in the gut at that time point. These results demonstrated that (a) *B. subtilis* can be successfully transferred to the gut through water, and (b) *Pseudomonas* cannot be successfully transferred to the gut irrespective of its abundance in the source water. Members of the genus *Pseudomonas* are non-sporulating, aerobic Gram-negative rods observed in a wide range of terrestrial and aquatic environments in addition to plant and animal-associated ecosystems. Consistent with this broad environmental distribution, these bacteria exhibit metabolic versatility (Pérez et al., 2010; Özen and Ussery, 2012). In fish, *Pseudomonas* is commonly observed in the faeces and gut of both salt and fresh water species (Austin, 2006; Cahill, 1990). Various phenomena, such as the competitive exclusion of *Pseudomonas* by *B. subtilis*, differences in the ecological preference/adaptability of the two species in the gut and host selectivity for *Bacillus* but not *Pseudomonas*, may have played a role in the recovery of *B. subtilis* but not *Pseudomonas* from the guts of tilapia larvae during probiotic treatment.

In the present study, *B. subtilis* was only transiently detected and thus was not included in the stable larval microbiota. One week after the larvae were exposed to conventional aquaculture conditions (day 21), the abundance of *B. subtilis* was already below detection level. This finding underscores the challenge of successfully colonizing the fish gut with a probiotic strain. The presence of this strain in the gut can be expected only until a few days after probiotic discontinuation, consistent with previous studies reporting that probiotic strains added through water or feed could be detected in the guts of fish and shrimp

for only a few days after discontinuing the probiotic application (Kim and Austin, 2006; Vaseeharan and Ramasamy, 2003; Sharifuzzaman et al., 2014; Zhang et al., 2009; Robertson et al., 2000). In a recent study, Standen et al. (2015) observed that the presence of a multi-species probiotic containing *Lactobacillus reuteri*, *Bacillus subtilis*, *Enterococcus faecium* and *Pediococcus acidilactici* gradually declined after probiotic cessation. However, the detection of each probiotic strain in the gut varied between 6 and 18 days after reverting to the control diet, suggesting that the persistence of probiotics in the gut is species-specific. Furthermore, the probiotic supplemented feed was administered to adult tilapia for eight weeks, whereas here, *B. subtilis* was administered to axenic tilapia larvae for one week. The dosage and duration of supplementation and the selection of the probiotic strain/s might influence colonization success, and the persistence of the probiotic might also depend on the developmental state of the animal (Pérez et al., 2010; Ramos et al., 2013; Gerritsen et al., 2011).

In the present study, the development of gut microbial communities in the two treatments revealed different patterns. The gut microbiota in the control treatment were more affected by spatial (tank) rather than temporal differences (time), i.e., the samples clustered according to replicate tank rather than sampling day (Figure 5.4c). Interestingly, the spatiotemporal patterns observed in gut bacterial communities were also observed in the water microbiota of the control treatment. In a previous study on tilapia larvae, we observed that tank replication determined the inter-individual variation of gut microbiota (Giatsis et al. 2014). Here, this finding was differently applied for each treatment. This difference could be associated with the initial contact of larvae with the probiotic strain. At the time larvae from the probiotic treatment were introduced to conventional conditions, their guts were already colonized with certain bacteria (primarily *B. subtilis*), whereas the larvae from the control treatment were introduced to conventional conditions while their guts were germ-free.

The successful transfer via water and the high relative abundance of the probiotic strain in the gut indicate that it is conceivable to inoculate the gut community with bacteria during early gut development. At the end of the probiotic stage, four gut samples were collected from both probiotic chambers. The observed inter-individual variation in the abundance of the probiotic strain in the gut suggests that the results should be interpreted with caution. Studies on the use of probiotics in humans and animals have also reported high inter-individual variation, even for identically treated groups (Garcia-Mazcorro et al., 2011; Baxter et al., 2015; Montesi et al., 2005; Ling et al., 2013). The abundance of a probiotic strain in the gut or faeces is neither clear-cut proof of successful probiotic use nor evidence of probiosis, primarily reflecting the difficulty in establishing the precise relationships between the health benefits and the presence and/or relative abundance of a specific microbe (except for specific pathogens) (Gerritsen et al., 2011). Notably, inter-individual variation could certainly mask treatment effects by either type-I or type-II errors. Thus,

more data points (higher statistical power) should be included in future studies to verify whether the observed correlations are maintained.

The observed low persistence of the probiotic strain in the gut could indicate a lack of ecological preference or adaptability of the probiotic strain in the gut and/or host selectivity against the probiotic. Nevertheless, the gut communities remained different between treatments, even after discontinuation of the probiotic and despite receiving the same diet and living in water containing similar microbial profiles (Figure 5.5).

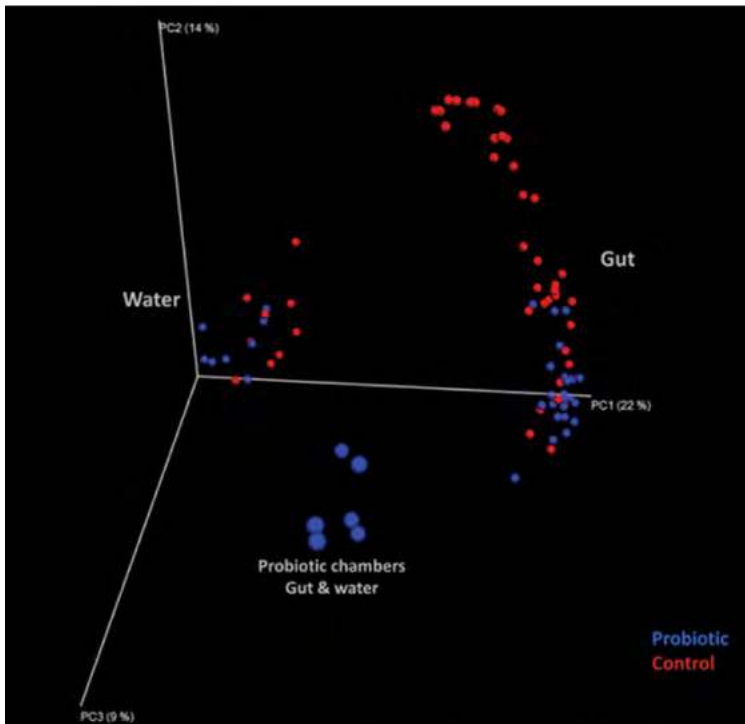


Figure 5.5. Principal coordinate analysis (PCoA) of the gut (right) and water (left) microbial communities from all sampling days (14, 21 and 28) and treatments (probiotic and control). The blue spheres along the PC3 (centre) correspond to water and gut samples from the probiotic chambers on day 14. This analysis was based on the square root-transformed relative abundance data of the OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray Curtis index. The percentage of total variation explained by each of the three first PCo axes is shown in the parentheses.

It is doubtful (although there is no clear-cut evidence) that the presence of *Pseudomonas* in the probiotic chambers induced the observed probiotic legacy effects, as *Pseudomonas*, although present in the water, was nearly absent from the larval gut. It is more likely that the initial presence of *B. subtilis* led to a different sequence of events of bacterial colonization, reflecting the synergistic or antagonistic interactions between the bacteria already present and/or other bacteria entering the gut. It is also likely that the transition from axenic to either conventional or probiotic conditions differentially modulated the

immune response and mucosal innate immunity of the larvae. The responses of IgA, cytokine production and the development of mucosal T-regulatory cells were likely reduced in germ-free animals through the activation of TLR-dependent pathways (Gomez de Agüero et al., 2016; Fagundes et al., 2012; Kubinak and Round, 2012). TLR9 was expressed on the colonic apical surface in wild type but not germ-free mice (Ewaschuk et al., 2007). These results demonstrated that the gut microbiota alters the way the host reacts to infectious stimuli or particular bacterial taxa (Neish, 2014) entering the gut, and this difference could also be the case in the present study. Differences in the initial priming of the immune system in the probiotic group are certainly among the potential mechanisms (Thompson et al., 2010; Corthésy et al., 2007).

After the discontinuation of probiotic administration, differences in the gut microbiota between treatments primarily reflected differences in the relative abundance of the genera *Nocardia*, *Mycobacterium*, *Rhodococcus*, *Rhodanobacter*, *Halomonas* and *Ralstonia*. Most of these genera have been identified in previous studies on tilapia larvae and other fish species. The genus *Rhodococcus* has been reported in the guts of tilapia, sole, red rock fish, Norwegian mackerel, USA smelt, rainbow trout and shrimp (Giatsis et al., 2015; Kim et al., 2007; Sanchez et al., 2012; Spanggaard et al., 2000; Huber et al., 2004), and the genus *Nocardia* has been reported in the guts of tilapia (Kohl et al., 2014; Giatsis et al., 2015) and Atlantic salmon (Bakke-McKellep et al., 2007). *Ralstonia* has been observed in the guts of seabass (Carda-Diéguez et al., 2014), rainbow trout (Kim et al., 2007; Navarrete et al., 2012; Pond et al., 2006), yellow catfish (Wu et al., 2010), zebrafish (Rawls et al., 2004) and shrimp (Durand et al., 2010). Furthermore, members of the genus *Halomonas* have been reported in the guts of Arctic charr (Ringo et al., 2006), Atlantic cod (Fjellheim et al., 2011), Midas cichlids (Franchini et al., 2014), queen conch (Carrascal et al., 2014) and Artemia brine shrimp (Tkavc et al., 2011; Riddle et al., 2013). These findings indicate that some of the predominant genera observed in the present study could represent common members of the gut microbiota of tilapia larvae or fish in general, suggesting that (a) host-specificity for particular microbial taxa is modulated by selective pressures within the host gut, and (b) these taxa are involved in major metabolic functions in the fish gut. Host-selective capabilities have been revealed in axenic zebrafish (*Danio rerio*) that received a faecal transplant derived from mice. The implanted mouse community subsequently shifted towards a state resembling a native zebrafish community (Rawls et al., 2006). In addition, zebrafish originating from the wild shared a core gut microbiota with those reared in captivity, demonstrating a host-specific microbial community in the gut (Roeselers et al., 2011). The observed differences in the abundance of these genera within and between studies could reflect a certain degree of influence of the environmental microbiota (i.e., available bacteria, including the probiotic strain used in the present study), community-level interactions and dietary interventions, underlying powerful organizing principles in community composition.

To what extent post-treatment gut microbial uniformity or distinctness reflects a sustained effect of the probiotic remains unknown. We suggest that future studies focus on the long-term effects of probiotic legacy during the early developmental stages of animals. To observe a general phenomenon, future experiments are needed to determine how this effect compares with that of antibiotic or dietary interventions. It has been suggested that legacy effects in humans play a role in defining the microbial structure during early life stages, and these effects can be minimized based on the diet of the host (Turnbaugh et al., 2009). If this idea also applies to animals, then the early administration of a probiotic strain, accompanied by continuous prebiotic administration, could further extend probiotic residence in the gut, even after its discontinuation.

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Supplementary material

Table S5.1. Antibiotics and antifungals used for the preparation of the incubation medium. All components were diluted in autoclaved synthetic freshwater (ASF) to the concentration indicated in the table. Original protocol has been obtained by Situmorang et al. (2014) and modifications have been applied regarding the addition of Gentamycin in the incubation medium.

Name	Concentration	Dosage	Description	Source
Kanamycin Sulphate	Sigma T7783	1 mL L ⁻¹	Broad spectrum antibiotic	(Situmorang et al., 2014)
Rifampicin	Sigma K1377	1 mL L ⁻¹	Broad spectrum antibiotic	(Situmorang et al., 2014)
Ampicillin sodium salt	Sigma A0166	1 mL L ⁻¹	Broad spectrum antibiotic	(Situmorang et al., 2014)
Trimethoprim	Sigma 89307	1 mL L ⁻¹	Bacteriostatic antibiotic	(Situmorang et al., 2014)
Gentamicin	Sigma G1397	50 µL L ⁻¹	Gram-negative antibiotic	Rosco NeoSensitabs
Amphotericin B	Sigma A9528	100 µL L ⁻¹	Polyene antifungal	(Situmorang et al., 2014)
Fluorescent Brightener 28	Sigma F3543	2.5 mL L ⁻¹	Antifungal	(Situmorang et al., 2014)

Table S5.2. Meta-data of gut (G) and water (W) samples from the control (C) and probiotic (P) treatment. Microbial communities were characterized on day (D) 14, 21 & 28 in the probiotic chamber (P-CH) and/or the active suspension tanks. Two replicate probiotic chambers and three replicate active suspension tanks were sampled per treatment (C1-3 & P1-3) from which a water and four replicate gut samples (G1-4) were analysed.

No	Sample_ID	Treatment	Culture system	Biome	Day	Tank	Replicate
1	P-CH1.D14.W	Probiotic	Probiotic chamber	Water	14	1	-
2	P-CH2.D14.W	Probiotic	Probiotic chamber	Water	14	2	-
3	C1.D14.W	Control	Active suspension	Water	14	1	-
4	C2.D14.W	Control	Active suspension	Water	14	2	-
5	C3.D14.W	Control	Active suspension	Water	14	3	-
6	P1.D14.W	Probiotic	Active suspension	Water	14	1	-
7	P2.D14.W	Probiotic	Active suspension	Water	14	2	-
8	P3.D14.W	Probiotic	Active suspension	Water	14	3	-
10	C1.D21.W	Control	Active suspension	Water	21	1	-
11	C2.D21.W	Control	Active suspension	Water	21	2	-
12	C3.D21.W	Control	Active suspension	Water	21	3	-
13	P1.D21.W	Probiotic	Active suspension	Water	21	1	-
14	P2.D21.W	Probiotic	Active suspension	Water	21	2	-
15	P3.D21.W	Probiotic	Active suspension	Water	21	3	-
17	C1.D28.W	Control	Active suspension	Water	28	1	-
18	C2.D28.W	Control	Active suspension	Water	28	2	-
19	C3.D28.W	Control	Active suspension	Water	28	3	-
20	P1.D28.W	Probiotic	Active suspension	Water	28	1	-
21	P2.D28.W	Probiotic	Active suspension	Water	28	2	-
22	P3.D28.W	Probiotic	Active suspension	Water	28	3	-
24	C1.D14.G1	Control	Active suspension	Whole gut	14	1	1
25	C1.D14.G2	Control	Active suspension	Whole gut	14	1	2
26	C1.D14.G3	Control	Active suspension	Whole gut	14	1	3
27	C1.D14.G4	Control	Active suspension	Whole gut	14	1	4
28	C2.D14.G1	Control	Active suspension	Whole gut	14	2	1
29	C2.D14.G2	Control	Active suspension	Whole gut	14	2	2
30	C2.D14.G3	Control	Active suspension	Whole gut	14	2	3
31	C2.D14.G4	Control	Active suspension	Whole gut	14	2	4
32	C3.D14.G1	Control	Active suspension	Whole gut	14	3	1

