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Effect of rearing systems and dietary probiotic supplementation on the growth and gut microbiota of Nile tilapia (*Oreochromis niloticus*) larvae

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ABSTRACT

The establishment of the early-life gut microbiota plays an important role in fish development and influences the host's health status and growth performance. Different rearing conditions can impact the initial colonization of the gut microbiota, while the addition of probiotics may also affect such colonization. However, how this may affect fish larvae survival and growth remains largely unexplored. In this study, 3-day old Nile tilapia embryos were hatched until 9 days post fertilization (dpf) in three systems, including one flow-through system (FTS) and two identical recirculating aquaculture systems (RAS). When feeding started at 10 dpf, tilapia larvae in the FTS and one of the RAS were fed with a control diet, while larvae in the second RAS were fed with the control diet coated with *B. subtilis* spores (RASB). The feeding trial lasted 26 days, from larvae to fry stage, during which the survival, growth performance and gut microbiota were analyzed. The larvae reared in FTS showed significantly lower survival than those in RAS and RASB, while no differences were observed in fish growth and apparent feed conversion ratio between treatments. Different rearing systems resulted in different gut microbiota compositions, which strongly correlated with the survival rate and standard body length at harvest. *Cetobacterium* was enriched in RAS and RASB, while was barely detected in the gut of FTS-reared tilapia fry. Probiotic supplementation increased the relative abundance of beneficial *Bacillus* in fish gut. Our findings indicate that rearing fish larvae in RAS supports better survival compared to FTS, while dietary probiotic supplementation further modulates the gut bacterial composition and stimulates presence of beneficial bacteria during early life.

1. Introduction

The stable production of high-quality juveniles is a bottleneck for many species in aquaculture. Fish larvae are susceptible due to their immature immune system and high disease susceptibility, which results in high mortality during larval rearing (Zapata et al., 2006). The unpredictability of mortality and individual growth causes low reproductivity in larvae cultivation, even when similar nutrition and physiochemical water quality are applied (Verner-Jeffreys et al., 2004). Common problems in aquaculture occur by naturally present opportunistic bacteria that may become pathogenic when the host immune system is weakened by environmental stress. The colonization of gut microbes in fish starts when the yolk sac is consumed, and the mouth opens for external feeding (Egerton et al., 2018). The early life colonizing of microbes in the gut can facilitate the maturation of the digestive tract and nutrient digestion, the further development of innate immunity (Rawls et al., 2004), as well as the prevention of pathogen invasion, which influences the growth and health of fish larvae (Nayak, 2010; Rawls et al., 2004; Vadstein et al., 2013). Therefore, the selective establishment of beneficial microbiota in the gut is crucial for the stable production of healthy fish larvae.

The microbial colonization of the fish gut is mainly influenced by rearing water and feed, apart from the selective pressure from the fish-host itself (Dehler et al., 2017; Giatsis et al., 2015). Recent studies showed that the gut microbiota of fish larvae, such as zebrafish, are more similar to the surrounding environment than adult fish, which indicates the great importance of the early-life rearing environment (Stephens et al., 2016; Xiao et al., 2021). For instance, Nile tilapia (*Oreochromis niloticus*) larvae reared in recirculating aquaculture system (RAS) and active suspensions tanks showed distinct gut microbiota composition (Giatsis et al., 2014). Moreover, the similarity between the bacterial communities in tilapia gut and rearing water was reported between 4% and 8%, while a strong correlation in the bacterial community compositions was also observed (Giatsis et al., 2015). On the other hand, feed may have a more negligible effect on gut microbiota composition since feed showed less similarity with the gut microbiota

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than the water in both tilapia and cod larvae culture (Bakke et al., 2013; Giatsis et al., 2015). However, a comprehensive understanding of how rearing environment and feed influence the gut microbial colonization of tilapia larvae and to which extent it can affect fish survival and growth is lacking.

Traditionally, flow-through systems (FTS) are used for fish larvae culture (Attramadal et al., 2012). In FTS, the nutrient load and microbial density are continuously diluted due to water exchange, which has been reported to select for fast-growing bacteria, known as r-strategists (Attramadal et al., 2014; Attramadal et al., 2012). On the other hand, RAS has lower water exchange level than FTS, thus maintaining a constant nutrient load (organic matter) and microbial density. RAS allows maintaining a stable microbial community composition in the water selective to the growth of slow-growing bacteria, known as K-strategists (Attramadal et al., 2014). Opportunistic bacteria are characterized as r-strategists, which often can affect negatively fish health, while K-strategists can deal better with perturbations in nutrient availability and are considered harmless for fish survival (Vadstein et al., 2018a). A study in Atlantic cod showed that larvae reared in RAS performed better than those reared in FTS when fed with live feed, despite the water quality being inferior to FTS (Attramadal et al., 2012). Besides, higher survival and specific growth rate (SGR) were observed in larvae reared in RAS compared to larvae reared in FTS during dry feed period, which implies that larvae could be more robust when cultured in RAS during early life (Attramadal et al., 2014, Attramadal et al., 2012). However, to which extend RAS and FTS systems influence the gut microbiota establishment and performance of freshwater fish species remains largely unknown. A comparison between marine and freshwater larvae would be interesting since freshwater larvae like Nile tilapia do not require natural food (e.g. microalgae, artemia and rotifer) at first feeding. This fact results in a low disturbance of the microbiota in the rearing water, making Nile tilapia a suitable model species to test the effect of rearing systems.