No	Sample_ID	Treatment	Culture system	Biome	Day	Tank	Replicate
33	C3.D14.G2	Control	Active suspension	Whole gut	14	3	2
34	C3.D14.G3	Control	Active suspension	Whole gut	14	3	3
35	C3.D14.G4	Control	Active suspension	Whole gut	14	3	4
36	C1.D21.G1	Control	Active suspension	Whole gut	21	1	1
37	C1.D21.G2	Control	Active suspension	Whole gut	21	1	2
38	C1.D21.G3	Control	Active suspension	Whole gut	21	1	3
39	C1.D21.G4	Control	Active suspension	Whole gut	21	1	4
40	C2.D21.G1	Control	Active suspension	Whole gut	21	2	1
41	C2.D21.G2	Control	Active suspension	Whole gut	21	2	2
42	C2.D21.G3	Control	Active suspension	Whole gut	21	2	3
43	C2.D21.G4	Control	Active suspension	Whole gut	21	2	4
44	C3.D21.G1	Control	Active suspension	Whole gut	21	3	1
45	C3.D21.G2	Control	Active suspension	Whole gut	21	3	2
46	C3.D21.G3	Control	Active suspension	Whole gut	21	3	3
47	C3.D21.G4	Control	Active suspension	Whole gut	21	3	4
48	C1.D28.G1	Control	Active suspension	Whole gut	28	1	1
49	C1.D28.G2	Control	Active suspension	Whole gut	28	1	2
50	C1.D28.G3	Control	Active suspension	Whole gut	28	1	3
51	C1.D28.G4	Control	Active suspension	Whole gut	28	1	4
52	C2.D28.G1	Control	Active suspension	Whole gut	28	2	1
53	C2.D28.G2	Control	Active suspension	Whole gut	28	2	2
54	C2.D28.G3	Control	Active suspension	Whole gut	28	2	3
55	C2.D28.G4	Control	Active suspension	Whole gut	28	2	4
56	C3.D28.G1	Control	Active suspension	Whole gut	28	3	1
57	C3.D28.G2	Control	Active suspension	Whole gut	28	3	2
58	C3.D28.G3	Control	Active suspension	Whole gut	28	3	3
59	C3.D28.G4	Control	Active suspension	Whole gut	28	3	4
60	P1.D14.G1	Probiotic	Probiotic chamber	Whole gut	14	1	1
61	P1.D14.G2	Probiotic	Probiotic chamber	Whole gut	14	1	2
62	P2.D14.G1	Probiotic	Probiotic chamber	Whole gut	14	2	3
63	P2.D14.G2	Probiotic	Probiotic chamber	Whole gut	14	2	4
64	P1.D21.G1	Probiotic	Active suspension	Whole gut	21	1	1
65	P1.D21.G2	Probiotic	Active suspension	Whole gut	21	1	2
66	P1.D21.G3	Probiotic	Active suspension	Whole gut	21	1	3
67	P1.D21.G4	Probiotic	Active suspension	Whole gut	21	1	4
68	P2.D21.G1	Probiotic	Active suspension	Whole gut	21	2	1
69	P2.D21.G2	Probiotic	Active suspension	Whole gut	21	2	2
70	P2.D21.G3	Probiotic	Active suspension	Whole gut	21	2	3
71	P2.D21.G4	Probiotic	Active suspension	Whole gut	21	2	4
72	P3.D21.G1	Probiotic	Active suspension	Whole gut	21	3	1
73	P3.D21.G2	Probiotic	Active suspension	Whole gut	21	3	2
74	P3.D21.G3	Probiotic	Active suspension	Whole gut	21	3	3
75	P3.D21.G4	Probiotic	Active suspension	Whole gut	21	3	4
80	P1.D28.G1	Probiotic	Active suspension	Whole gut	28	1	1
81	P1.D28.G2	Probiotic	Active suspension	Whole gut	28	1	2
82	P1.D28.G3	Probiotic	Active suspension	Whole gut	28	1	3
83	P1.D28.G4	Probiotic	Active suspension	Whole gut	28	1	4
84	P2.D28.G1	Probiotic	Active suspension	Whole gut	28	2	1
85	P2.D28.G2	Probiotic	Active suspension	Whole gut	28	2	2
86	P2.D28.G3	Probiotic	Active suspension	Whole gut	28	2	3
87	P2.D28.G4	Probiotic	Active suspension	Whole gut	28	2	4
88	P3.D28.G1	Probiotic	Active suspension	Whole gut	28	3	1
89	P3.D28.G2	Probiotic	Active suspension	Whole gut	28	3	2
90	P3.D28.G3	Probiotic	Active suspension	Whole gut	28	3	3
91	P3.D28.G4	Probiotic	Active suspension	Whole gut	28	3	4

Table S5.3. Similarity percentages (SIMPER) analysis of gut microbiota on day 14. Table indicates the foremost three characteristic OTUs from each group contributing to the discrimination between the control and probiotic treatment. Contribution values indicate the importance of each OTU (percentage) in increasing Bray Curtis dissimilarity between the two treatments.

Similarity Percentages - species contributions

Data type: Abundance

Resemblance: Bray-Curtis dissimilarity

Groups Control & Probiotic

Average dissimilarity = 96.82

Genus (OTUs)	Group Control	Group Probiotic	Av. Dissimilarity	Contrib%	Cum.%
	Av. Abundance	Av. Abundance			
Bacillus (814)	0	47.95	23.97	24.76	24.76
Nocardia (754)	12.45	0.12	6.16	6.37	31.13
Mycobacterium (643)	11.53	0.18	5.67	5.86	36.99
Rhodococcus (741)	9.21	0	4.61	4.76	41.75
Rhodanobacter (340)	0.34	8.2	3.94	4.07	45.81
Halomonas (496)	0.25	3.6	1.69	1.74	47.55

Table S5.4. Permutational MANOVA test and pairwise comparisons of gut microbiota for main factors effect and interaction terms. Analysis is based on Bray Curtis similarity of square root transformed relative abundance data. Permutation method was used on unrestricted permutations of raw data and 999 permutations. A pseudo-F statistic was computed for each permutation and the P (perm) values give the proportion of permuted pseudo-F statistics that are equal to or greater than the original (un-permuted) pseudo-F statistic. A multivariate analogue to the univariate t-statistic was used for the pairwise comparisons. The effect on gut microbiota was tested for factors "treatment" (control and probiotic) and "day" (day 21 and 28). df: Degrees of freedom, SS and MS: Sum and mean of the squares.

PERMANOVA main test results						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Treatment	1	7269.3	7269.3	8.8805	0.001	998
Day	1	6784.7	6784.7	8.2885	0.001	999
Treatment x Day	1	2036.1	2036.1	2.4874	0.012	999
Residual	44	36017	818.57			
Total	47	52107				
PERMANOVA pairwise comparisons						
Term 'Treatment x Day' for pairs of levels of factor 'Treatment'						
Within level 'Day 21' of factor 'Day'						
Groups	df	t	P(perm)	Unique perms		
Control vs. Probiotic	22	2.1573	0.001	996		
Within level 'Day 28' of factor 'Day'						
Control vs. Probiotic	22	2.5883	0.001	999		
Term 'Treatment x Day' for pairs of levels of factor 'Day'						
Within level 'Control' of factor 'Treatment'						
Day 21 vs 28	22	1.9031	0.006	996		
Within level 'Probiotic' of factor 'Treatment'						
Day 21 vs 28	22	2.6979	0.001	998		

Table S5.5. Multivariate permutation dispersion (PermDisp) testing for heterogeneity of community structure within each group. Analysis is based on square root relative abundance OTU data. Analysis tested the null hypothesis of no difference between groups' dispersion by computing the dissimilarity of each sample from its group centroid and assessing by permutation analysis whether these dissimilarities differ between the two groups. Significant effects on group dispersion were tested for factor "Treatment" (control & probiotic) on experimental days 21 and 28.

Day 21 of factor "Treatment"	F-statistic	Sample Size	Average Dispersion	
Control		12	28.25 (0.73)	
Probiotic		12	23.84 (1.32)	
Deviation from centroid		t	df	P (perm)
Control vs. Probiotic	8.4965	2,915	22	0.011

Day 28 of factor "Treatment"	F-statistic	Sample Size	Average Dispersion	
Control		12	27.22 (0.81)	
Probiotic		12	24.79 (1.21)	
Deviation from centroid		t	df	P (perm)
Control vs. Probiotic	2.7827	1,6681	22	0.121

Table S5.6. Permutational MANOVA table testing for differences in water microbiota between the two treatments on each experimental day. Analysis was based on Bray Curtis similarity of square root transformed relative abundance data. Permutation method was used on unrestricted permutations of raw data. A pseudo-F statistic was computed for each permutation and the P (perm) values give the proportion of permuted pseudo-F statistics that are equal to or greater than the original (un-permuted) pseudo-F statistic. SS and MS: Sum and mean of the squares, df: degrees of freedom, P(MC): P-values calculated based on 9999 Monte Carlo permutations drawn from the theoretical asymptotic permutation distribution.

PERMANOVA table for factor "Treatment" (Probiotic vs Control)							
Water Day 14							
Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms	P(MC)
Treatment	1	1440.9	1440.9	1.3695	0.19	10	0.29
Residual	4	4208.3	1052.1				
Total	5	5649.2					
Water Day 21							
Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms	P(MC)
Treatment	1	1197	1197	1.2964	0.313	10	0.311
Residual	4	3693.3	923.33				
Total	5	4890.4					
Water Day 28							
Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms	P(MC)
Treatment	1	1440.9	1440.9	1.3695	0.19	10	0.29
Residual	4	4208.3	1052.1				
Total	5	5649.2					

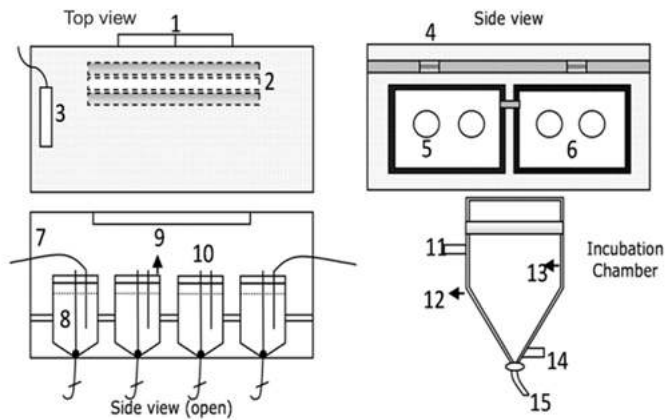


Figure S5.1. Customized cabinet used in probiotic stage. Dimensions LxWxH: 1500x65x70 (mm). (1) HEPA filter (0.3 μm), (2) UV (1 x 30W Ultraviolet 253.7 nm) and daylight (TLD-30W) lamps; (3) Built-in electric sockets, (4) Airtight locks, (5) Gloves, (6) Plexiglas doors (UV-protection), (7) air flow inlet (0.25 μm filter), (8) Custom-built glass chamber (incubation), \varnothing 12 cm, V: 2L, (9) PVC chamber lid \varnothing 16 cm, (10) Bottom sieve remover, (11) Heated water inlet, (12 & 13) Outer/inner double wall glass, (14) Heated water outlet and (15) Discharge valve.

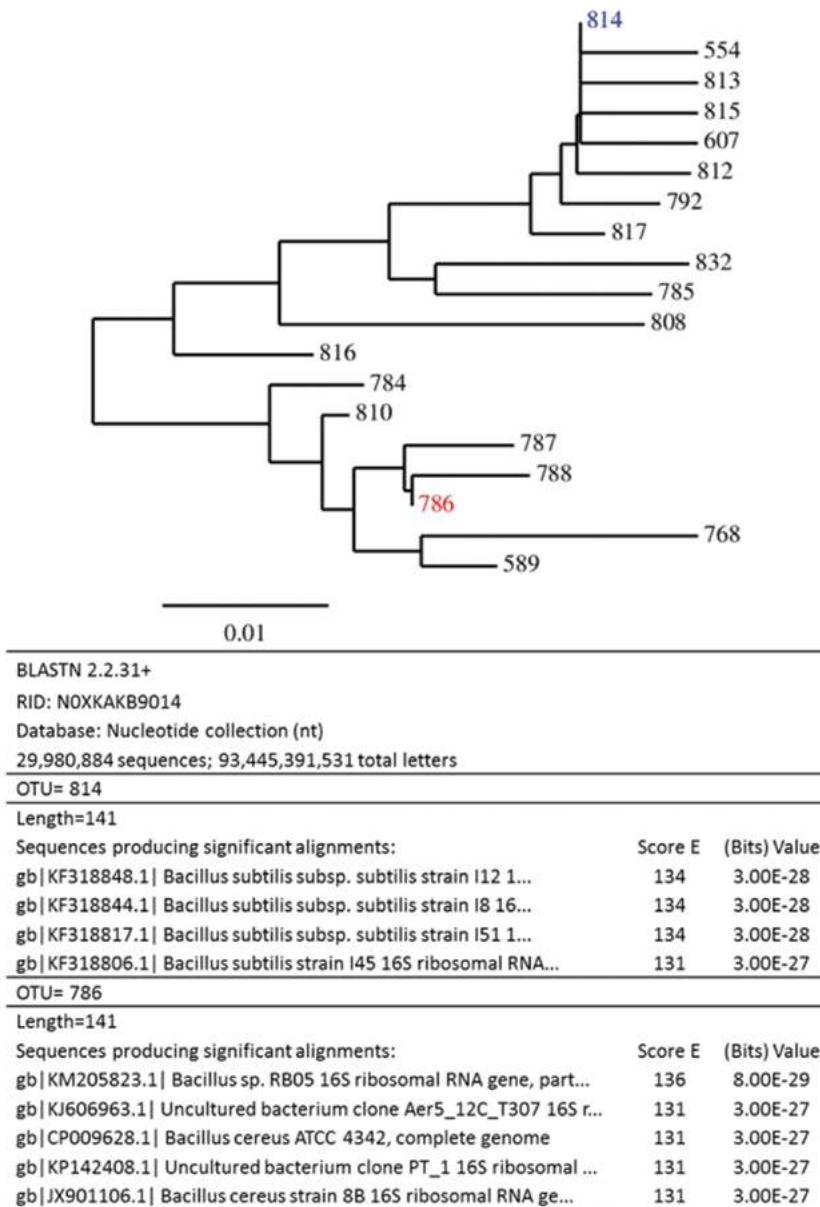
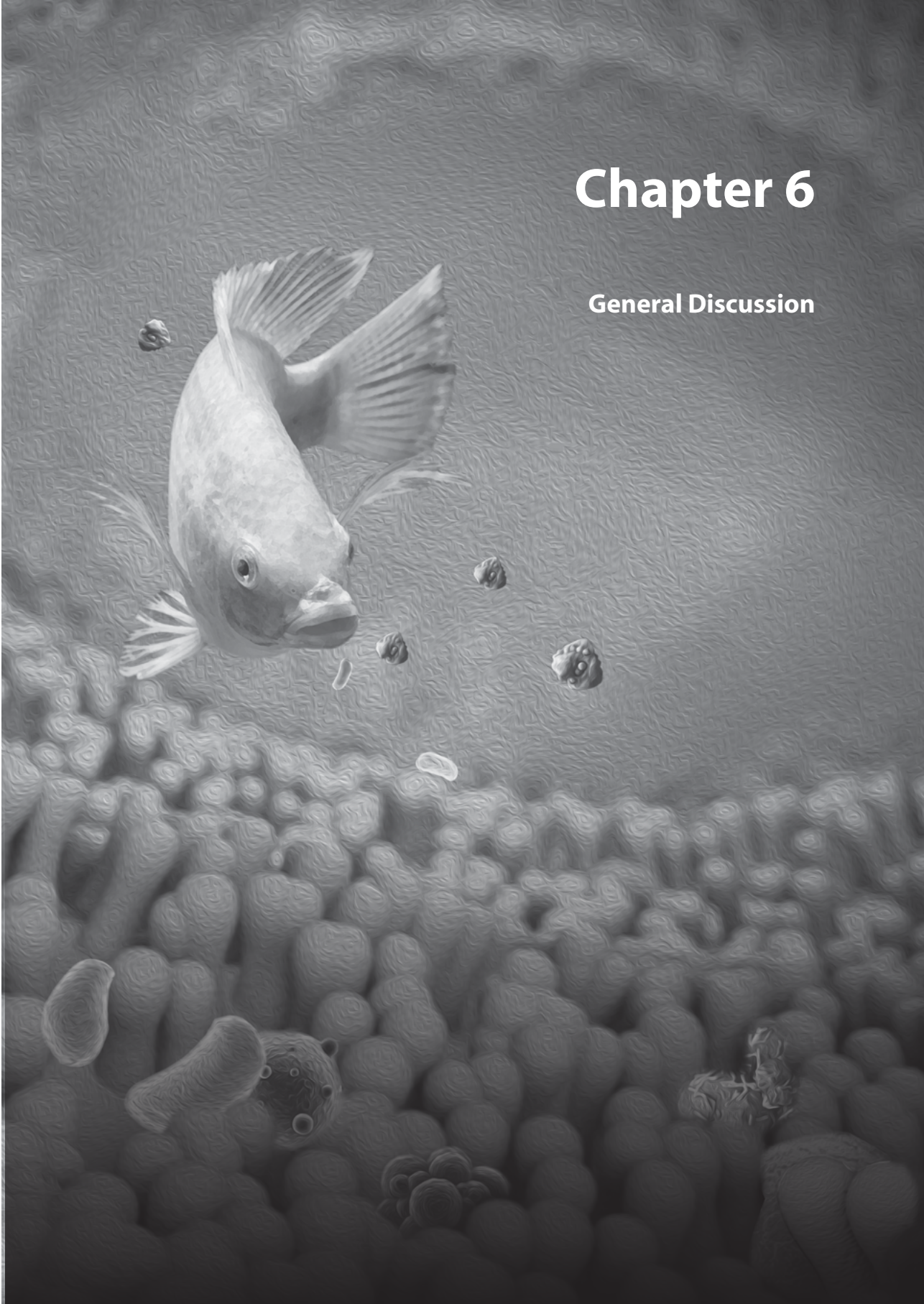


Figure S5.2. Phylogenetic tree phylogram of *Bacillus* related OTUs and BLAST analysis results for the two most abundant OTUs. Numbers at the branch's end indicate the OTU ID. OTU 814 (blue) is the added *B. subtilis* and 786 (red) is the second most abundant OTU member of the genus *Bacillus* belonging to another species, as indicated in the table. Tree branches give an estimate of a phylogeny where branch lengths are proportional to the amount of inferred evolutionary change.

Chapter 6

General Discussion



6.1 Introduction

Aquaculture has evolved as the fastest growing food-producing sector over the last 20 years (FAO, 2014). Nevertheless, the aquaculture production sector is still facing major bottlenecks such as suboptimal growth and high mortality (Attramadal et al., 2012). Fish-microbe interaction has been suggested to be a key factor related to the aforementioned limitations (Vadstein et al., 2013). Exploration of the possible beneficial roles of intestinal microbiota in fish started very recently looking into various aspects, such as nutrient metabolism, innate immune response and epithelial proliferation (Roeselers et al., 2011).