The use of probiotics, which are beneficial microbes that can modulate the microbial community of its host, improve feed utilization and reduce disease susceptibility, has been proposed as a strategy for sustainable aquaculture (Dawood et al., 2018; Hoseinifar et al., 2018; Verschuere et al., 2000). It has been demonstrated that probiotics increase the survival of marine fish larvae (Gomez-Gil et al., 2000). Among the commonly used probiotic species, *Bacillus* species show better properties, owing to their sporulation capacity and the ability to produce antimicrobial substances in aquaculture (Kuebutornye, 2019). To date, many studies have shown the probiotic effect of *Bacillus* spp. on Nile tilapia, including improvement in survival, nutrient digestion and growth, as well as enhancement of immune response and disease resistance (Abarike et al., 2018; Apún-Molina et al., 2009; Galagarza et al., 2018; Liu et al., 2017; Selim and Reda, 2015). Besides, the modulatory effect of *Bacillus* spp. on the predominant gut microbiota of tilapia was previously investigated by denaturing gradient gel electrophoresis (DGGE) (Guimarães et al., 2021; Hassaan et al., 2021; Tachibana et al., 2021; Wang et al., 2017). Nowadays, next-generation sequencing allows exploring how different treatments affect the gut microbiota composition of aquatic species with high resolution, including Nile tilapia (Boyd et al., 2020; Hallali et al., 2018; Maas et al., 2021). For instance, Giatsis et al. (2016) showed that a water bath with *B. subtilis* for 7 days could significantly change the gut microbiota of tilapia larvae and reduce the inter-individual variation between replicate tanks. Therefore, the importance of probiotic addition during first feeding to effectively colonize the larval gut with beneficial bacteria is worth investigating.

Considering the differences in the rearing environment between RAS and FTS, as described above, we hypothesize that tilapia larvae reared in RAS will develop a different gut microbiota and show better survival and growth than those reared in FTS. Besides, we hypothesize that dietary probiotic supplementation will further enrich beneficial bacteria in the fish gut and improve fish performance. In this study, two rearing systems, namely FTS and RAS, were tested for Nile tilapia larvae culture. To test the impact of *B. subtilis* as dietary probiotic in RAS treatment, a control diet and a control + *B. subtilis* coated diet (RASB) were applied. The effect of the three treatments (FTS, RAS and RASB) on survival, growth performance and gut microbiota were evaluated in Nile tilapia larvae, starting from first feeding, for 26 days.

2. Material and methods

This experiment was carried out under the registration code of 2017.W-0077.003 which was approved by the Dutch Central Animal Experiments Committee and Animal Welfare Body of Wageningen University. This experiment was part of a larger project testing the effect of microbial rearing conditions during incubation and larvae period on tilapia performance and gut microbiota until 4 months old.

2.1. Experimental setup and feed preparation

Three microbial rearing environments were tested for Nile tilapia larvae culture, namely FTS + control diet, RAS + control diet and RAS + *B. subtilis* coated diet (RASB). The experimental setup, including rearing systems and diets are visualized in Fig. 1. In FTS, tap water was firstly degassed of nitrogen in a sump (Fig. 1A) before flowing to each fish tanks and outflow water from the fish tanks was discharged from the system. The two replicate RAS (Fig. 1B and C) were not connected to each other, however, had the same size and shape of all system components and the same water flow rate through filters and aquaria. Besides, the biofilters for the two RAS were primed for a month before the start of the experiment with biofilm from a mature recirculating system holding Nile tilapia. The three systems shared the same water supply and were operated at 27 °C ± 1 °C. Each system contained three replicate 70-L tanks (water volume 60-L) to culture hatched embryos. The experiment started with 3 days post fertilization (dpf) embryos (TilAqua International, Velden, the Netherlands) which were reared for 33 days (7 days in incubators and 26 days in tanks). To start the experiment, one batch of 2835 male Nile tilapia embryos coming from a mix of different spawns, was equally divided over 3 incubators. At 10 dpf, swim-up larvae from each incubator were counted and randomly divided between the 3 tanks connecting to their respective rearing system. After the transfer, the incubators were disconnected from their culture system. The water flow through each tank was set at approximately 2 L/min.

In this study, a commercial tilapia diet (F-0.5 GR Pro Aqua Brut-Trouw Nutrition, France), containing 57% protein, 15% crude fat, 8.5% carbohydrate and 11% ash, was used as control diet and the experimental diet was produced by coating the control diet with *B. subtilis* spores. The capacity of *B. subtilis* to produce endospores makes it possible to supply spores to fish through feed. The *B. subtilis* strain (Microbiologics 0269P, Mijdrecht, The Netherlands) was grown on Luria-Bertani (LB) medium, containing 15 g L^{-1} bacteriological agar type E, 10 g L^{-1} Tryptone, 10 g L^{-1} NaCl, and 5 g L^{-1} yeast extract powder, at 37 °C for 24 h. The single bacterial colony was cultured on Difco Sporulation Media (DSM) with the following modification (Monteiro et al., 2005, 2014): nutrient broth 8 g L^{-1}, KCl 1 g L^{-1}, $MnCl_2$ (10 mM) 1 mL L^{-1} and $MgSO_4$ (1 M) 1 mL L^{-1} was sterilized at 121 °C for 30 min. To 1 L of DSM, the following filter sterilized solutions were added: $CaCl_2$ (1 M) 0.5 mL, and FeSO4 (1 mM) 1 mL. The DSM was placed on a shaking incubator (150 rpm) at 37 °C for 48 h. After that, the DSM were pasteurized for 20 min at 80 °C water bathed to kill the live bacteria so that *B. subtilis* spores can be harvested. To quantify the density of spores, the 10^{-5} and 10^{-6} dilutions of DSM were incubated in LB medium at 37 °C for 24 h before counting the colony forming unit (CFU). Prior to use, the DSM were centrifuged at 4500 *g* for 30 min to pellet the spores and stored at 4 °C. The *B. subtilis* spores pellet was resolved in PBS at the concentration of 10^9 CFU mL^{-1}. Then the solution was sprayed on feed at the ratio of 100 mL: 1 kg of feed using a vacuum coater (Dinnissen Pegasus®-10VC, Sevenum, The Netherlands) to get 10^8 CFU/ g feed. The selected dose was according to previous recommendations (Liu et al., 2017; Won et al., 2020). The control diet was