The underlying mechanisms that drive spatio-temporal dynamics of abundance and diversity of intestinal microbiota have not received sufficient attention. Traditional microbial management techniques (e.g. antibiotics, hygiene protocols), and more recently probiotic administration, have been applied empirically (Martínez Cruz et al., 2012) and still lack adequate and consistent scientific evidence to validate the proposed health benefits of microbial management. In order to design innovative and effective microbial management strategies, we need to elucidate the metabolic potential of microbial diversity, disentangle the host-microbe interactions and clarify the environmental impact on gut microbial assembly.

Intestinal colonization is largely a stochastic process, with successful bacteria being those that happened to be present at the right moment and place (Fjellheim et al., 2007). However, environmental, nutritional and physiological influences can lead to the disturbance of diversity, abundance and/or functionality of the gut microbiota (Verschuere et al., 1997; De Schryver and Vadstein, 2014). The microbial composition in the gut is also influenced by microbiota in the environment and by the host itself (Rawls et al., 2006; Navarrete et al., 2012; Verschuere et al., 2000).

The main objective of this thesis was to deepen our understanding on the principal factors driving gut microbiota development. In particular, we determined the impact of rearing environment (e.g. free-living bacteria), early life microbial exposure and probiotic use on the microbial composition and abundance inside the gut of developing tilapia larvae. The sequencing libraries presented in this thesis represent the most comprehensive collection of 16S rRNA gene sequences from gut microbiota of a commercial fish species and its environment. This chapter discusses the research needed to implement more sustainable and applicable microbial management strategies for aquaculture. In addition, a meta-analysis is presented in which our main findings are compared to publically available gut microbiome datasets of different species. In this way, we aimed to provide a broader perspective on the forces that structure gut microbiota, including free-living communities from different environments.

6.2 Temporal variation of gut microbiota

The gut microbiota of fish larvae evolves fast into a complex and dynamic community, which differs by location in the gastro-intestinal tract. Differences in the microbial communities over time are defined as temporal variation. In **chapter 2** we showed that time was the most significant factor related to microbial shifts in the fish gut. Temporal variation of fish gut microbiota is more pronounced during early development when the fish gut is not yet fully developed and the immune system is immature (Gatesoupe, 1999). Thus, early microbial fluctuations in the fish gut are primarily driven by ontogenetic changes (i.e. genetic, physiological), whereas adaptation to nutritional and environmental situations should be expected at later developmental stages (Nayak, 2010b; Romero and Navarrete, 2006; Navarrete et al., 2012; Li et al., 2012b).

The observed rapid and stochastic changes of microbiota in the gut over time suggest that results should be interpreted carefully since observations of start and endpoint do not provide information about the temporal variation in between. Additionally, ontogeny associated to the alimentary canal (morphology and function), immune system, feeding habits (behavior and type) and habitat preference (fresh or saltwater, pelagic or demersal) can be very different between fish species (Hansen and Olafsen, 1999; Bowden et al., 2005; Zapata et al., 2006; Tytler et al., 1990; Noakes and Barlow, 1976; German et al.). Thus, ontogenetic traits should be considered carefully when gut microbial shifts are studied over different developmental stages or between different fish species.

6.3 The impact of feed and water microbiota on gut microbial composition

Regarding within rearing system tank-to-tank variation, our results suggest that variation of gut microbiota between individuals is low as far as fish receive the same diet and share the same water (**Chapter 2**). On the contrary, studies with marine species show that variation between identically reared individuals could be higher than the one observed here (Bakke et al., 2013; Fjellheim et al., 2011; Gatesoupe and Covès, 2012). This can possibly be attributed to the fact that in the studies with marine species larvae are fed live feed, which was not the case in our studies. Since feed associated bacteria affect gut microbiota in fish larvae (McIntosh et al., 2008; Gatesoupe and Covès, 2012; Brunvold et al., 2007), higher batch-to-batch variation should be expected in larvae fed live feed, compared to larvae receiving formulated feed.

Water and feed microbiota are the only two microbial sources for fish. We found that gut microbiota from individuals reared in replicate production systems differed significantly even though systems were handled identically and larvae were fed the same feed. Observed differences of water microbiota between replicate systems is the only plausible

explanation for the observed differences in the fish gut communities. Therefore, we recommend to investigate treatment effects (e.g. different diets) on gut microbiota within the same system (fish share the same water source), rather than between replicate systems. In fish production facilities, this would mean that producers should not always expect similar microbial composition neither in the gut of fish nor in the water of systems managed similarly.

The effect of different rearing techniques i.e. recirculating (RAS) vs active suspension (AS) systems on the bacterial community composition of the larvae was evaluated in **chapters 2 and 3**. We found that the choice of culture system strongly determined the composition and development of bacterial communities in the water, which in turn, triggered changes in the fish gut communities. Our data showed that the water microbiota had a greater impact on the composition of gut communities than feed microbiota, which concurs with the findings of Bakke et al. (2013). These results can be of great significance for aquaculture production as the choice of production system could, to a certain degree, determine the composition of the microbiota in the gut. Thus, one could explore the possibility of water microbial management customized for each production system (e.g. use of probiotics) to realize health benefits for the host and/or environmental benefits by improving water quality.

Stochastic factors also influenced early colonization in the gut. Stochastic phenomena imply that the composition of the microbiota is determined by probability. Because of this stochasticity, sampling power and pooling of samples should be taken into consideration. To obtain a reliable representation of the gut microbial composition, sufficient numbers of individuals need to be sampled. In many studies, individual gut or larvae samples are pooled, running the risk that inter-individual variation could be completely masked. Analyzing individuals is recommended as long as the causes for the inter-individual variability are insufficiently understood. The minimum sample size for hypothesis testing should be estimated from beforehand and the method used for such power calculations should be explained carefully in scientific papers and reports.

The small impact of feed microbiota compared to the impact of water microbiota on gut microbiota found in **chapters 2 and 3** could be a consequence of the fact that tilapia larvae were fed artificial diets that all passed through the same processing steps and which were partially sterilized during processing (e.g. heating, extrusion). However, when deliberately making large contrasts in feed microbiota by mixing commercial feed with different types of sludge as described in **chapter 4**, feed-associated bacteria did affect the gut microbiota. Contrarily to the previous chapters, gut microbiota shared a much higher number of OTUs (operational taxonomic units) with feed than with water microbiota. There are two elements which might explain this outcome. First, the microbial load of the sludge-based diets used in the **chapter 4** was higher and more diverse compared to

the commercial diet. This could explain why gut microbiota of larvae fed the control diet were mainly influenced by the water rather than by the feed microbiota. Second, to make the sludge-based diets, sludge produced under aerobic, methanogenic and denitrifying conditions was mixed with the commercial diet, which resulted in a higher relative species abundance and diversity in feed microbiota than for the water microbiota. Therefore, we believe that the level of (environmental) influence on the gut microbiota highly depends on the degree of diversity and abundance of the microbiota in the environment as well as the metabolic and ecological compatibility of the latter inside the fish gut.

In a study with chicken, Yin et al., (2010) suggested that, at a very early stage of life, bacterial succession in the gut can be influenced by a combination of exposure to proper bacterial inocula and host selective pressures. Depending on the production system, fish can have access to taxonomically and metabolically diverse microbial communities which in turn have a higher or lower chance in adapting to the gut habitat. Nile tilapia has an omnivorous feeding strategy including detritivory (El-Sayed, 2006). The close association with sediments during feeding may suggest that sediment associated microbes are taken up by Nile tilapia. The fact that anaerobic (obligate and facultative) bacteria are among the principal colonizers of the fish gut indicates the preference of these bacterial groups for anaerobic habitats. To which extent species in sediment microbiota could successively establish themselves in the fish gut, depends on their adaptability within the gut environment and the host's preferences. Our experiment with sludge-based diets suggests to further exploring this route.

Summarizing, this shows that the microbial assembly in the fish gut is a skewed selection of feed and water bacteria, as a result of a random sampling from the available environmental bacterial pool, determined by the historical contingency (timing and order of species inflow), species interactions within the gut and host preferences. Understanding how microbiota in water, feed and gut interact still needs further research. Insights are still too limited to be applied in improving the design, microbial management and nutrient cycling of fish rearing systems. Future research should first focus on disentangling the key processes governing selection and propagation of water and feed microbiota, which ultimately will become important species of the microbial community in the adult fish gut.

6.4 The impact of probiotic use on gut composition during early development

Probiotics have been shown to reduce the risk and severity of infections caused by bacterial pathogens (Gatesoupe, 1999; Martínez Cruz et al., 2012) and have been successfully used as immunostimulants (Cain and Swan, 2010; Lepage et al., 2012; Song et al., 2014) and as growth promoters in fish and shrimp (Pérez-Sánchez et al., 2014; Merrifield et al., 2010). Currently, delivery of probiotics through feed in larval stages is the most common strategy for management of larval gut microbiota in aquaculture (Nayak, 2010a). Here, the successful transfer of a probiotic strain via the water into the gut indicates that inoculation of the gut community with bacteria during early gut development is conceivable. To enhance the colonization success of the probiotic inside a “virgin” gut ecosystem, we maintained the larvae under axenic conditions before exposure to a probiotic strain. Although for research purposes the use of gnotobiotic or axenic systems is an excellent tool to extend our understanding on host-microbe interactions and microbial community assembly, in commercial production of fish larvae maintaining axenic conditions is practically impossible. Nevertheless, an early application of probiotics during the yolk sac stage could increase the chances for a successful colonization of the larval gut.

Further, in **chapter 5** we observed two interesting patterns. First, the final concentration of probiotic cells in the water was different between replicate tanks, although the same number of probiotic cells was added daily. Second, the gut microbiota of the larvae was always dominated by *B. subtilis*, the probiotic administered in our study. These findings demonstrate that application of probiotics through water led to successful transfer of the strain into the gut. Nevertheless, the abundance in the water was not a good predictor of the abundance in the fish gut.

After discontinuing the administration of the probiotic strain, its abundance declined. This could indicate a lack of adaptability of the probiotic strain inside the gut and/or strong host selectivity against the probiotic strain. It means that successful colonization of a probiotic strain in the fish gut remains very challenging. The presence of the strain in the gut can only be expected during the period of supplementation or shortly after discontinuation (Kim and Austin, 2006; Vaseeharan and Ramasamy, 2003; Sharifuzzaman et al., 2014; Zhang et al., 2009; Robertson et al., 2000). Dosage and duration of probiotic supplementation may influence colonization success, but the persistence of the probiotic after entering the gut remains largely a black box. Possible factors influencing persistence include the probiotic species, the host species, environmental factors and the developmental stage of the fish (Pérez et al., 2010; Ramos et al., 2013; Standen et al., 2015). Following a similar approach as in fecal microbiota transplantation (FMT), (Khoruts and Sadowsky, 2011), transmitting donor phenotypes of interest could help us understand the mechanisms of colonization and persistence of microbial transplants in the recipient gut.

Regardless of the low survival of the probiotic strain, gut microbiota remained different between treatments even after the discontinuation of probiotic administration and even though the fish were receiving the same diet and swimming in water with a similar microbial profile. The initial presence of *B. subtilis* may have led to a different sequence of events of bacterial colonization due to synergistic or antagonistic interactions the probiotic strain and/or other bacteria entering the gut. We suggest that future studies should focus on the long term effects of probiotic exposure during early developmental stages of fish. It has been suggested that in humans, legacy effects can play a role in defining microbial structure, but that these effects can be quickly minimized by the diet of the host (Turnbaugh et al., 2009b). If this applies also to fish, we suggest that early administration of a probiotic strain/s during the larval stage, accompanied by a continuous prebiotic administration (synbiotics) or dietary management could further extend the probiotic residence in the gut even after its discontinuation.

6.5 Meta-analyses of the fish gut microbiota

Several publications have focused on the impact of nutritional and environmental changes on fish gut microbial communities. In this context, the meta-analysis study of Sullam et al. (2012) has been a valuable contribution towards identifying factors determining microbial assembly inside the fish gut. Authors suggested that gut microbial composition is strongly correlated with species trophic level and habitat salinity. Comparison of gut microbiota with other habitats revealed that fish gut communities are often similar to those of other animals and rarely reflect the communities of their surrounding habitats. However, in that meta-analysis data from both culture-dependent and independent methods were used which could introduce apparent variation between samples and/or different studies.

To determine the relative importance of factors on shaping the microbial communities in the fish gut, we performed a meta-analysis of 16S rRNA gene sequence data from 12 different studies on gut microbiota of different fish species. The meta-analysis incorporated datasets from the three studies included in this thesis, unpublished datasets from two of our previous studies on tilapia larvae and seven publically available gut microbiome datasets from studies performed in trout, carp, zebrafish and tilapia. Data in these studies were generated by using different sequencing platforms (454 Titanium, Illumina HiSeq 2000 and Ion-torrent) and primers targeting different regions of the 16S rRNA gene.

6.5.1 Methodology

Datasets were selected through a review of published studies on fish gut microbiota and their environments. Only data from freshwater species were used due to the lack of publically available data on gut microbiome of saltwater fish species generated by sequencing platforms other than Sanger sequencing. We initially analyzed datasets generated only by the Illumina sequencing platform i.e. from the two studies included in this thesis (**chapter 3 and 4**) and from another two of our unpublished studies on tilapia larvae. From that dataset we sought to determine the relationship between gut microbial communities and those present in their feed and water. The next step was to analyze all available datasets with sequences originating from the gut without including data from non-gut samples. We examined the phylogenetic similarity among gut bacteria of the same and different fish species from different habitats, diets, facilities and developmental stage. Sample metadata and study supplementary information are shown in Table S6.1, 6.2 and 6.3.

Sequencing data were analyzed using NG-Tax, an in-house pipeline for 16S rRNA amplicon analysis, including the prediction of sequence regions not covered by the original sequence data to improve comparability across different platforms (Ramiro Garcia et al., 2016). Statistical analyses were performed using Primer 6 (version 6.1.11) (Primer-E Ltd., Plymouth, United Kingdom) and its PERMANOVA add-on package (Gorley and Clarke, 2006).

6.5.2 Relationships between gut, water and feed microbiota: Illumina dataset of this thesis

We sought to determine the relationship between gut microbial communities of tilapia larvae with those present in the water and feed. Unweighted Unifrac was used to examine phylogenetic distance between fish gut and environmental microbial communities. Principal coordinates analysis (PCoA) showed that communities from different environments i.e. gut, water, and feed were significantly different (PERMANOVA test: $P_{\text{perm}} = 0.001$), (Fig. 6.1a). We also found that gut microbial communities were less dispersed than the corresponding water or feed communities between experiments ($P_{\text{perm}}: 0.001$). Within the illumina datasets, the lowest variation of gut communities was observed between samples from the probiotic study and the highest in the study where the sludge-based diets were used (Fig. 6.1b), ($P_{\text{perm}}: 0.001$).

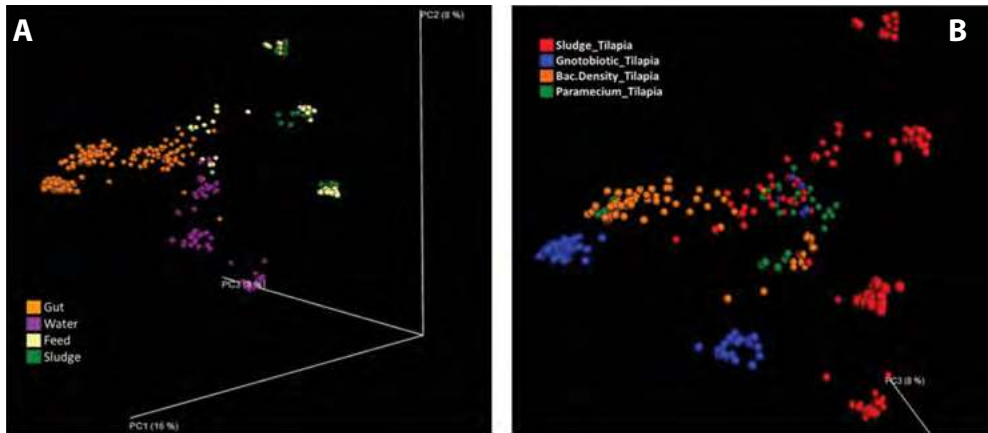


Figure 6.1 Principal coordinates analysis of microbiota from different sample types and studies. Relative distances of all points represent the relative dissimilarities of microbiota between samples as measured by Unweighted Unifrac. Spheres are color coded by: (A) Sample type; gut (orange), water (purple), feed (yellow) or sludge (green), and (B) Study; Sludge (red), Gnotobiotic (blue), Bac.Density (orange) or Paramecium (green). The percentage of total variation that is explained by each PCo axis is given in parentheses.

6.5.3 Relationship of fish gut microbiota between different fish species

A total of about 16.9M reads were selected from twelve next generation sequencing libraries on fish gut microbiota resulting in 2417 unique sequences. Among these sequences the dominant phylum (average abundance from all studies) was generally Proteobacteria (Fig. 6.2). In contrast, Fusobacteria was the most abundant phylum in the study of different carps raised in ponds (Li et al., 2014a), Firmicutes in the gut of tilapia fed with probiotics (Standen et al., 2015), and Actinobacteria, Planctomycetes and Firmicutes were the most dominant bacterial phyla in the gut of tilapia larvae in 3/5 of our studies i.e. the “Gnotobiotic”, “Bac.Density” and “RAS-AS” studies (Fig. 6.2).

Based on unweighted Unifrac, a PCoA was performed and the 10 most discriminant genera were plotted in a biplot (Fig. 6.3). Gut samples from the probiotic study (Probiotics_Tilapia in Fig. 6.3) consisted mainly of species belonging to the genera *Nocardia*, *Bacillus* and *Rhodococcus*. In our unpublished study in tilapia larvae (“**Bac.Density_Tilapia**”), the larval gut was enriched with species from the genus *Paenibacillus*, whereas larvae fed with sludge-based diets (“**Sludge_Tilapia**”) tended to have more species from the genus *Ralstonia*. From the studies which were principally discriminated along the PCoA1, species from the genera *Bradyrhizobium* and *Sphingomonas* (41 to 93% of the total abundance) outnumbered all other species in the gut of rainbow trout, the only carnivore fish species of this dataset.

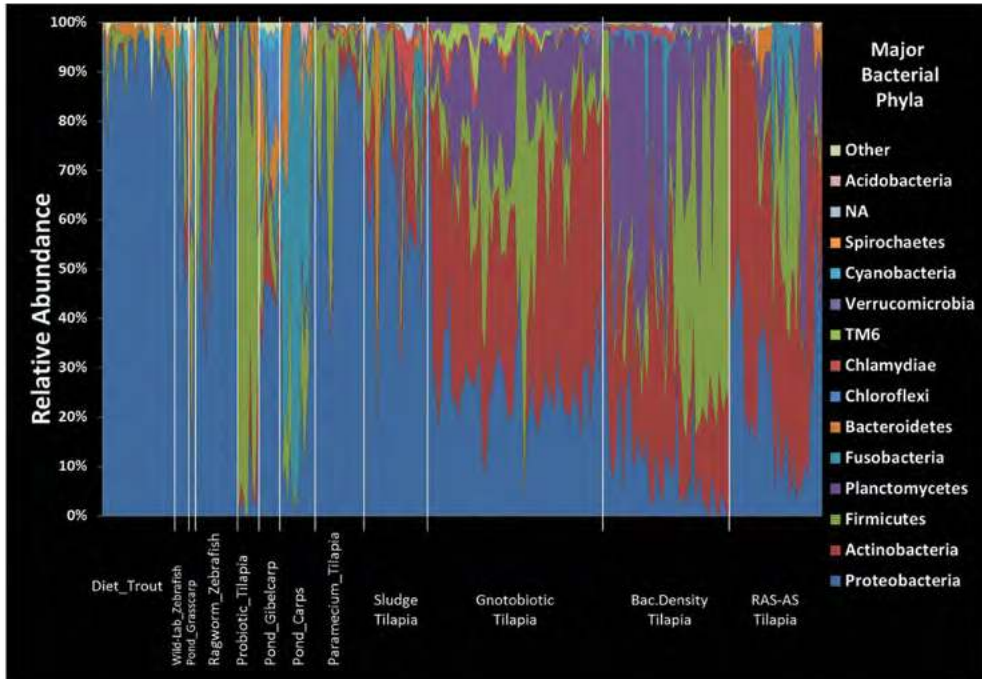


Figure 6.2. Area plot with percentage relative abundance of main bacterial phyla (y-axis, right) found in the gut samples of individuals from the different studies (x-axis)

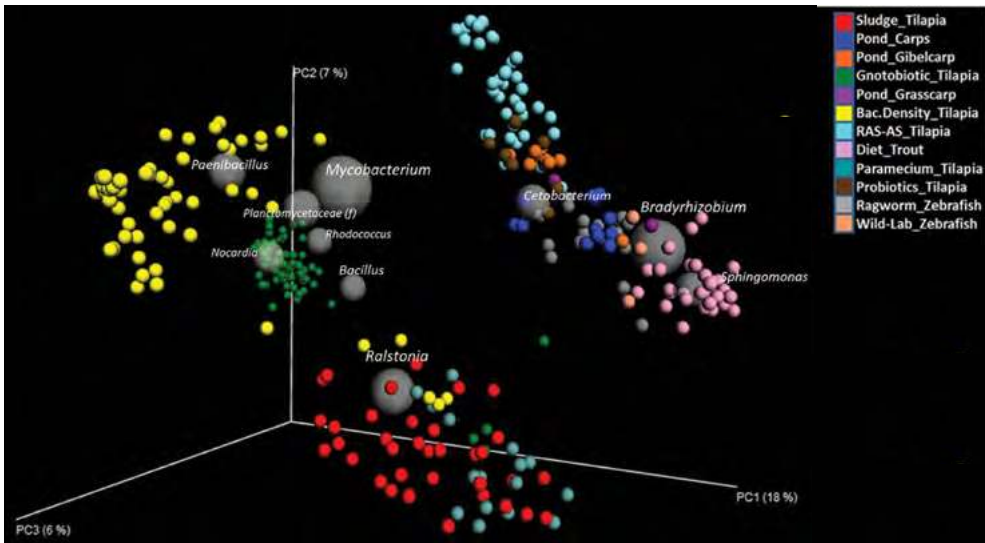


Figure 6.3. Principal coordinates biplot of gut microbiota of individuals from the different studies. Relative distances of all points represent the relative dissimilarities of gut microbiota between samples as measured by Unweighted Unifrac. Grey spheres represent the 10 most discriminant genera in explaining the observed variation between samples (colored spheres) and their size is proportional to the relative contribution of the genus in discriminating its space associated samples. The percentage of total variation that is explained by each PCo axis is given in parentheses.

6.5.4 Final Reflection

The meta-analysis showed that the gut microbiota has only a limited overlap within those found in their surrounding environment. This is in agreement with Wong and Rawls, (2012) who stated that communities inside the gut do not directly reflect the ones present in their habitat. Obviously, the pressure responsible for the selection of microbiota in the gut highly depends on the host (Li et al., 2012b). Further, regardless of the different treatments applied across our studies, gut communities clustered closely together, compared to microbiota in water, feed and sludge. This indicates that gut is a habitat which is less amenable to manipulation than water or feed. The host contributes to a fairly stable environment in the gut, leading to a homeostatic interaction between host and gut microbiota. On the other hand, it also shows that strict selection criteria operate inside the gut regarding which bacteria flourish and which are extirpated. Habitat related selective pressures are attributed to pH, gastric and bile secretions, nutrients availability and digestive enzymes activity, as well as host immune responses (Hansen and Olafsen, 1999; Verschuere et al., 1997; Sullam et al., 2012).

The lower variation of gut microbiota observed between samples from the probiotic study could be associated with the plasticity of gut microbiota in returning to a stable state after termination of a perturbation. Compared to the rest of our studies, the antibiotic and later probiotic treatments were terminated after one week, and from that point onwards, fish were receiving the same diet and swimming in water with a similar microbial profile. Thus, the time window of our observations could have coincided with the post-perturbation period of microbial community recovery. In that study, phylogenetically related genera tend to be abundant across most of the gut samples after discontinuation of the treatments. This resulted in a lower dispersion of gut samples when Unifrac distance was used (**meta-analysis**). Without considering the phylogenetic similarity of the taxa present in the gut, at a fine taxonomic resolution, gut microbiota was more clearly clustered between treatments as shown in **chapter 5**. These results have both methodological and biological inference. Measures of β -diversity should be chosen carefully depending on the research question. Additionally, phylogenetically different bacteria can develop in the gut; yet, the functionality of these different communities might be highly similar, or vice versa. Literature regarding relationship between genetic and functional diversity of bacteria are controversial (Mouchet et al., 2012 and literature cited therein), indicating that future research should focus on unraveling this relationship in more detail.

The most abundant phylum in all analyzed studies was that of Proteobacteria, indicating that the majority of fish gut bacteria are members of this taxon. This corroborates the results of (Rawls et al., 2006; Nayak, 2010b). Apparently, Proteobacteria are well adapted to the fish gut habitat, despite phylogenetic differences between their hosts, habitat heterogeneity and differences in hypotheses tested, resulting in highly different treatments. Differences between the prevalent phyla were mostly due to the dominance of the phyla Actinobacteria, Planctomycetes and Firmicutes. These results disclose a separation between our own

datasets and those acquired from other studies. Interlocation variation (variation between labs or facilities) of gut microbiota has been previously reported in zebrafish (Roeselers et al., 2011) but also in lab-reared mice (Friswell et al., 2010). The causes of the observed patterns could include differences in the choice of rearing system, water properties, handling and diets used.

It should be noted that almost half of the datasets incorporated in the meta-analysis were originated from our studies on tilapia larvae where the same methodology was used. This suggests that extrapolation of results from the meta-analysis presented here should be interpreted with caution due to the higher relative weight of the own work to the complete dataset. Nevertheless, within our own datasets spatial variation was observed between the "AS-RAS"- "Gnotobiotic"- "Bac.Density" and the "Paramecium"- "Sludge" studies. In particular, we found that *Mycobacterium* was the most discriminant genus in the larval gut of the first three studies. This difference could be attributed to the use of flow-through systems in the latter studies where the microbial load of the water was significantly reduced (lower carrying capacity due to water exchange and reduction of water microbial load due to UV sterilization).

Our phylogenetic analysis of gut communities revealed, in most cases, a clear clustering by study. To this end, differences in microbiota composition between experiments could be related to the experimental treatments and their effects on gut communities. However, also differences in methodological approaches could explain the differences found in microbiota composition. Choice of PCR primers, 16S rRNA region targeted, DNA extraction protocol, and sequencing platforms have been previously associated with significant differences between studies in human gut microbiome (Lozupone et al., 2013). It will therefore be important that future studies comparing gut microbiota of different fish species should be consistent with respect to the methods used for sample collection and analysis (Wong and Rawls, 2012).

Our results revealed a significant effect of rearing environment on gut microbiota. This observation was only valid within our facilities but not throughout the studies included in the meta-analysis. No significant effect of different rearing environments was observed in gut communities between animals raised in artificial or natural habitats (Wong and Rawls, 2012; Roeselers et al., 2011). In these studies, authors revealed that gut bacterial community membership in fish is mostly associated with their trophic level and matches their respective phylogenetic relationships, as has been also proposed for mammals (Ley et al., 2008). Here, we observed no shared constituents of the gut microbiota between the same fish species of the different studies. The clear dominance of the two genera (*Bradyrhizobium* and *Sphingomonas*) unique for the only carnivore fish species of this dataset (rainbow trout) could indicate host-microbe relationships attributed to the host's trophic level. Nonetheless, to draw clear conclusions it is important in the future to include additional fish from taxa, trophic levels and water salinities not included in this study.

6.6 Concluding remarks

The aim of microbial management is to steer gut microbiota towards a desirable state. To do so, we need to understand the ecological mechanisms and forces in the larval gut that regulate community composition. The results presented in this thesis have contributed to this knowledge and facilitate the development of safe and effective methods for manipulating gut microbial composition to promote the health of fish. Guidelines for optimization of experimental design and methodological conformity are given together with suggestions and ideas for future research themes.

A major outcome of our work is that the initial microbial contact is leading in determining gut microbial assembly. This encourages the development of strategies to steer initial colonization through the microbial management of water and feed. Gut microbiota of larvae can be manipulated towards improved intestinal function and immune development through the application of probiotics at the critical early developmental stage. Live prey can be an effective way of delivering probiotics but also prebiotics to the larvae through bio-encapsulation and/or bio-enrichment. The use of a selected mixture of beneficial strains combined with dietary ingredients (synbiotics) may prove more effective in different situations and more stable over time compared to a single strain probiotic.

The reduction of wild fish stocks, the high demand for fish protein and the need to develop sustainable aquaculture production methods are challenges which offer also opportunities. The areas of research interest have been defined and the technology is slowly shifting towards the era that next generation sequencing will become routine. The coming years will be an exciting time for fish microbiome research.

Supplementary material

Table S6.1. Metadata of all sample types (gut, water, feed and sludge) which were included in the meta-analysis. Only samples from own datasets are presented and only where Illumina HiSeq 2000 was used.

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
Aerob.D0.WATER	AACCAGAA	1	p	Sludge_Tilapia	WATER	index_8nt_2	1	Aerobic
Methanog.D0.WATER	AACCATGC	1	p	Sludge_Tilapia	WATER	index_8nt_3	2	Methanogenic
Denitrif.D0.WATER	AACGGCGC	1	p	Sludge_Tilapia	WATER	index_8nt_16	3	Denitrifying
Control.D0.WATER	AAGGAGCG	1	p	Sludge_Tilapia	WATER	index_8nt_42	4	Control
Aerob.inlet.D8.WATER	ACCAATTA	1	p	Sludge_Tilapia	WATER	index_8nt_70	5	Aerobic
Aerob.tank.D8.WATER	ACGGGAAT	1	p	Sludge_Tilapia	WATER	index_8nt_82	6	Aerobic
Methanog.inlet.D8.WATER	ACGCTCGC	1	p	Sludge_Tilapia	WATER	index_8nt_101	7	Methanogenic
Methanog.tank.D8.WATER	AGACCTGC	1	p	Sludge_Tilapia	WATER	index_8nt_138	8	Methanogenic
Denitrif.inlet.D8.WATER	AGCAGATT	1	p	Sludge_Tilapia	WATER	index_8nt_157	9	Denitrifying
Denitrif.tank.D8.WATER	AGCTTCGC	1	p	Sludge_Tilapia	WATER	index_8nt_167	10	Denitrifying
Control.inlet.D8.WATER	AGGTCGAA	1	p	Sludge_Tilapia	WATER	index_8nt_174	11	Control
Control.tank.D8.2.WATER	AGGTTATG	1	p	Sludge_Tilapia	WATER	index_8nt_175	12	Control
Aerob.inlet.D16.WATER	AGTCGCGC	1	p	Sludge_Tilapia	WATER	index_8nt_181	13	Aerobic
Aerob.tank.D16.WATER	AGTTATGC	1	p	Sludge_Tilapia	WATER	index_8nt_183	14	Aerobic
Methanog.inlet.D16.WATER	ATATCCGC	1	p	Sludge_Tilapia	WATER	index_8nt_204	15	Methanogenic
Methanog.tank.D16.WATER	ATGCCGGA	1	p	Sludge_Tilapia	WATER	index_8nt_223	16	Methanogenic
Denitrif.inlet.D16.WATER	ATCGGATT	1	p	Sludge_Tilapia	WATER	index_8nt_224	17	Denitrifying
Denitrif.tank.D16.WATER	CAACCTCT	1	p	Sludge_Tilapia	WATER	index_8nt_236	18	Denitrifying
Control.inlet.D16.WATER	CAACGAGG	1	p	Sludge_Tilapia	WATER	index_8nt_237	19	Control
Control.tank.D16.WATER	CATAAGCG	1	p	Sludge_Tilapia	WATER	index_8nt_272	20	Control
Aerob.D8.out.repeat	CATGATGC	1	p	Sludge_Tilapia	WATER	index_8nt_283	21	Aerobic
Methanog.D8.out.repeat	CATTGCCA	1	p	Sludge_Tilapia	WATER	index_8nt_287	22	Methanogenic
Denitrif.D8.out.repeat	CCATGGCG	1	p	Sludge_Tilapia	WATER	index_8nt_304	23	Denitrifying
Control.D8.out.repeat	CCATATG	1	p	Sludge_Tilapia	WATER	index_8nt_305	24	Control
Aerob.D16.out.repeat	CCGTACTA	1	p	Sludge_Tilapia	WATER	index_8nt_319	25	Aerobic