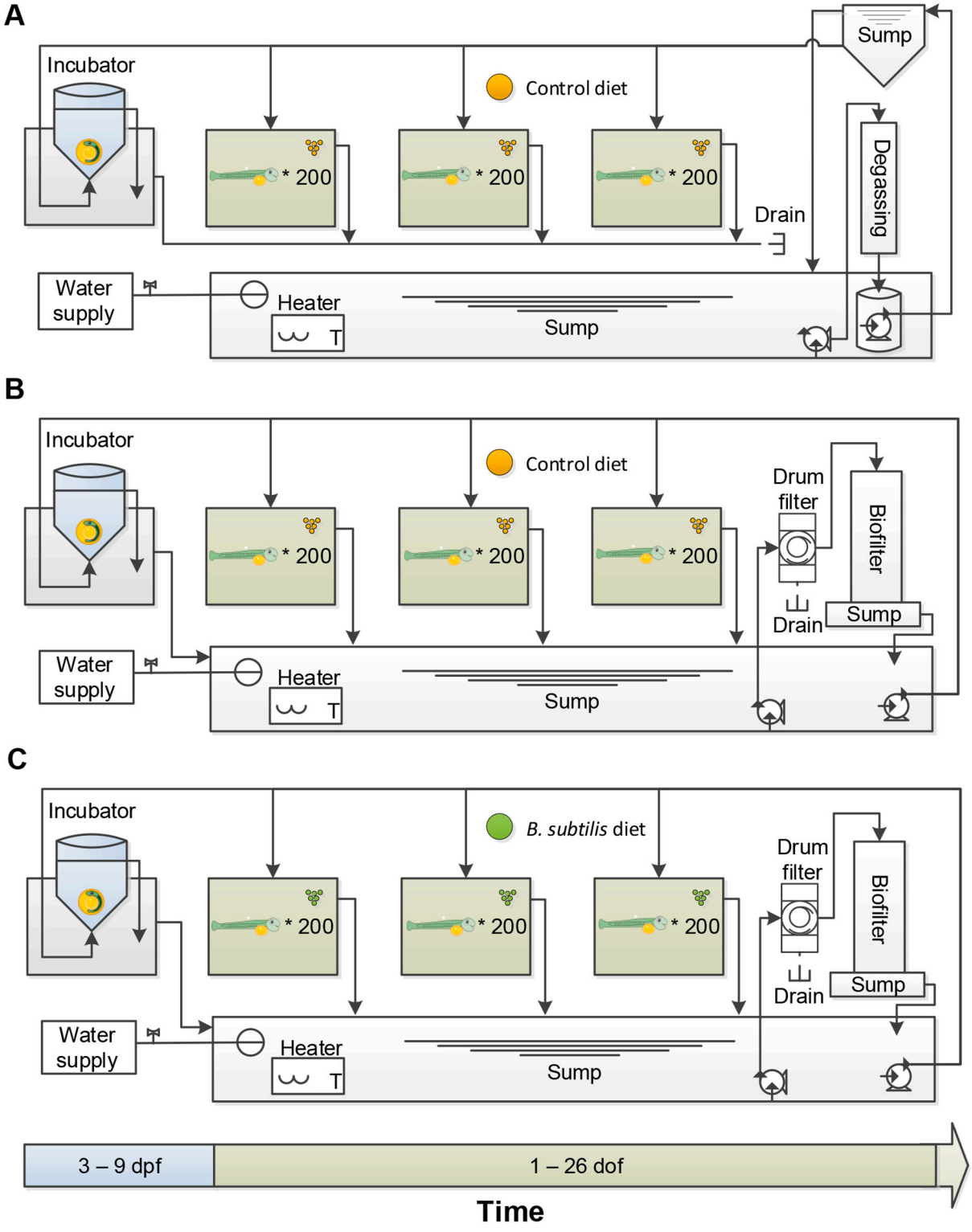

Fig. 1. Schematic of the experimental setup, (a) flow-through system (FST) + control diet, (b) recirculating aquaculture system (RAS) + control diet, (c) RAS + *B. subtilis* coated diet (RASB). Nile tilapia eggs were hatched in an incubator starting from 3 dpf until 9 dpf when they can freely swim. After incubation, the incubator was disconnected from the rearing system, and the larvae were equally divided over three tanks within the same system. Feeding started at 10 dpf and was continued for 26 days. dpf, days post fertilization; dof, days of feeding.

prepared by spraying with only PBS solution at the ratio of 100 mL: 1 kg of feed. The two types of feed were pre-dried in separate ovens for 12 h at 37 °C. After pre-drying, the dry matter (DM) content of diet was measured by drying at 103 °C for 4 h until constant weight. Feeding started at 10 dpf which was referred as 1 day of feeding (dof), and was continued for 26 days. At 5 dof, the number of tilapia larvae in each tank was reduced to 200 (i.e. 3.3 larvae/L) and batch weighed. The average individual body weight was then calculated and assumed to be the initial body weight of tilapia larvae. Fish were fed three times a day for the first 14 days (Giatsis et al., 2014), and then changed to two times at the

feeding rate of 20 g DM $kg^{-0.8}$ d^{-1} (9–19% body weight/d). In this study, fish were fed at restricted feeding level (El-Sayed, 2002; Santiago et al., 1987), and feeding amount was adjusted daily according to the number of fish that survived in each tank. Dissolved oxygen was maintained at >7 mg/L in the tank of each system.

2.2. Water quality measurement and fish sampling

Water samples were collected from the common outlet of the FTS and from the large sump in each RAS (Fig. 1B and C) to measure the water physiochemical quality. The pH, conductivity, temperature (°C) and water supplementation in each system were measured every day. The total ammonia nitrogen (TAN), nitrite (NO_2^--N) and nitrate (NO_3^--N) in the water samples were measured three times a week using Merck Spectroquant Test kits. The number of dead larvae was recorded three times a day and dead larvae were removed immediately from the tank. On 15 dof, salt was added to all systems to increase the salinity to 1.0 ppt, which was a standard procedure to reduce the fish mortality.

On 27 dof, all the surviving fish from each tank were counted and batch weighted to calculated the average final body weight (g), survival (%), apparent feed conversion ratio (FCR) and specific growth rate (SGR, %/d) using the following formulas: Survival = $100 \times N_f/N_i$, Apparent FCR = Total feeding / ($W_f \times N_f - W_i \times N_i$), SGR = $100 \times (\mathrm{Ln}W_f - \mathrm{Ln}W_i)/t$, where N_i and N_f are the initial and final number of fish per tank, W_i (g) and W_f (g) are the initial and final average individual body weight of fish, t is the duration of the experimental period in days. Besides, 3 fish per tank were randomly collected for gut microbiota analysis and euthanized with overdosed 2-Phenoxyethanol solution in water from the corresponding system. To collect the microbiota attached to the gut mucosa, all fish were not fed during the night prior to the day of sampling. The individual body weight (BW, g) and standard body length (SBL, cm) of sampled fish were measured. The fish samples were stored at −80 °C for gut dissection.

2.3. Gut DNA isolation and high throughout sequencing

The 27 sampled fish were first rinsed wit 70% ethanol and sterile water, then the whole gut was removed under a dissection microscope according to Giatsis et al. (2014). The gut samples were individually flash frozen in liquid nitrogen and stored at −80 °C. The gut samples were subjected to lysis by lysozyme buffer and proteinase K before DNA extraction using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). The harvested DNA was quantified using the nano drop spectrophotometer. Sequencing of the PCR-amplified V4 region of 16S rRNA, using primers 515F (5′-CTAGTGCCAGCMGCCGCGGTAA −3′) and 806R (5′-CTAGGACTACHVGGGTWTCTAAT-3′), was performed using a MiSeq PE300 Next Generation system (Illumina) by Genome Quebec, following the company's protocol. Blank samples without DNA template were used as controls. Sequences are submitted to SRA under the access number PRJNA748076.

2.4. Data analysis

The effect of different rearing systems on the water quality parameters, fish growth performance predictors and gut alpha-diversity indices were tested by one-way ANOVA (IBM, SPSS version 25), when normality (Shapiro-Wilk test) and homogeneity (Levene's test) of variance were verified; otherwise rank-transformation was applied. The effect of different rearing systems on the water quality and cumulative mortality was tested by repeated measure ANOVA. Differences between treatments were compared using Tukey HSD when the effect was significant ($P < 0.05$).

An open-source software package, DADA2, was applied to model and correct Illumina-sequenced amplicon errors (Callahan et al., 2016). Data were demultiplexed into forward and reverse reads according the barcode sequence into sample identity, and trimming was performed. For the forward reads and based on the quality profiles, the first 250 nucleotides were kept and the rest were trimmed, while for the reverse reads, the last 220 nucleotides were kept. DADA2 resolves differences at the single-nucleotide level and the end product is an amplicon sequence variant table, recording the number of times each amplicon sequence variant (ASV) was observed in each sample (100% sequence identity). Taxonomy was assigned using the Ribosomal Database Project Classifier (Wang et al., 2007) against the 16S gene reference SILVA database (138 version) (McLaren, 2020). One gut sample from RAS (Seq16) and one gut sample from RASB (Seq33) were removed from analysis since the rarefication curve were not reaching the plateau. The ASV table of the remaining 25 gut samples were subjected to an online platform (https://www.microbiomeanalyst.ca/) to calculate the alpha diversity indices including Chao 1, ACE, Shannon and Simpson after removing the taxa with <2 counts and < 10% prevalence (Dhariwal et al., 2017). Linear Discriminant Analysis (LDA) was applied to calculate the effect size (LEfSe) of each differentially abundant taxa identified by non-parametric factorial Kruskal-Wallis sum-rank test. Principle Coordinate Analysis (PCoA) was conducted to show the beta diversity of the gut samples according to Bray-Curtis distance. The statistical analysis of the gut microbial community was performed by Primer software (Version 6). In detail, the correlation between the gut microbial community and the final fish BW and SBL were analyzed by Distance Linear Model (DistLM). The effect of rearing environments and replicate tanks on the gut microbial composition were analyzed by two-way PERMANOVA.

3. Results

3.1. Water quality maintenance and fish survival

The hatching rate of Nile tilapia eggs were 82%, 73% and 78% in the incubator for the FTS, RAS and RASB treatment, respectively. The three rearing systems shared the same source for water supplementation, and no significant differences in pH, TAN and NO_2-N was observed between the systems (Table 1). The average temperature in the three systems was maintained at 26.2 ± 0.2 °C. Nitrate was found higher in the two RAS than in FTS. Still, the water quality in the three rearing systems were maintained within preset limits for the growth of Nile tilapia larvae. However, mortality started at 6 dof and followed the trend of FTS > RAS > RASB (Fig. 2). The repeated measure ANOVA showed that the cumulative mortality was significantly higher in FTS ($P = 0.002$), while RAS and RASB had similar cumulative mortality over time. Between 1 and 14 dof, the salinity of the three systems were similar at 0.1 ppt. At 15 dof, salt was added to all systems to elevate the water salinity to 1.0 ppt, which did not reduce fish mortality. According to the regulation of experimental animal ethics, this experiment was terminated on 26 dof, when the cumulative mortality in the experiment became too high.

3.2. Fish growth performance in the larvae to fry stage

Feeding was adjusted daily according to the average number of surviving larvae per tank within each treatment. The amount of feed (mg/d/fish) fed to the different treatments were similar (Table 2). All

Table 1
The physiochemical water quality in the three systems between 1 and 26 dof.

	FTS	RAS	RASB	SEM	*P* value
pH	8.2	7.6	7.5	0.015	ns
TAN (mg/L)	<0.01	<0.01	<0.01	na	na
NO_2-N (mg/L)	0.00	0.01	0.01	0.002	ns
NO_3-N (mg/L)	0.0^a	4.3^b	1.9^b	0.556	**
Water supplement (m^3/d)	7.31^b	0.04^a	0.06^a	0.446	***

dof, days of feeding; SEM, standard error of mean; ns, not significant; na, not applied; ** $P < 0.01$, *** $P < 0.001$, the superscript letter indicates the significance between treatments. FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet.