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
Methanog.D16.out.repeat	CCGTCTGC	1	p	Sludge_Tilapia	WATER	index_8nt_320	26	Methanogenic
Denitrif.D16.out.repeat	CGCAAGCT	1	p	Sludge_Tilapia	WATER	index_8nt_351	27	Denitrifying
Control.D16.out.repeat	CGGAAGAG	1	p	Sludge_Tilapia	WATER	index_8nt_371	28	Control
Aerob.A.D0.SLUDGE	CTAGAGA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_395	29	Aerobic
Aerob.B.D0.SLUDGE	CTAGGCG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_399	30	Aerobic
Aerob.C.D0.SLUDGE	CTCAGTCT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_401	31	Aerobic
Methanog.A.D0.SLUDGE	CTCGGTA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_409	32	Methanogenic
Methanog.B.D0.SLUDGE	CTGTGAA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_426	33	Methanogenic
Methanog.C.D0.SLUDGE	CTTGCAG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_436	34	Methanogenic
Denitrif.A.D0.SLUDGE	CTTGGCCT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_437	35	Denitrifying
Denitrif.B.D0.SLUDGE	GAACCGTT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_438	36	Denitrifying
Denitrif.C.D0.SLUDGE	GAACGTAT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_440	37	Denitrifying
Aerob.A.D8.SLUDGE	GAAC TAAG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_441	38	Aerobic
Aerob.B.D8.SLUDGE	GAAGCTCG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_443	39	Aerobic
Aerob.C.D8.SLUDGE	GAGTTATA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_468	40	Aerobic
Methanog.A.D8.SLUDGE	GATGAATG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_474	41	Methanogenic
Methanog.B.D8.SLUDGE	GATGATAA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_475	42	Methanogenic
Methanog.C.D8.SLUDGE	GATGCCT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_477	43	Methanogenic
Denitrif.A.D8.SLUDGE	GCCAGGTT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_497	44	Denitrifying
Denitrif.B.D8.SLUDGE	GCCTTAAG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_505	45	Denitrifying
Denitrif.C.D8.SLUDGE	GCTAATCT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_519	46	Denitrifying
Aerob.A.D16.SLUDGE	GGAGCGCA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_532	47	Aerobic
Aerob.B.D16.SLUDGE	GGAGTATG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_534	48	Aerobic
Aerob.C.D16.SLUDGE	GGTACCAA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_551	49	Aerobic
Methanog.A.D16.SLUDGE	GGTAGAAT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_553	50	Methanogenic
Methanog.B.D16.SLUDGE	GTCCGCAA	1	p	Sludge_Tilapia	SLUDGE	index_8nt_577	51	Methanogenic
Methanog.C.D16.SLUDGE	GTTAAGTT	1	p	Sludge_Tilapia	SLUDGE	index_8nt_583	52	Methanogenic
Denitrif.A.D16.SLUDGE	GTTCTACG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_587	53	Denitrifying
Denitrif.B.D16.SLUDGE	TATTGCGC	1	p	Sludge_Tilapia	SLUDGE	index_8nt_617	54	Denitrifying
Denitrif.C.D16.SLUDGE	TCAGCGAG	1	p	Sludge_Tilapia	SLUDGE	index_8nt_622	55	Denitrifying

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
Aerob.1.D0.FEED	TCATTCGG	1	p	Sludge_Tilapia	FEED	index_8nt_628	56	Aerobic
Aerob.2.D0.FEED	TCCGTATA	1	p	Sludge_Tilapia	FEED	index_8nt_633	57	Aerobic
Aerob.3.D0.FEED	TCCTCCGC	1	p	Sludge_Tilapia	FEED	index_8nt_637	58	Aerobic
Aerob.4.D0.FEED	TCGAATAA	1	p	Sludge_Tilapia	FEED	index_8nt_640	59	Aerobic
Methanog.1.D0.FEED	TCGATATT	1	p	Sludge_Tilapia	FEED	index_8nt_641	60	Methanogenic
Methanog.2.D0.FEED	TCTATTCC	1	p	Sludge_Tilapia	FEED	index_8nt_650	61	Methanogenic
Methanog.3.D0.FEED	TGACTCAA	1	p	Sludge_Tilapia	FEED	index_8nt_661	62	Methanogenic
Methanog.4.D0.FEED	TGATCTCA	1	p	Sludge_Tilapia	FEED	index_8nt_665	63	Methanogenic
Denitrif.1.D0.FEED	TGCCCTGG	1	p	Sludge_Tilapia	FEED	index_8nt_671	64	Denitrifying
Denitrif.2.D0.FEED	TGGTAATT	1	p	Sludge_Tilapia	FEED	index_8nt_684	65	Denitrifying
Denitrif.3.D0.FEED	TGGTATGA	1	p	Sludge_Tilapia	FEED	index_8nt_685	66	Denitrifying
Denitrif.4.D0.FEED	AACCAAGAA	2	p	Sludge_Tilapia	FEED	index_8nt_2	1	Denitrifying
Control.1.D0.FEED	AACCATGC	2	p	Sludge_Tilapia	FEED	index_8nt_3	2	Control
Control.2.D0.FEED	AACGGCGC	2	p	Sludge_Tilapia	FEED	index_8nt_16	3	Control
Control.3.D0.FEED	AAGGAGGG	2	p	Sludge_Tilapia	FEED	index_8nt_42	4	Control
Control.4.D0.FEED	ACCAATTA	2	p	Sludge_Tilapia	FEED	index_8nt_70	5	Control
Aerob.1.D8.FEED	ACCGGAAT	2	p	Sludge_Tilapia	FEED	index_8nt_82	6	Aerobic
Aerob.2.D8.FEED	ACGCTCGC	2	p	Sludge_Tilapia	FEED	index_8nt_101	7	Aerobic
Aerob.3.D8.FEED	AGACCTGC	2	p	Sludge_Tilapia	FEED	index_8nt_138	8	Aerobic
Aerob.4.D8.FEED	AGCAGATT	2	p	Sludge_Tilapia	FEED	index_8nt_157	9	Aerobic
Methanog.1.D8.FEED	AGCTTCGC	2	p	Sludge_Tilapia	FEED	index_8nt_167	10	Methanogenic
Methanog.2.D8.FEED	AGGTCGAA	2	p	Sludge_Tilapia	FEED	index_8nt_174	11	Methanogenic
Methanog.3.D8.FEED	AGGTTATG	2	p	Sludge_Tilapia	FEED	index_8nt_175	12	Methanogenic
Methanog.4.D8.FEED	AGTCGGCG	2	p	Sludge_Tilapia	FEED	index_8nt_181	13	Methanogenic
Denitrif.1.D8.FEED	AGTTATGC	2	p	Sludge_Tilapia	FEED	index_8nt_183	14	Denitrifying
Denitrif.2.D8.FEED	ATATCCGC	2	p	Sludge_Tilapia	FEED	index_8nt_204	15	Denitrifying
Denitrif.3.D8.FEED	ATGCCCGA	2	p	Sludge_Tilapia	FEED	index_8nt_223	16	Denitrifying
Denitrif.4.D8.FEED	ATGCGGAT	2	p	Sludge_Tilapia	FEED	index_8nt_224	17	Denitrifying
Control.1.D8.FEED	CAACCTCT	2	p	Sludge_Tilapia	FEED	index_8nt_236	18	Control
Control.2.D8.FEED	CAACGAGG	2	p	Sludge_Tilapia	FEED	index_8nt_237	19	Control

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
Control.3.D8.FEED	CATAAGCG	2	p	Sludge_Tilapia	FEED	index_8nt_272	20	Control
Control.4.D8.FEED	CATGATGC	2	p	Sludge_Tilapia	FEED	index_8nt_283	21	Control
Aerob.1.D16.FEED	CATTGCCA	2	p	Sludge_Tilapia	FEED	index_8nt_287	22	Aerobic
Aerob.2.D16.FEED	CCATGCCG	2	p	Sludge_Tilapia	FEED	index_8nt_304	23	Aerobic
Aerob.3.D16.FEED	CCATTATG	2	p	Sludge_Tilapia	FEED	index_8nt_305	24	Aerobic
Aerob.4.D16.FEED	CCGTACTA	2	p	Sludge_Tilapia	FEED	index_8nt_319	25	Aerobic
Methanog.1.D16.FEED	CCGTCTGC	2	p	Sludge_Tilapia	FEED	index_8nt_320	26	Methanogenic
Methanog.2.D16.FEED	CGCAAGCT	2	p	Sludge_Tilapia	FEED	index_8nt_351	27	Methanogenic
Methanog.3.D16.FEED	CGGAAGAG	2	p	Sludge_Tilapia	FEED	index_8nt_371	28	Methanogenic
Methanog.4.D16.FEED	CTAGGAGA	2	p	Sludge_Tilapia	FEED	index_8nt_395	29	Methanogenic
Denitrif.1.D16.FEED	CTCAGCGC	2	p	Sludge_Tilapia	FEED	index_8nt_399	30	Denitrifying
Denitrif.2.D16.FEED	CTCAGTCT	2	p	Sludge_Tilapia	FEED	index_8nt_401	31	Denitrifying
Denitrif.3.D16.FEED	CTCGCGTA	2	p	Sludge_Tilapia	FEED	index_8nt_409	32	Denitrifying
Denitrif.4.D16.FEED	CTGCTGAA	2	p	Sludge_Tilapia	FEED	index_8nt_426	33	Denitrifying
Control.1.D16.FEED	CTTGCAG	2	p	Sludge_Tilapia	FEED	index_8nt_436	34	Control
Control.2.D16.FEED	CTTGGCCT	2	p	Sludge_Tilapia	FEED	index_8nt_437	35	Control
Control.3.D16.FEED	GAACCGTT	2	p	Sludge_Tilapia	FEED	index_8nt_438	36	Control
Control.4.D16.FEED	GAACGTAT	2	p	Sludge_Tilapia	FEED	index_8nt_440	37	Control
Common.1.D0.GUT	GAACCTAAG	2	p	Sludge_Tilapia	GUT	index_8nt_441	38	Common
Common.2.D0.GUT	GAAGCTCG	2	p	Sludge_Tilapia	GUT	index_8nt_443	39	Common
Common.3.D0.GUT	GAGTTATA	2	p	Sludge_Tilapia	GUT	index_8nt_468	40	Common
Common.4.D0.GUT	GATGATAA	2	p	Sludge_Tilapia	GUT	index_8nt_475	42	Common
Aerob.1.D8.GUT	GATGCCT	2	p	Sludge_Tilapia	GUT	index_8nt_477	43	Aerobic
Aerob.2.D8.GUT	GCCAGGTT	2	p	Sludge_Tilapia	GUT	index_8nt_497	44	Aerobic
Aerob.3.D8.GUT	GCCTTAAG	2	p	Sludge_Tilapia	GUT	index_8nt_505	45	Aerobic
Aerob.4.D8.GUT	GCTAATCT	2	p	Sludge_Tilapia	GUT	index_8nt_519	46	Aerobic
Methanog.1.D8.GUT	GGAGCGCA	2	p	Sludge_Tilapia	GUT	index_8nt_532	47	Methanogenic
Methanog.2.D8.GUT	GGAGTATG	2	p	Sludge_Tilapia	GUT	index_8nt_534	48	Methanogenic
Methanog.3.D8.GUT	GGTACCAA	2	p	Sludge_Tilapia	GUT	index_8nt_551	49	Methanogenic
Methanog.4.D8.GUT	GGTAGAAT	2	p	Sludge_Tilapia	GUT	index_8nt_553	50	Methanogenic

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
Denitrif.1.D8.GUT	GTCCGAA	2	p	Sludge_Tilapia	GUT	index_8nt_577	51	Denitrifying
Denitrif.2.D8.GUT	GTTAAGTT	2	p	Sludge_Tilapia	GUT	index_8nt_583	52	Denitrifying
Denitrif.3.D8.GUT	GTTCACG	2	p	Sludge_Tilapia	GUT	index_8nt_587	53	Denitrifying
Denitrif.4.D8.GUT	TATTGCGC	2	p	Sludge_Tilapia	GUT	index_8nt_617	54	Denitrifying
Control.1.D8.GUT	TCAGCGAG	2	p	Sludge_Tilapia	GUT	index_8nt_622	55	Control
Control.2.D8.GUT	TCATTCCG	2	p	Sludge_Tilapia	GUT	index_8nt_628	56	Control
Control.3.D8.GUT	TCCGTATA	2	p	Sludge_Tilapia	GUT	index_8nt_633	57	Control
Control.4.D8.GUT	TCCTCCGC	2	p	Sludge_Tilapia	GUT	index_8nt_637	58	Control
Aerob.1.D16.GUT	TCGAATAA	2	p	Sludge_Tilapia	GUT	index_8nt_640	59	Aerobic
Aerob.2.D16.GUT	TCGATATT	2	p	Sludge_Tilapia	GUT	index_8nt_641	60	Aerobic
Aerob.3.D16.GUT	TCTATTCC	2	p	Sludge_Tilapia	GUT	index_8nt_650	61	Aerobic
Aerob.4.D16.GUT	TGACTCAA	2	p	Sludge_Tilapia	GUT	index_8nt_661	62	Aerobic
Methanog.1.D16.GUT	TGATCTCA	2	p	Sludge_Tilapia	GUT	index_8nt_665	63	Methanogenic
Methanog.2.D16.GUT	TGCCTGCG	2	p	Sludge_Tilapia	GUT	index_8nt_671	64	Methanogenic
Methanog.3.D16.GUT	TGGTAATT	2	p	Sludge_Tilapia	GUT	index_8nt_684	65	Methanogenic
Methanog.4.D16.GUT	TGGTATGA	2	p	Sludge_Tilapia	GUT	index_8nt_685	66	Methanogenic
Denitrif.1.D16.GUT	TTAGGATG	2	p	Sludge_Tilapia	GUT	index_8nt_693	67	Denitrifying
Denitrif.2.D16.GUT	AACCAGAA	3	p	Sludge_Tilapia	GUT	index_8nt_2	1	Denitrifying
Denitrif.3.D16.GUT	AACCATGC	3	p	Sludge_Tilapia	GUT	index_8nt_3	2	Denitrifying
Denitrif.4.D16.GUT	AACGGCGC	3	p	Sludge_Tilapia	GUT	index_8nt_16	3	Denitrifying
Control.1.D16.GUT	AAGGAGCG	3	p	Sludge_Tilapia	GUT	index_8nt_42	4	Control
Control.2.D16.GUT	ACCAATA	3	p	Sludge_Tilapia	GUT	index_8nt_70	5	Control
Control.3.D16.GUT	ACCGGAAT	3	p	Sludge_Tilapia	GUT	index_8nt_82	6	Control
Control.4.D16.GUT	ACGCTCGC	3	p	Sludge_Tilapia	GUT	index_8nt_101	7	Control
CH1.D14.W	AGACCTGC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_138	8	Gnotobiotic
CH2.D14.W	AGCAGATT	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_157	9	Gnotobiotic
AX1.D14.W	AGCTTCGC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_167	10	Axenic
AX2.D14.W	AGTGCGAA	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_174	11	Axenic
AX3.D14.W	AGGTTATG	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_175	12	Axenic
GN1.D14.W	AGTCGCC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_181	13	Gnotobiotic

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
GN2.D14.W	AGTTATGC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_183	14	Gnotobiotic
GN3.D14.W	ATATCCGC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_204	15	Gnotobiotic
GN4.D14.W	ATGCCGGA	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_223	16	Gnotobiotic
AX1.D21.W	ATCGGATT	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_224	17	Axenic
AX2.D21.W	CAACCTCT	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_236	18	Axenic
AX3.D21.W	CAACGAGG	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_237	19	Axenic
GN1.D21.W	CATAAGCG	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_272	20	Gnotobiotic
GN2.D21.W	CATGATGC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_283	21	Gnotobiotic
GN3.D21.W	CATTGCCA	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_287	22	Gnotobiotic
GN4.D21.W	CCATGGCC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_304	23	Gnotobiotic
AX1.D28.W	CCATTATG	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_305	24	Axenic
AX2.D28.W	CCGTACTA	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_319	25	Axenic
AX3.D28.W	CCGTCTGC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_320	26	Axenic
GN1.D28.W	CGCAAGCT	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_351	27	Gnotobiotic
GN2.D28.W	CGGAAGAG	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_371	28	Gnotobiotic
GN3.D28.W	CTAGGAGA	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_395	29	Gnotobiotic
GN4.D28.W	CTCAGGCC	3	p	Gnotobiotic_Tilapia	WATER	index_8nt_399	30	Gnotobiotic
AX1.D14.G1	CTCAGTCT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_401	31	Axenic
AX1.D14.G2	CTCGCGTA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_409	32	Axenic
AX1.D14.G3	CTGCTGAA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_426	33	Axenic
AX1.D14.G4	CTTGCGAG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_436	34	Axenic
AX2.D14.G1	CTTGCCCT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_437	35	Axenic
AX2.D14.G2	GAACCGTT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_438	36	Axenic
AX2.D14.G3	GAACGTAT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_440	37	Axenic
AX2.D14.G4	GAACATAAG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_441	38	Axenic
AX3.D14.G1	GAAGCTCG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_443	39	Axenic
AX3.D14.G2	GAGTTATA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_468	40	Axenic
AX3.D14.G3	GATGATAA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_475	42	Axenic
AX3.D14.G4	GATGCCGT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_477	43	Axenic
AX1.D21.G1	GCCAGGTT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_497	44	Axenic