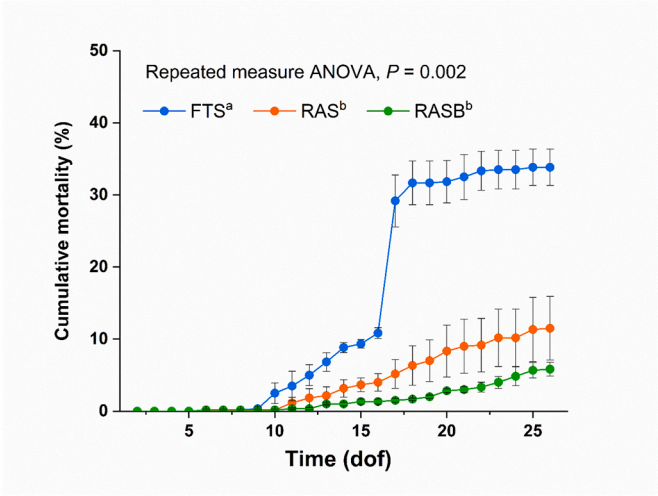

Fig. 2. The cumulative mortality of Nile tilapia larvae in the three treatments during 1–26 dof. Values are presented as average ± standard error. The superscript letters indicate the significant difference between treatments. dof, day of feeding; FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet.

Table 2
The Nile tilapia larvae growth performance between 5 and 26 dof.

Treatment	FTS	RAS	RASB	SEM	*P* value
Feeding (mg/d/fish)	13.8	14.8	14.9	0.18	na
BW_i (g)	0.013	0.014	0.014	0.002	ns
BW_f (g)	0.44	0.47	0.45	0.04	ns
WG (g)	0.434	0.452	0.437	0.15	ns
SGR (%)	16.2	15.8	15.7	0.60	ns
FCR	0.98	0.91	0.93	0.12	ns
Survival rate (%)	61.7[a]	86.3[b]	90.3[b]	4.96	**

BW_i, initial individual body weight; BW_f, final individual body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SEM, standard error of mean; na, not applicable; ns, not significant; dof, days of feeding, FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet; ** $P < 0.01$.

treatments had a similar average individual body weight at 5 dof. Rearing system and probiotic supplementation had no effect on the final individual body weight at 26 dof. Also, the SGR and apparent FCR during the experiment were similar between treatments ($P > 0.05$). The survival on 26 dof in FTS (62%) was significantly lower than survival in RAS (86%) and RASB (90%).

3.3. Gut microbial community composition

The alpha-diversity indices including richness (observed ASVs), Chao1, ACE, Shannon, Simpson and Fisher were not different between treatments ($P > 0.05$). Numerically, there was trend that FTS < RAS < RASB in bacterial richness (i.e. richness, Chao1 and ACE indices) and diversity (i.e. Shannon index) in the gut of tilapia fry at 26 dof.

To examine the unique and shared ASVs, we defined as the presence of an ASV in a treatment when its prevalence was higher than 33%, meaning that it is present in at least one fish within each tank. Venn diagram analysis indicated that 69 ASVs were shared between the three treatments (Fig. 3a). Besides, RAS had 56 ASVs uniquely shared with RASB but not with FTS, while only 17 ASVs were uniquely shared between FTS and RAS, but not with RASB. FTS had the highest number of unique ASVs (121) that were only present in this treatment. As core genera were defined taxa that had higher than 87% in prevalence and 0.01% in relative abundance (RA) in each of the three treatments (Fig. 3b). Our analysis showed that one genus, namely *Plesiomonas*, was shared among all three treatments, while *Escherichia Shingella* showed high prevalence in RAS, and *Gemmobater* and *Bacillus* were prevalent in the RASB treatment. No core genus was solely identified in FTS treatment.

Looking at the phylum level, Proteobacteria, Actinobacteriota and Planctomycetota were the dominating phyla in the gut of tilapia fry from the three treatments, which accounted for 87% of the total population (Fig. 4a). On the genus level, *Plesiomonas* showed high average relative abundance in all treatments (FTS = 24%, RAS = 23%, RASB = 15%) (Fig. 4b). *Cetobacterium* spp. occupied on average 5.0% of the total relative abundance in RAS and 4.9% in RASB, while it was barely detected in FTS (RA < 0.02%). Besides, *Shinella* showed high abundance in one of the tanks from FTS (Tank C; RA = 24%), and *Gordonia* showed high abundance in RASB (RA = 21%).

The gut bacterial community showed distinct distribution among the three treatments according to PCoA diagram (Fig. 5). The first axis of