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
AX1.D21.G2	GCCTTAAG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_505	45	Axenic
AX1.D21.G3	GCTAATCT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_519	46	Axenic
AX1.D21.G4	GGAGCGCA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_532	47	Axenic
AX2.D21.G1	GGAGTATG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_534	48	Axenic
AX2.D21.G2	GGTACCAA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_551	49	Axenic
AX2.D21.G3	GGTAGAAT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_553	50	Axenic
AX2.D21.G4	GTCCGCAA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_577	51	Axenic
AX3.D21.G1	GTTAAGTT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_583	52	Axenic
AX3.D21.G2	GTTCTACG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_587	53	Axenic
AX3.D21.G3	TATTGCGC	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_617	54	Axenic
AX3.D21.G4	TCAGCGAG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_622	55	Axenic
AX1.D28.G1	TCATTCCG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_628	56	Axenic
AX1.D28.G2	TCCGTATA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_633	57	Axenic
AX1.D28.G3	TCCTCCGC	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_637	58	Axenic
AX1.D28.G4	TCGAATAA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_640	59	Axenic
AX2.D28.G1	TCGATATT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_641	60	Axenic
AX2.D28.G2	TCTATTCC	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_650	61	Axenic
AX2.D28.G3	TGACTCAA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_661	62	Axenic
AX2.D28.G4	TGATCTCA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_665	63	Axenic
AX3.D28.G1	TGCCCTCG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_671	64	Axenic
AX3.D28.G2	TGGTAATT	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_684	65	Axenic
AX3.D28.G3	TGGTATGA	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_685	66	Axenic
AX3.D28.G4	TTAGGATG	3	p	Gnotobiotic_Tilapia	GUT	index_8nt_693	67	Axenic
GN1.D14.G1	AACCAGAA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_2	1	Gnotobiotic
GN1.D14.G2	AACCATGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_3	2	Gnotobiotic
GN2.D14.G1	AACGGCGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_16	3	Gnotobiotic
GN2.D14.G2	AAGGAGCG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_42	4	Gnotobiotic
GN1.D21.G1	ACCAATTA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_70	5	Gnotobiotic
GN1.D21.G2	ACCGGAAT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_82	6	Gnotobiotic
GN1.D21.G3	ACGCTCGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_101	7	Gnotobiotic

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
GN1.D21.G4	AGACCTGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_138	8	Gnotobiotic
GN2.D21.G1	AGCAGATT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_157	9	Gnotobiotic
GN2.D21.G2	AGCTTCGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_167	10	Gnotobiotic
GN2.D21.G3	AGGTCGAA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_174	11	Gnotobiotic
GN2.D21.G4	AGGTTATG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_175	12	Gnotobiotic
GN3.D21.G1	AGTCGGCG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_181	13	Gnotobiotic
GN3.D21.G2	AGTTATGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_183	14	Gnotobiotic
GN3.D21.G3	ATATCCGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_204	15	Gnotobiotic
GN3.D21.G4	ATGCCGGA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_223	16	Gnotobiotic
GN4.D21.G1	ATGCGGAT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_224	17	Gnotobiotic
GN4.D21.G2	CAACCTCT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_236	18	Gnotobiotic
GN4.D21.G3	CAACGAGG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_237	19	Gnotobiotic
GN4.D21.G4	CATAAGCG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_272	20	Gnotobiotic
GN1.D28.G1	CATGATGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_283	21	Gnotobiotic
GN1.D28.G2	CATTGCCA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_287	22	Gnotobiotic
GN1.D28.G3	CCATGGCG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_304	23	Gnotobiotic
GN1.D28.G4	CCATTATG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_305	24	Gnotobiotic
GN2.D28.G1	CCGTACTA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_319	25	Gnotobiotic
GN2.D28.G2	CCGTCTGC	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_320	26	Gnotobiotic
GN2.D28.G3	CGCAAGCT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_351	27	Gnotobiotic
GN2.D28.G4	CGGAAGAG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_371	28	Gnotobiotic
GN3.D28.G1	CTAGGAGA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_395	29	Gnotobiotic
GN3.D28.G2	CTCAGGCG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_399	30	Gnotobiotic
GN3.D28.G3	CTCAGTCT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_401	31	Gnotobiotic
GN3.D28.G4	CTCGGTA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_409	32	Gnotobiotic
GN4.D28.G1	CTGCTGAA	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_426	33	Gnotobiotic
GN4.D28.G2	CTTGGCAG	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_436	34	Gnotobiotic
GN4.D28.G3	CTTGGCCT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_437	35	Gnotobiotic
GN4.D28.G4	GAACCGTT	4	p	Gnotobiotic_Tilapia	GUT	index_8nt_438	36	Gnotobiotic
LW/D1.W	GAACGTAT	4	p	Bac.Density_Tilapia	WATER	index_8nt_440	37	Low_water

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
HW.D1.W	GAACAAAG	4	p	Bac.Density_Tilapia	WATER	index_8nt_441	38	High_water
LW.LF1.D24.W	GAAGCTCG	4	p	Bac.Density_Tilapia	WATER	index_8nt_443	39	Lowwater_Lowfeed
LW.LF2.D24.W	GAGTTATA	4	p	Bac.Density_Tilapia	WATER	index_8nt_468	40	Lowwater_Lowfeed
LW.LF3.D24.W	GATGATAA	4	p	Bac.Density_Tilapia	WATER	index_8nt_475	42	Lowwater_Lowfeed
LW.HF1.D24.W	GATCGGCT	4	p	Bac.Density_Tilapia	WATER	index_8nt_477	43	Lowwater_Highfeed
LW.HF2.D24.W	GCCAGGTT	4	p	Bac.Density_Tilapia	WATER	index_8nt_497	44	Lowwater_Highfeed
LW.HF3.D24.W	GCCTTAAG	4	p	Bac.Density_Tilapia	WATER	index_8nt_505	45	Lowwater_Highfeed
HW.LF1.D24.W	GCTAATCT	4	p	Bac.Density_Tilapia	WATER	index_8nt_519	46	Highwater_Lowfeed
HW.LF2.D24.W	GGAGCGCA	4	p	Bac.Density_Tilapia	WATER	index_8nt_532	47	Highwater_Lowfeed
HW.LF3.D24.W	GGAGTATG	4	p	Bac.Density_Tilapia	WATER	index_8nt_534	48	Highwater_Lowfeed
HW.HF1.D24.W	GGTACCAA	4	p	Bac.Density_Tilapia	WATER	index_8nt_551	49	Highwater_Highfeed
HW.HF2.D24.W	GGTAGAAT	4	p	Bac.Density_Tilapia	WATER	index_8nt_553	50	Highwater_Highfeed
HW.HF3.D24.W	GTCCGCAA	4	p	Bac.Density_Tilapia	WATER	index_8nt_577	51	Highwater_Highfeed
CB.D0.G1	GTTAAGTT	4	p	Bac.Density_Tilapia	GUT	index_8nt_583	52	Initial
CB.D0.G2	GTTCTACG	4	p	Bac.Density_Tilapia	GUT	index_8nt_587	53	Initial
CB.D0.3	TATTGCGC	4	p	Bac.Density_Tilapia	GUT	index_8nt_617	54	Initial
CB.D0.4	TCAGCGAG	4	p	Bac.Density_Tilapia	GUT	index_8nt_622	55	Initial
LW.HF1.D24.G1	TCATTCCG	4	p	Bac.Density_Tilapia	GUT	index_8nt_628	56	Lowwater_Highfeed
LW.HF1.D24.G2	TCCGTATA	4	p	Bac.Density_Tilapia	GUT	index_8nt_633	57	Lowwater_Highfeed
LW.HF1.D24.G3	TCTCCCGC	4	p	Bac.Density_Tilapia	GUT	index_8nt_637	58	Lowwater_Highfeed
LW.HF1.D24.G4	TCGAATAA	4	p	Bac.Density_Tilapia	GUT	index_8nt_640	59	Lowwater_Highfeed
LW.HF2.D24.G1	TCGATATT	4	p	Bac.Density_Tilapia	GUT	index_8nt_641	60	Lowwater_Highfeed
LW.HF2.D24.G2	TCTATTCC	4	p	Bac.Density_Tilapia	GUT	index_8nt_650	61	Lowwater_Highfeed
LW.HF2.D24.G3.I4	TGACTCAA	4	p	Bac.Density_Tilapia	GUT	index_8nt_661	62	Lowwater_Highfeed
LW.HF2.D24.G4.I4	TGATCTCA	4	p	Bac.Density_Tilapia	GUT	index_8nt_665	63	Lowwater_Highfeed
LW.HF3.D24.G1.I4	TGCCTGGC	4	p	Bac.Density_Tilapia	GUT	index_8nt_671	64	Lowwater_Highfeed
LW.HF3.D24.G2	TGGTAATT	4	p	Bac.Density_Tilapia	GUT	index_8nt_684	65	Lowwater_Highfeed
LW.HF3.D24.G3	TGGTATGA	4	p	Bac.Density_Tilapia	GUT	index_8nt_685	66	Lowwater_Highfeed
LW.HF3.D24.G4	TTAGGATG	4	p	Bac.Density_Tilapia	GUT	index_8nt_693	67	Lowwater_Highfeed
LW.LF1.D24.G1	AACCAGAA	5	p	Bac.Density_Tilapia	GUT	index_8nt_2	1	Lowwater_Lowfeed

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
LW.LF1.D24.G2	AACCATGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_3	2	Lowwater_Lowfeed
LW.LF1.D24.G3	AACGGCGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_16	3	Lowwater_Lowfeed
LW.LF1.D24.G4	AAGGAGCG	5	p	Bac.Density_Tilapia	GUT	index_8nt_42	4	Lowwater_Lowfeed
LW.LF2.D24.G1	ACCAATTA	5	p	Bac.Density_Tilapia	GUT	index_8nt_70	5	Lowwater_Lowfeed
LW.LF2.D24.G2	ACGGGAAT	5	p	Bac.Density_Tilapia	GUT	index_8nt_82	6	Lowwater_Lowfeed
LW.LF2.D24.G3	ACGCTGCG	5	p	Bac.Density_Tilapia	GUT	index_8nt_101	7	Lowwater_Lowfeed
LW.LF2.D24.G4	AGACCTGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_138	8	Lowwater_Lowfeed
LW.LF3.D24.G1	AGCAGATT	5	p	Bac.Density_Tilapia	GUT	index_8nt_157	9	Lowwater_Lowfeed
LW.LF3.D24.G2	AGCTTCGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_167	10	Lowwater_Lowfeed
LW.LF3.D24.G3	AGGTCGAA	5	p	Bac.Density_Tilapia	GUT	index_8nt_174	11	Lowwater_Lowfeed
LW.LF3.D24.G4	AGGTTATG	5	p	Bac.Density_Tilapia	GUT	index_8nt_175	12	Lowwater_Lowfeed
HW.HF1.D24.G1	AGTCGCGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_181	13	Highwater_Highfeed
HW.HF1.D24.G2	AGTTATGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_183	14	Highwater_Highfeed
HW.HF1.D24.G3	ATATCCGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_204	15	Highwater_Highfeed
HW.HF1.D24.G4	ATGCCGGA	5	p	Bac.Density_Tilapia	GUT	index_8nt_223	16	Highwater_Highfeed
HW.HF2.D24.G1	ATCGGATT	5	p	Bac.Density_Tilapia	GUT	index_8nt_224	17	Highwater_Highfeed
HW.HF2.D24.G2	CAACCTCT	5	p	Bac.Density_Tilapia	GUT	index_8nt_236	18	Highwater_Highfeed
HW.HF2.D24.G3	CAACGAGG	5	p	Bac.Density_Tilapia	GUT	index_8nt_237	19	Highwater_Highfeed
HW.HF2.D24.G4	CATAAGCG	5	p	Bac.Density_Tilapia	GUT	index_8nt_272	20	Highwater_Highfeed
HW.HF3.D24.G1	CATGATGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_283	21	Highwater_Highfeed
HW.HF3.D24.G2	CATTGCCA	5	p	Bac.Density_Tilapia	GUT	index_8nt_287	22	Highwater_Highfeed
HW.HF3.D24.G3	CCATGCGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_304	23	Highwater_Highfeed
HW.HF3.D24.G4	CCATTATG	5	p	Bac.Density_Tilapia	GUT	index_8nt_305	24	Highwater_Highfeed
HW.LF1.D24.G1	CCGTACTA	5	p	Bac.Density_Tilapia	GUT	index_8nt_319	25	Highwater_Lowfeed
HW.LF1.D24.G2	CCGTCTGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_320	26	Highwater_Lowfeed
HW.LF1.D24.G3	CGCAAGCT	5	p	Bac.Density_Tilapia	GUT	index_8nt_351	27	Highwater_Lowfeed
HW.LF1.D24.G4	CGGAAGAG	5	p	Bac.Density_Tilapia	GUT	index_8nt_371	28	Highwater_Lowfeed
HW.LF2.D24.G1	CTAGGAGA	5	p	Bac.Density_Tilapia	GUT	index_8nt_395	29	Highwater_Lowfeed
HW.LF2.D24.G2	CTCAGCGC	5	p	Bac.Density_Tilapia	GUT	index_8nt_399	30	Highwater_Lowfeed
HW.LF2.D24.G3	CTCAGTCT	5	p	Bac.Density_Tilapia	GUT	index_8nt_401	31	Highwater_Lowfeed

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
HWLF2.D24.G4	CTCGGTA	5	p	Bac.Density_Tilapia	GUT	index_8nt_409	32	Highwater_Lowfeed
HWLF3.D24.G1	CTGCTGAA	5	p	Bac.Density_Tilapia	GUT	index_8nt_426	33	Highwater_Lowfeed
HWLF3.D24.G2	CTTGCAG	5	p	Bac.Density_Tilapia	GUT	index_8nt_436	34	Highwater_Lowfeed
HWLF3.D24.G3	CTTGGCCT	5	p	Bac.Density_Tilapia	GUT	index_8nt_437	35	Highwater_Lowfeed
HWLF3.D24.G4	GAACCGTT	5	p	Bac.Density_Tilapia	GUT	index_8nt_438	36	Highwater_Lowfeed
STNF.STNW.G1	GAACGTAT	5	p	Paramecium_Tilapia	GUT	index_8nt_440	37	Stainfeed_stainwater
STNF.STNW.G2	GAACCTAAG	5	p	Paramecium_Tilapia	GUT	index_8nt_441	38	Stainfeed_stainwater
STNF.STNW.G3	GAAGCTCG	5	p	Paramecium_Tilapia	GUT	index_8nt_443	39	Stainfeed_stainwater
STNF.STNW.G4	GAGTTATA	5	p	Paramecium_Tilapia	GUT	index_8nt_468	40	Stainfeed_stainwater
STRF.STNW.G1	GATGATAA	5	p	Paramecium_Tilapia	GUT	index_8nt_475	42	Sterilefeed_stainwater
STRF.STNW.G2	GATGCCT	5	p	Paramecium_Tilapia	GUT	index_8nt_477	43	Sterilefeed_stainwater
STRF.STNW.G3	GCCAGGTT	5	p	Paramecium_Tilapia	GUT	index_8nt_497	44	Sterilefeed_stainwater
STRF.STNW.G4	GCCTTAAG	5	p	Paramecium_Tilapia	GUT	index_8nt_505	45	Sterilefeed_stainwater
STNF.STRW.G1	GCTAATCT	5	p	Paramecium_Tilapia	GUT	index_8nt_519	46	stainfeed_sterilewater
STNF.STRW.G2	GGAGCGCA	5	p	Paramecium_Tilapia	GUT	index_8nt_532	47	stainfeed_sterilewater
STNF.STRW.G3	GGAGTATG	5	p	Paramecium_Tilapia	GUT	index_8nt_534	48	stainfeed_sterilewater
STNF.STRW.G4	GGTACCAA	5	p	Paramecium_Tilapia	GUT	index_8nt_551	49	stainfeed_sterilewater
NF.STNW.G1	GGTAGAAT	5	p	Paramecium_Tilapia	GUT	index_8nt_553	50	nonfed_stainwater
NF.STNW.G2	GTCCGCAA	5	p	Paramecium_Tilapia	GUT	index_8nt_577	51	nonfed_stainwater
NF.STNW.G3	GTTAAGTT	5	p	Paramecium_Tilapia	GUT	index_8nt_583	52	nonfed_stainwater
NF.STNW.G4	GTTCTACG	5	p	Paramecium_Tilapia	GUT	index_8nt_587	53	nonfed_stainwater
STN.BROTH.1	TATTGGCG	5	p	Paramecium_Tilapia	Bacteria	index_8nt_617	54	Stain_bacteria
STN.BROTH.2	TCAGCGAG	5	p	Paramecium_Tilapia	Bacteria	index_8nt_622	55	Stain_bacteria
Fed.para.1	TCATTCCG	5	p	Paramecium_Tilapia	Paramecium	index_8nt_628	56	Fed_paramecium
Fed.para.2	TCCGTATA	5	p	Paramecium_Tilapia	Paramecium	index_8nt_633	57	Fed_paramecium
Fed.para.3	TCCTCCGC	5	p	Paramecium_Tilapia	Paramecium	index_8nt_637	58	Fed_paramecium
NFed.para.1	TCGAATAA	5	p	Paramecium_Tilapia	Paramecium	index_8nt_640	59	Nonfed_paramecium
NFed.para.2	TCGATATT	5	p	Paramecium_Tilapia	Paramecium	index_8nt_641	60	Nonfed_paramecium
NFed.para.3	TCTATTCCG	5	p	Paramecium_Tilapia	Paramecium	index_8nt_650	61	Nonfed_paramecium
LWHF2.D24.3I5	TGACTCAA	5	p	Bac.Density_Tilapia	GUT	index_8nt_661	62	Lowwater_Highfeed

#SampleID	Barcode Sequence	Library Number	Direction	Study	Sample Type	Barcode ID	Barcode	Treatment
LWHF2.D24.4.15	TGATCTCA	5	p	Bac.Density_Tilapia	GUT	index_8nt_665	63	Lowwater_Highfeed
LWHF3.D24.1.15	TGCCTGCG	5	p	Bac.Density_Tilapia	GUT	index_8nt_671	64	Lowwater_Highfeed
ASwaterF1	TGGTAATT	5	p	Paramecium_Tilapia	water	index_8nt_684	65	Active suspension
ASwaterF2	TGGTATGA	5	p	Paramecium_Tilapia	water	index_8nt_685	66	Active suspension
ASwaterF3	TTAGGATG	5	p	Paramecium_Tilapia	water	index_8nt_693	67	Active suspension

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
4509019.3	AACGATCC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509030.3	ATGCGGTT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509041.3	CTCACTGT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509022.3	ACAGACTC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509033.3	CAGTAGTC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509044.3	GACAACTC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509021.3	AAGGCCTT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509032.3	CACACAGT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509043.3	GAACTGGA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509023.3	ACCTACCT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509034.3	CCAATTCC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509045.3	GAGAGACT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509016.3	AGGAACCA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509027.3	CGCCAATA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509038.3	GGAAITGG	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509020.3	AATTCGGG	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509031.3	CAACTGCA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509042.3	CTGTGACA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509024.3	ACGAAGCA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509035.3	CCGGAATT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454

Table S6.2. Metadata of all gut samples included in the meta-analysis from own and publically available datasets.