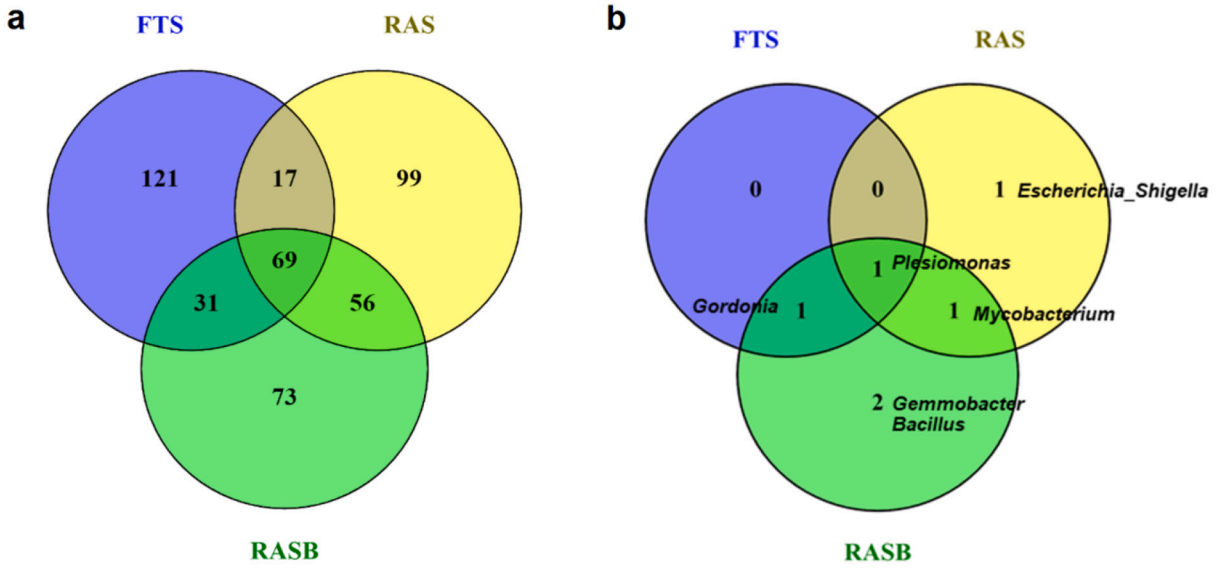

Fig. 3. Venn diagram showing (a) the shared ASV (prevalence >33% in each treatment) and (b) the core genera (prevalence >87% in each treatment, relative abundance >0.01%) in the three treatments. FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet.

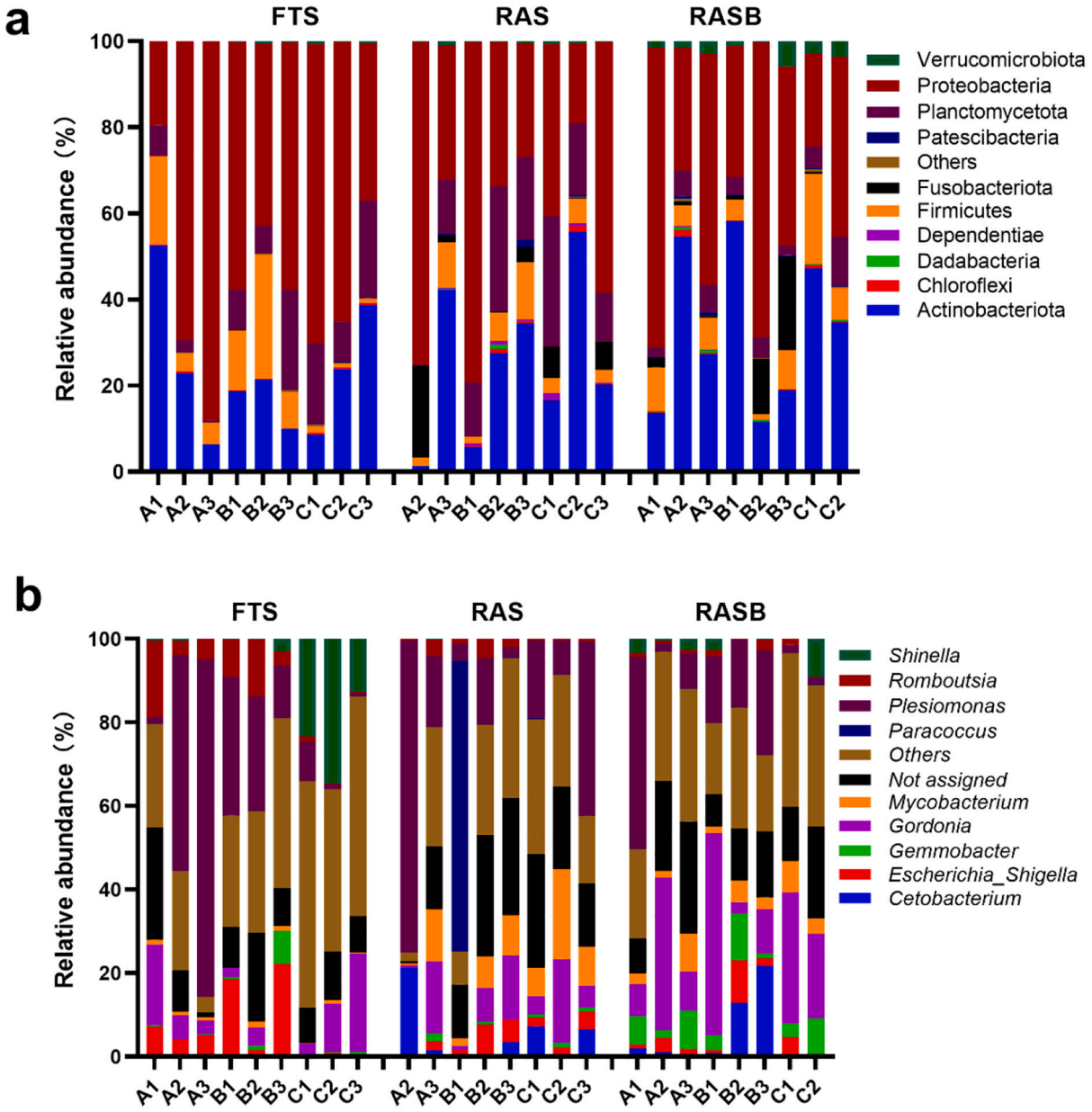

Fig. 4. The relative abundance of the top 10 (a) phyla and (b) genera, in all gut samples. The letters A, B and C represent replicate tanks within each treatment and the numbers 1, 2 and 3 represent the sampled fish within one replicate tank. FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet.

PCoA explained 19.5% of total variation, which was due to the difference in the rearing system (i.e. FTS and RAS). The second axis of PCoA explained 14% of the total variation, which was associated with the dietary supplementation of *B. subtilis* in RASB. The pairwise PERMANOVA results indicated that both FTS vs RAS ($P = 0.001$) and RAS vs RASB ($P = 0.001$) had a different bacterial composition in the gut of tilapia fry (Table S1). Besides, PERMANOVA analysis within treatment showed a tank effect ($P = 0.002$) on gut bacterial community composition (Table S2a). We further investigated separately the tank effect within each treatment using PERMANOVA (Table S2b, c and d), which revealed no differences between the replicate tanks in RAS ($P = 0.179$) and RASB ($P = 0.15$), while tank effect was observed in FTS ($P = 0.006$). PCoA diagram also showed that the gut bacterial community from the three fish cultured in FTS tank C was not clustered with the fish from FTS tank A and B. Moreover, DistLM analysis showed that BW ($P = 0.027$), SBL ($P = 0.006$) and survival ($P = 0.001$) had significant correlations with the gut bacterial community composition (Table S3a). The BW, SBL and survival rate could explain up to 13.5% of the total variation in the gut bacterial composition (Table S3b). Since BW and SBL were strongly correlated, the marginal test showed no significant difference when adding BW as a variable. To be noticed, SBL and survival of tilapia were positively correlated with the two RAS treatments and negatively correlated with the FTS treatments (Fig. 5).

At last, the genera significantly enriched in each treatment were selected by LDA and their relative abundances were shown in Fig. 6. A total of 39 genera were detected significantly enriched in the gut from each of the three treatment. In detail, FTS was enriched with *Shinella* (RA = 8.3%) and *Hyphomicrobium* (RA = 1.6%). RAS was enriched with *Paracoccus* (RA = 8.7%), *Mycobacterium* (RA = 8.7%) and *Cetobacterium* (RA = 5.0%). RASB was enriched with *Gemmobacter* (RA = 5.7%) and *Bacillus* (RA = 4.0%).

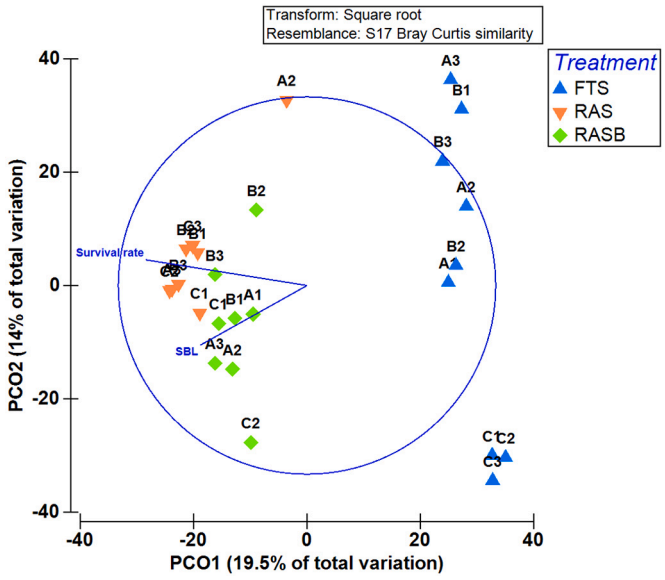

Fig. 5. Principal Co-ordinate analysis (PCoA) diagram showing the bacterial distribution in the three treatments according to Bray-Curtis distance. The letters A, B and C represent replicate tanks within each treatment and the numbers 1, 2 and 3 represent the sampled fish within one replicate tank. Analysis of Bray-Curtis distance showed significantly different (PERMANOVA, Pseudo F-statistic = 3.3137, $P = 0.001$, based on 934 permutations) between the three treatments. SBL, standard body length; FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet.

4. Discussion

Environmental rearing conditions during early life and diet determine the microbial community composition and structure in the fish intestine (Giatsis et al., 2015; Siriyappagouder et al., 2018; Vestrum et al., 2020; Yan et al., 2012; Yukgehnaish et al., 2020). The assembly of gut microbiota further influences fish larvae' immunological and histological development (Vadstein et al., 2013), which plays a crucial rule in fish health and growth. Our study demonstrated the feasibility of modulating the bacterial community in the fish gut by creating different rearing systems or by dietary probiotic supplementation during early life, which could influence survival and lead to a healthy gut microbiota composition.

4.1. Rearing system affected mortality and gut microbiota of tilapia fry

The water quality in the three rearing systems was optimal for the growth of Nile tilapia (FAO, 2012). Due to the continuous water exchange, FTS consumed 160 times more water than RAS (Table 1). The nitrate concentration (NO_3^--N) reached 4.3 and 1.9 mg/L in RAS and RASB, respectively, due to nitrification in the trickling filter of RAS (Greiner and Timmons, 1998). Supplementation of *Bacillus* spp. in the feed or water was shown to enhance the water quality by reducing the ammonia and nitrate concentrations in the systems (Elsabagh et al., 2018; Mohammadi et al., 2020; Zokaeifar et al., 2014). A trend for a lower nitrate concentration in RASB than RAS was observed in our experiment during the later experimental period. However, this difference could also be due to the numerically higher water exchange in RASB when compared with RAS. Therefore, the effect of dietary supplementation of *B. subtilis* on the water quality needs future research with a more extended experimental period.