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
4509046.3	GCAATACC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509026.3	AGCTGATC	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509037.3	CGATAAGG	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509048.3	GCTAGGAA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509025.3	AGACTGT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509036.3	CCTTCCA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509047.3	GCCGAAT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509017.3	ATATCCGG	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509028.3	CGTAGGTT	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509039.3	GGCGAATA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509018.3	ATCCGCAA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509029.3	CTACCTTG	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
4509040.3	GGTACGAA	7	Diet_Trout	Adult	Flow_through	Rainbow_trout	Whole_gut	454
ERX004488	AAAAAAAA	8	Wild-Lab_Zebrafish	Adult	Static	Zebrafish	Digesta	454
ERX004489	CCCCCCCC	8	Wild-Lab_Zebrafish	Adult	Static	Zebrafish	Digesta	454
ERX004490	GGGGGGGG	8	Wild-Lab_Zebrafish	Adult	Static	Zebrafish	Digesta	454
ERR042931	AAAAAAAA	9	Pond_Grasscarp	Adult	Pond	Grass_Carp	Digesta	454
ERR042934	CCCCCCCC	9	Pond_Grasscarp	Adult	Pond	Grass_Carp	Digesta	454
ERR042935	GGGGGGGG	9	Pond_Grasscarp	Adult	Pond	Grass_Carp	Mucosa	454
ERR042937	TTTTTTTT	9	Pond_Grasscarp	Adult	Pond	Grass_Carp	Digesta	454
ERR358524	AAAAAAAA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358525	AAAAAAAA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358526	AAAAAAAAAG	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358527	AAAAAAAAAT	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358528	AAAAAACA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358529	AAAAAACC	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358530	AAAAAACG	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358531	AAAAAACT	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358532	AAAAAAGA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358533	AAAAAAGC	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
ERR358534	AAAAAAGG	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358535	AAAAAAGT	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358536	AAAAAATA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358537	AAAAAATC	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358538	AAAAAATG	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358539	AAAAAATT	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358540	AAAAACAA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
ERR358541	AAAAAGAA	10	Ragworm_Zebrafish	Larvae	Flow_through	Zebrafish	Whole_gut	454
mgm4621988.3.050	AAAAAAA	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621989.3.050	AAACCCC	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621990.3.050	AAAAGGGG	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621991.3.050	AAAATTTT	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621992.3.050	CCCCAAAA	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621993.3.050	CCCCCCCC	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621994.3.050	CCCCGGGG	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
mgm4621995.3.050	CCCTTTT	11	Probiotic_Tilapia	Fingerlings	Recirculating	Tilapia	Digesta	lon_torrent
DRR001611	AAAAAAA	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Mucosa	454
DRR001612	AAAAAAC	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001613	AAAAAAG	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001614	AAAAAAT	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001615	AAAAACA	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001616	AAAAACC	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001617	AAAAACG	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001618	AAAAACT	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001619	AAAAAGA	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001620	AAAAAGC	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
DRR001621	AAAAAGG	12	Pond_Gibelcarp	Adult	Pond	Gibel_carp	Digesta	454
07Ra.2.1	AACCAAC	6	RAS-AS_Tilapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Ra.2.10	AACCAAG	6	RAS-AS_Tilapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Ra.2.4	AACCATCG	6	RAS-AS_Tilapia	Larvae	Recirculating	Tilapia	Whole_gut	454

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
07Ra.4.2	AACCATGC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Ra.4.7	AACCGCAT	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Ra.4.9	AACCGCTA	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Rb.3.10	AACCGGAA	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Rb.3.3	AACCGGTT	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Rb.3.7	AACCTACG	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Rb.4.10	AACCTAGC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Rb.4.3	AACCTTCC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07Rb.4.5	AACCTTGG	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
07AS.4.1	AAGGAACG	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
07AS.4.6	AAGGAAGC	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
07AS.4.7	AAGCATCC	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
07As.5.2	AACGATGG	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
07AS.5.8	AACGCCAT	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
07AS.5.9	AACGCCCTA	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
42Ra.2.10	AACGTTGC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Ra.2.7	AAGCAACG	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Ra.2.9	AAGCAAGC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Ra.4.1	AAGCATCC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Ra.4.4	AAGCATGG	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Ra.4.5b	AAGCCGAA	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Rb.3.10	AAGCCGTT	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Rb.3.3	AAGCGCAA	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Rb.3.9	AAGCGCTT	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Rb.4.2	AAGCGGAT	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Rb.4.6	AAGCGGTA	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42Rb.4.8	AAGTACC	6	RAS-AS_Tlapia	Larvae	Recirculating	Tilapia	Whole_gut	454
42AS.4.3	AAGCTAGG	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
42AS.4.7	AAGCTTCG	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
42AS.4.8	AAGCTTGC	6	RAS-AS_Tlapia	Larvae	Active_suspension	Tilapia	Whole_gut	454

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
42AS.5.10	ACACCTCT	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
42AS.5.4	ACACCTGA	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
42AS.5.5	ACAGGACT	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
00AS.4.5	ACACGAGA	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
00AS.4.9	ACACGTCA	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
00AS.5.3	ACACGTGT	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
00AS.5.8	ACACTCAG	6	RAS-AS_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	454
SAMD00013620	TGFACTCG	13	Pond_Carps	Adult	Pond	Grass_carp	Digesta	454
SAMD00013625	ACAGTCAT	13	Pond_Carps	Adult	Pond	Grass_carp	Digesta	454
SAMD00013618	ACAGTGGC	13	Pond_Carps	Adult	Pond	Grass_carp	Digesta	454
SAMD00013619	CATAGCAT	13	Pond_Carps	Adult	Pond	Grass_carp	Mucosa	454
SAMD00013626	ACATATAG	13	Pond_Carps	Adult	Pond	Crucian_carp	Digesta	454
SAMD00013624	ACATCTAT	13	Pond_Carps	Adult	Pond	Crucian_carp	Digesta	454
SAMD00013622	ACATGATG	13	Pond_Carps	Adult	Pond	Crucian_carp	Digesta	454
SAMD00013623	ATCAGACA	13	Pond_Carps	Adult	Pond	Crucian_carp	Mucosa	454
SAMD00013628	CAGTGCGT	13	Pond_Carps	Adult	Pond	Bighead_carp	Digesta	454
SAMD00013621	CATACATG	13	Pond_Carps	Adult	Pond	Bighead_carp	Digesta	454
SAMD00013627	CATACGAT	13	Pond_Carps	Adult	Pond	Bighead_carp	Digesta	454
SAMD00013629	CAGTAGAC	13	Pond_Carps	Adult	Pond	Bighead_carp	Digesta	454
Common.1.D0.GUT	GAAGTAAG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Mucosa	454
Common.2.D0.GUT	GAAGCTCG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Common.3.D0.GUT	GAGTATA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Common.4.D0.GUT	GATGATAA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.1.D8.GUT	GATGCGCT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.2.D8.GUT	GCCAGGTT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.3.D8.GUT	GCCTTAAG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.4.D8.GUT	GCTAATCT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.1.D8.GUT	GGAGCGCA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.2.D8.GUT	GGAGTATG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.3.D8.GUT	GGTACCAC	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.4.D8.GUT	GGTAGAAT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
Denitrif.1.D8.GUT	GTCCGCAA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.2.D8.GUT	GTTAAGTT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.3.D8.GUT	GTTACTAGG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.4.D8.GUT	TATTGCGC	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.1.D8.GUT	TCAGCGAG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.2.D8.GUT	TCATTCGG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.3.D8.GUT	TCCGTATA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.4.D8.GUT	TCCTCCGC	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.1.D16.GUT	TCGAATAA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.2.D16.GUT	TCGATATT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.3.D16.GUT	TCTATTGG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Aerob.4.D16.GUT	TGACTCAA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.1.D16.GUT	TGATCTCA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.2.D16.GUT	TGCCCTGCG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.3.D16.GUT	TGGTAATT	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Methanog.4.D16.GUT	TGGTATGA	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.1.D16.GUT	TTAGGATG	2	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.2.D16.GUT	AACCAGAA	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.3.D16.GUT	AACCATGC	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Denitrif.4.D16.GUT	AAGGGCGC	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.1.D16.GUT	AAGGAGCG	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.2.D16.GUT	ACCAATTA	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.3.D16.GUT	ACCGGAAT	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
Control.4.D16.GUT	ACGCTCGC	3	Sludge_Tilapia	Larvae	Flow_through	Tilapia	Whole_gut	illumina_Hiseq
AX1.D14.G1	CTCAGTCT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D14.G2	CTCGCGTA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D14.G3	CTGCTGAA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D14.G4	CTTGGGAG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D14.G1	CTTGGCCT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D14.G2	GAACCGTT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
AX2.D14.G3	GAACGTAT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D14.G4	GAACTAAG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D14.G1	GAAGCTCG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D14.G2	GAGTATA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D14.G3	GATGATAA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D14.G4	GATGCGCT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D21.G1	GCCAGGTT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D21.G2	GCCTTAAG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D21.G3	GCTAATCT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D21.G4	GGAGCGCA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D21.G1	GGAGTATG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D21.G2	GGTACCAC	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D21.G3	GGTAGAAT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D21.G4	GTCCGCAA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D21.G1	GTTAAGTT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D21.G2	GTTCTAGG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D21.G3	TATTGCGC	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D21.G4	TCAGCGAG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D28.G1	TCATTCCG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D28.G2	TCCGTATA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D28.G3	TCCTCCGC	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX1.D28.G4	TCGAATAA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D28.G1	TCGATATT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D28.G2	TCTATTCC	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D28.G3	TGACTCAA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX2.D28.G4	TGATCTCA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D28.G1	TGCTCGGC	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D28.G2	TGGTAATT	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D28.G3	TGGTATGA	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
AX3.D28.G4	TTAGGATG	3	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
GN1.D14.G1	AACCAGAA	4	Gnotobiotic_Tillapia	Larvae	Probiotic	Tilapia	Whole_gut	illumina_Hiseq
GN1.D14.G2	AACCATGC	4	Gnotobiotic_Tillapia	Larvae	Probiotic	Tilapia	Whole_gut	illumina_Hiseq
GN2.D14.G1	AAGCGGC	4	Gnotobiotic_Tillapia	Larvae	Probiotic	Tilapia	Whole_gut	illumina_Hiseq
GN2.D14.G2	AAGGAGCC	4	Gnotobiotic_Tillapia	Larvae	Probiotic	Tilapia	Whole_gut	illumina_Hiseq
GN1.D21.G1	ACCAATTA	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D21.G2	ACCGGAAT	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D21.G3	ACGCTCGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D21.G4	AGACCTGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D21.G1	AGCAGATT	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D21.G2	AGCTTCGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D21.G3	AGGTCGAA	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D21.G4	AGGTTATG	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D21.G1	AGTCGGCC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D21.G2	AGTTATGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D21.G3	ATATCCGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D21.G4	ATGCCGGA	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D21.G1	ATGCGATT	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D21.G2	CAACCTCT	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D21.G3	CAACGAGG	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D21.G4	CATAAGCC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D28.G1	CATGATGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D28.G2	CATTGCCA	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D28.G3	CCATGCGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN1.D28.G4	CCATTATG	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D28.G1	CCGFACTA	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D28.G2	CCGTCTGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D28.G3	CGCAAGCT	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN2.D28.G4	CGGAAGAG	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D28.G1	CTAGGAGA	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D28.G2	CTCAGCGC	4	Gnotobiotic_Tillapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
GN3.D28.G3	CTCAGTCT	4	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN3.D28.G4	CTCGCGTA	4	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D28.G1	CTGCTGAA	4	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D28.G2	CTTGCGAG	4	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D28.G3	CTTGGCCT	4	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
GN4.D28.G4	GAACCGTT	4	Gnotobiotic_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
CB.D0.G1	GTTAAGTT	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
CB.D0.G2	GTTTACG	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
CB.D0.3	TATTGCC	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
CB.D0.4	TCAGCGAG	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF1.D24.G1	TCATTCCG	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF1.D24.G2	TCCGTATA	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF1.D24.G3	TCCTCCGC	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF1.D24.G4	TCGAATAA	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF2.D24.G1	TCGATATT	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF2.D24.G2	TCTATTCCG	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF2.D24.G3.I4	TGACTCAA	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF2.D24.G4.I4	TGATCTCA	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF3.D24.G1.I4	TGCCCTGGG	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF3.D24.G2	TGGTAATT	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF3.D24.G3	TGGTATGA	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.HF3.D24.G4	TTAGGATG	4	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F1.D24.G1	AACCAGAA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F1.D24.G2	AACCATGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F1.D24.G3	AACGGCCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F1.D24.G4	AAGGAGCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F2.D24.G1	ACCAATTA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F2.D24.G2	ACCGGAAT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F2.D24.G3	ACGCTCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWL.F2.D24.G4	AGACCTGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq

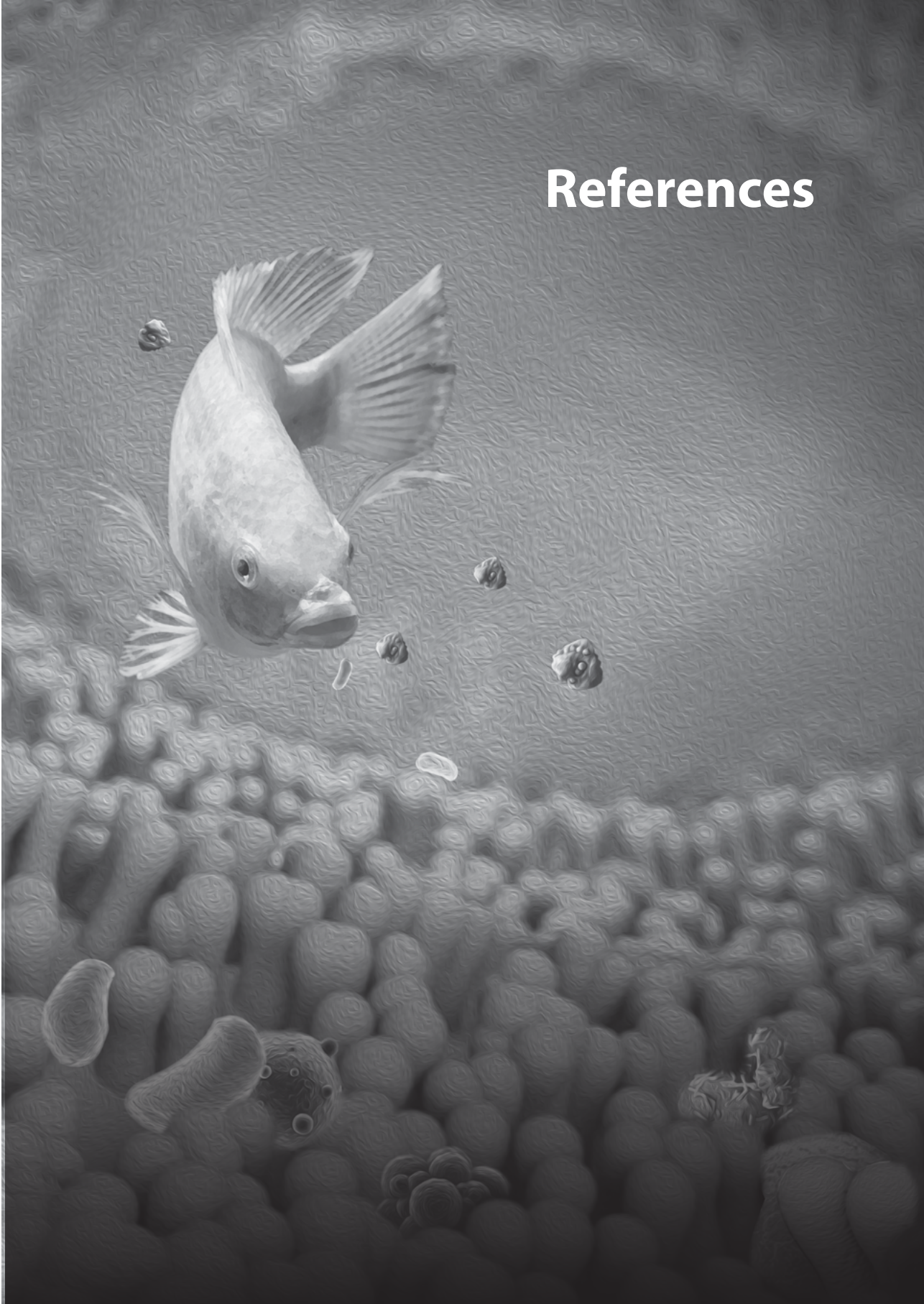
#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
LW.LF3.D24.G1	AGCAGATT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.LF3.D24.G2	AGTTCGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.LF3.D24.G3	AGGTCGAA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LW.LF3.D24.G4	AGGTTATG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF1.D24.G1	AGTCGGCC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF1.D24.G2	AGTTATGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF1.D24.G3	ATATCCGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF1.D24.G4	ATGCCGGA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF2.D24.G1	ATGCGATT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF2.D24.G2	CAACTCT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF2.D24.G3	CAACGAGG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF2.D24.G4	CATAAGCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF3.D24.G1	CATGATGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF3.D24.G2	CATTGCCA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF3.D24.G3	CCATGCCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.HF3.D24.G4	CCATTATG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF1.D24.G1	CCGTACTA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF1.D24.G2	CCGTCTGC	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF1.D24.G3	CGCAAGCT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF1.D24.G4	CGGAAGAG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF2.D24.G1	CTAGGAGA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF2.D24.G2	CTCAGCCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF2.D24.G3	CTCAGTCT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF2.D24.G4	CTCGCGTA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF3.D24.G1	CTGCTGAA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF3.D24.G2	CTTGCGAG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF3.D24.G3	CTTGGCCT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
HW.LF3.D24.G4	GAACCGTT	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
STNF:STNW.G1	GAAGTAT	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STNF:STNW.G2	GAACTAAG	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STNF:STNW.G3	GAAGCTCG	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq

#Sample_ID	Barcode Sequence	Library	Study	Age	Culture System	Species	Biome	Seq_Method
STNF.STNW.G4	GAGTTATA	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STRF.STNW.G1	GATGATAA	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STRF.STNW.G2	GATGCGCT	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STRF.STNW.G3	GCCAGGTT	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STRF.STNW.G4	GCCTTAAG	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STNF.STRW.G1	GCTAATCT	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STNF.STRW.G2	GGAGCGCA	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STNF.STRW.G3	GGAGTATG	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
STNF.STRW.G4	GGTACCAA	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
NF.STNW.G1	GGTAGAAT	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
NF.STNW.G2	GTCGCGAA	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
NF.STNW.G3	GTTAAGTT	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
NF.STNW.G4	GTTCTACG	5	Paramecium_Tilapia	Larvae	Static	Tilapia	Whole_gut	illumina_Hiseq
LWHF2.D24.3.I5	TGACTCAA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWHF2.D24.4.I5	TGATCTCA	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq
LWHF3.D24.1.I5	TGCCTGCG	5	Bac.Density_Tilapia	Larvae	Active_suspension	Tilapia	Whole_gut	illumina_Hiseq

Table S6.3. References and sequence databases of the studies included in the meta-analysis

Study	Study Reference	Sequence Database
Diet_Trout	(Wong et al., 2013)	http://metagenomics.anl.gov/?page=MetagenomeProject&project=2870
Wild-Lab_Zebrafish	(Roeselers et al., 2011)	http://www.ncbi.nlm.nih.gov/Traces/study/?acc=ERP000213
Pond_Grasscarp	(Wu et al., 2012b)	http://www.ebi.ac.uk/ena/data/view/ERP000842
Ragworm_Zebrafish	(Rurangwa et al., 2015)	http://www.ncbi.nlm.nih.gov/Traces/study/?acc=ERP004104
Probiotic_Tilapia	(Standen et al., 2015)	http://metagenomics.anl.gov/metagenomics.cgi?page=MetagenomeProject&project=12845#jobs
Pond_Gibelcarp	(Wu et al., 2013)	http://www.ncbi.nlm.nih.gov/Traces/study/?acc=DRP000498
Pond_Carps	(Li et al., 2014a)	http://www.ncbi.nlm.nih.gov/Traces/study/?acc=DRP001329
Paramecium_Tilapia	Unpublished data	NA
Sludge_Tilapia	(Giatsis et al., 2016, under review)	SRA/NCBI accession number SRP062681
Gnotobiotic_Tilapia	(Giatsis et al., 2016)	SRA/NCBI accession number SRP062676
Bac.Density_Tilapia	Unpublished data	NA
RAS-AS_Tilapia	(Giatsis et al., 2015)	http://www.ebi.ac.uk/ena/data/view/PRJEB4462

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Summary

Aquaculture has realized considerable growth over the past years while the world demand on seafood has been increasing. As aquaculture intensifies, the production sector needs to tackle major bottlenecks related to suboptimal growth and high and unpredictable mortality, especially in larval cultures. Attempts in overcoming those problems mostly focused on broodstock management, optimization of water quality, nutrition and manufacturing technology of the feeds. However, microbial processes might also be involved.

Fish-microbe interactions are closely related to overall fish health. Innate immune response and nutrient metabolism depend on microorganisms that colonize the gastrointestinal tract. Therefore it is important to understand the underlying mechanisms of microbial colonization in the fish gut, which will lead to a healthy and resilient microbial community (MC) and thus contribute to fish health.

The goal of this thesis was to investigate the role of water and feed microbial communities on shaping gut communities during early development of fish.

In **chapter 2** we characterized the variation in MC composition between identically reared individuals to determine the contribution of stochasticity to the overall variation. For this, we quantified the spatio-temporal variation of gut microbiota of Nile tilapia larvae, reared in recirculating or active suspension systems (RAS vs. AS). Individuals were reared for six weeks within the same or in replicate tanks, and within replicate and different production systems i.e. RAS and AS. Highly similar MCs developed in the gut when larvae shared the same water and diet. Rearing larvae in replicate production systems resulted in significantly different gut communities indicating that compositional replication of the MCs of an ecosystem is not fully predictable.

In **chapter 3** the relative contribution of water and feed microbiota to the development of gut MCs was evaluated. For this, we characterized the composition of gut microbiota of larvae reared in RAS and AS systems. Observed changes in gut MCs over time and between systems were highly correlated with shifts in water MCs. Differences in water physico-chemical properties corresponded with differences in water MC microbial composition. Mainly water MCs, and to a lesser degree feed MCs, were associated with changes in gut communities. Steering gut MCs could therefore be possible through water MC management tailored on the specifications of the rearing system in use.

The possibility of early life steering of gut communities via microbial manipulations of feed MCs was explored in **chapter 4**. We hypothesized that gut microbial composition is strongly shaped by selective pressures in the gut and by the MCs present in the water. Thus similar MCs should develop between treatments regardless the dietary treatments.

Fish larvae were fed a control feed or the control feed containing MCs derived from aerobic, methanogenic or denitrifying sludge reactors. We found that gut microbiota shared a much higher number of operational taxonomic units (OTUs) with microbiota in sludge-based feeds than with water, resulting in distinct gut MCs between treatments. Our findings suggest that Nile tilapia gut MC has a certain plasticity, which makes it amenable to interventions through proper feed microbial management.

In **chapter 5** we tested the imprinting effect of early exposure to the probiotic *Bacillus subtilis* on shaping gut MC composition even after the administration of the probiotic discontinues. For this, we constrained the initial contact with microbes from the environment by producing axenic tilapia larvae. Subsequently, the imprinting hypothesis was tested by exposing the axenic larvae to normal husbandry conditions. *B. subtilis* was detected in the gut during the administration period. Early life probiotic exposure affected gut MC composition during *B. subtilis* administration but also after discontinuing administration of the probiotic. Although *B. subtilis* abundance dropped below the detection limit within 2 weeks after stopping administration, our findings indicated that the early exposure to the probiotic strain via the water had a sustained impact on gut MC composition.

Finally, in **chapter 6** the main results of this thesis were discussed within the context of existing literature, and overall conclusions and practical implications for aquaculture production were presented. Additionally, a meta-analysis was performed with the dual purpose of examining (a) the phylogenetic similarity among gut MCs of the same and different fish species reared in different habitats, fed different diets and at different developmental stages and (b) the factors primarily shaping gut MCs. We showed that the selective pressure responsible in shaping gut MC composition highly depends on the host as gut communities clustered primarily together by host and to a lesser extent reflected differences in habitat and diet. The phylogenetic analysis of gut communities revealed a clear clustering by study indicating that manipulation of gut communities is conceivable. Study-to-study variation could be attributed to the methodology used for MC analysis highlighting also the importance of methodological uniformity when comparisons between studies are made.

Overall, this thesis provided fundamental knowledge on MC composition and development in aquaculture rearing systems. The insights generated are still too premature to fully explain, predict or steer MC composition. Additional studies are needed extending the experiments described here to other species and environments, executed following guidelines for optimization of experimental design and methodological conformity as suggested in this thesis. In the long run, this approach will facilitate the development of safe and effective methods for manipulating gut microbial composition to promote fish health in aquaculture rearing systems.

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About the author

Christos Giatsis was born on August 13th, 1981 in Athens, Greece. After high school he started in 2001 his studies on Agricultural technology at the Technological Institute of Messolonghi, Greece. He graduated in 2005 completing a B.Sc. thesis on "Environmental state of Messolonghi lagoon complex" which was carried out at the Aquaculture and Fisheries department. In 2008 he obtained his M.Sc. degree from the department of Applied Hydrobiology of the Agricultural University of Athens with a thesis entitled "Partial replacement of commercial diet by housefly pupae in Rainbow trout fingerlings". In 2009 he started his Ph.D. research at the Aquaculture and Fisheries group of Wageningen University as a part of the EU project "Promicrobe - Microbes as positive actors for more sustainable aquaculture". The results of his Ph.D. research are described in this thesis. In both 2013 and 2014 he collaborated as visiting scholar at Hagerman Fish Culture Experimental Station, University of Idaho, USA, working on "Examining the gut microbiome of rainbow trout fed plant protein diets". In the last year he has worked as a research assistant at the Aquaculture and Fisheries group of Wageningen University in a project entitled "Unlocking non-starch polysaccharides to improve feed utilization and probiotic benefits". He is currently employed as Project Head Probiotics of the Animal Nutrition and Care, by Evonik industries in Germany.



List of publications

- Giatsis C**, Sipkema D, Smidt H, Verreth J, Verdegem M. (2014). The Colonization Dynamics of the Gut Microbiota in Tilapia Larvae. *PLoS ONE* 9: e103641.
- Giatsis C**, Sipkema D, Smidt H, Heilig H, Benvenuti G, Verreth J, et al. (2015). The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Scientific reports* 5: 18206.
- Giatsis C**, Ramiro-Garcia J, Abernathy J, Verreth J, Smidt H, Sipkema D, Verdegem M (2016). Probiotic legacy on gut microbial assembly in fish larvae. *Scientific reports* (online).
- Giatsis C**, Verdegem M, Loo J van, Rohaan B, Verreth J, Smidt H, Ramiro-Garcia J, Sipkema D. (2016). Steering the gut microbiome of tilapia larvae with microbial diets (under review).
- Giatsis C**, Abernathy J, Loo J van, Borgos S. E, Degnes K.F, Aasen I.M, Untargeted metabolite profiling as a tool to understand the function of the gut microbiota in fish larvae (in preparation).
- Ramiro-Garcia J, Hermes GDA, **Giatsis C**, Sipkema D, Zoetendal EG, Schaap PJ, et al. (2016). NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. *F1000 Research* 5: 1791.
- Ramli Norulhuda M, Yusoff F.M, **Giatsis C**, Tan G.Y.A, Verreth J, Verdegem M. (2016). Inclusion effects of microalgae on water quality and bacterial community of recirculating aquaculture system (RAS) (submitted).

Training and Supervision Plan



Education and training		
The Basic Package	year	credits
WIAS Introduction Course	2009	
Course on philosophy of science and/or ethics	2010	
Introduction interview with WIAS scientific director and secretary: 27/04	2009	
Introduction interview with WIAS education coordinator: 16/04	2009	
Introduction interview with WIAS PhD students confidant: 16/04	2009	
Subtotal Basic Package		3.0
Scientific Exposure		
	year	credits
International conferences		
Aquaculture Europe, Porto, Portugal, Oct 6-8	2010	
Aquaculture Europe, Rhodes, Greece, Oct 18-21	2011	
Aquaculture Europe, Prague, Czech Republic, Sept 1-5	2012	
Aquaculture Europe, Rotterdam, Netherlands, Oct 20-23	2015	
US Trout Farmers Association & Idaho Aquaculture Association, Twin Falls, USA	2014	
Seminars and workshops		
Sole culture in the Netherlands, past, present and future, November 5	2010	
Resilience of fish in intensive systems, November 4	2010	
Presentations		
Oral presentation in Aquaculture Europe, Porto, Portugal, Oct 6-8	2010	
Oral presentation in Aquaculture Europe, Rhodes, Greece, Oct 18-21	2011	
Oral presentation in Prague, Czech Republic, Sept 1-5	2012	
Oral presentation in Rotterdam, Netherlands, Oct 20-23	2015	
WIAS Science Day-poster presentation	2010	
WIAS Science Day-poster presentation	2011	
WIAS Science Day-poster presentation	2012	
Subtotal Scientific Exposure		12.6
In-Depth Studies		
	year	credits
Disciplinary and interdisciplinary courses		
Larvita training School, Algarve, Portugal, Nov 22-26	2010	
Bionumerics-Applied maths course, Gent, Belgium Sept 5,6 & 7	2011	
Promicrobe course, Gent, Aug 20-22	2012	
Technology for novel fish feeds, Olhao, Portugal, Oct 26-29	2014	
Long term effects of diets low in fish oil and fish meal, Gran Canaria, Spain, Jan 13-15th	2016	
Advanced statistics courses		
Advanced Statistics course: Design of Experiments	2010	
MSc level courses		
Aquaculture production systems (APS)	2009	
Subtotal In-Depth Studies		13.4

Statutory Courses	year	credits
Use of Laboratory	2009	
Subtotal Statutory Courses		3.0
Professional Skills Support Courses	year	credits
Teaching and Supervising Thesis Students	2010	
Project and Time Management	2011	
Techniques for Writing and Presenting a Scientific Paper	2011	
Scientific Publishing	2011	
Reviewing a scientific paper	2011	
Subtotal Professional Skills Support Courses		4.2
Research Skills Training	year	credits
Preparing own PhD research proposal	2009	
Advanced molecular techniques, University of Idaho, USA	2014	
External training period		
Visiting scholar University of Idaho, USA	2013	
Subtotal Research Skills Training		11.0
Didactic Skills Training	year	credits
<i>Supervising practicals and excursions</i>		
Research master cluster YAS 60312 supervision	2010	
QAP course. Queens products excursion	2011	
<i>Supervising theses</i>		
MSc Student (major) Amin Muhamad	2009	
MSc Student (major) Paulo Mira Fernandes	2010	
MSc Student (major) Catarina Silva	2010	
MSc Student (major) Hera Suan	2010	
MSc Student (major) Papius Tibihika	2011	
MSc Student (major) Pandu Rizqi	2011	
MSc Student (minor) Pandu Rizqi	2012	
MSc Student (major) Ivan Tankowski	2012	
MSc Student (major) Bram Rohaan	2012	
MSc Student (major) Joost van Loo	2012	
Subtotal Didactic Skills Training		20.1
Management Skills Training	year	credits
<i>Organisation of seminars and courses</i>		
<i>Membership of boards and committees</i>		
Aquarius board member (EAS-SG Netherlands website administration)		
Subtotal Management Skills Training		3
Education and Training Total (minimum 30, maximum 60 credits)		70

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