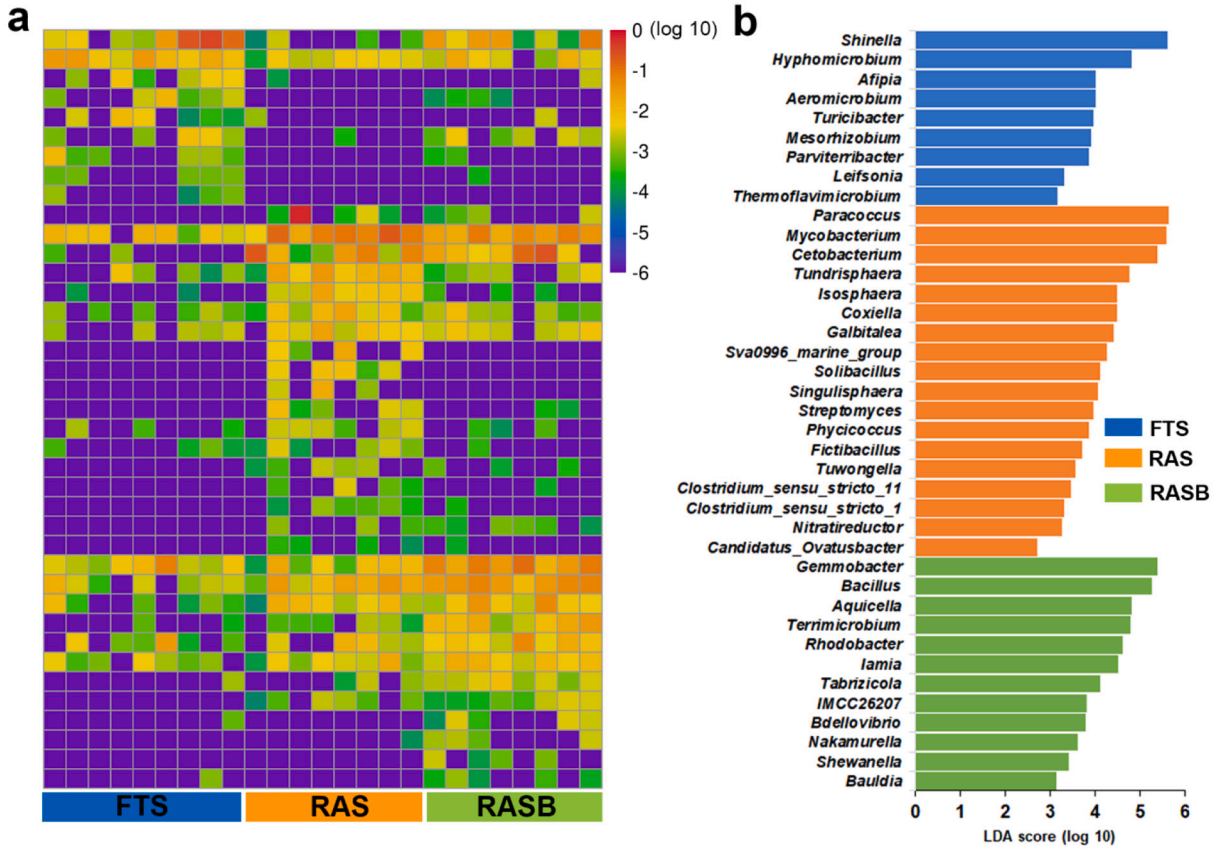

Fig. 6. The differentially relative abundant genera enriched in three treatment selected by LefSe. (a) Heatmap showing the relative abundance of differentially genus after logarithm transformation, (b) Bar plot showing the linear discriminant analysis (LDA) scores for the discriminating genera among the three treatments.

Our study showed that the rearing system (FTS vs RAS) had no significant effect on the growth of tilapia larvae (Table 2). At the same time, RAS significantly improved the survival rate of tilapia larvae compared with FTS (Fig. 2). This result is in line with results for Atlantic cod larvae that showed no significant differences in dry matter of body content between FTS and RAS, while cod larvae cultured in RAS had significantly higher survival than in FTS (Attramadal et al., 2014; Attramadal et al., 2012). According to the r/K strategist theory (Attramadal et al., 2014; Vadstein et al., 2018a), RAS has a more stable and diverse microbial community composition in the tank water than FTS, which is typically dominated by potentially pathogenic r-strategists in the water. The microbial matured water has improved marine larval survival in the early life stage (Vadstein et al., 2018b), which also applies to freshwater fish species like tilapia in the RAS and RASB treatment of this study.

In Atlantic cod rearing systems, FTS and RAS previously showed differences in the water microbial community due to the differences in water exchange (Vadstein et al., 2018a; Vestrum et al., 2020). A significantly higher hydraulic retention time in the RAS without ozone or UV disinfection increases the opportunity for slow-growing bacteria to stay longer in the system, as compared to FTS. In addition, the bacterial density in the water entering the rearing tanks of RAS is more than 10 times higher than that in FTS (Attramadal et al., 2014). Therefore, the difference in the microbial composition and bacterial loading of tank water between FTS and RAS might explain the differences in the fish gut microbiota. The fish sampled from different tanks within the same RAS showed similar gut microbiota composition in this study, in line with our previous study (Giatsis et al., 2014). However, fish sampled from different tanks within FTS showed different gut microbiota compositions (Table S2), according to the large number of unique ASVs detected in fish from FTS (Fig. 3). The high variability between individuals in FTS is also a characteristic of r-strategists, potentially explaining the significantly higher variability in the water microbial community between parallel tanks in FTS than RAS (Attramadal et al., 2012). However, it should be noted that the accumulation of uneaten feed and faecal waste may have resulted in the growth of heterotrophic bacterial biofilm on the tank bottom, which may also vary in composition between tanks. Such biofilms can be grazed by tilapia, potentially increasing individual variations in gut microbiota composition. Our study further demonstrated that RAS as a water microbial maturation strategy in larvae culture delivered a more stable and reproducible gut microbial community in tilapia gut than FTS.

4.2. Dietary probiotic supplementation altered fry gut microbiota but not growth

The growth-promoting effect of *Bacillus* spp. on tilapia is dose-dependent; for instance, dietary supply of *B. subtilis* at 4×10^7 CFU/g of feed had no effect on tilapia growth (Addo et al., 2017), while at a dosage of 10^8 CFU/g of feed or higher showed enhancement in the growth of tilapia (Mohammadi et al., 2020; Won et al., 2020; Zhu et al., 2019). Although *B. subtilis* can improve the growth and survival of juvenile or adult tilapia in some studies (Addo et al., 2017; Opiyo et al., 2019), dietary supplementation of *Bacillus* spores to 2 g tilapia fry for 8 weeks did not affect their growth (Sookchaiyaporn et al., 2020). In this study, dietary supplementation of *B. subtilis* at the dosage of 10^8 CFU/g did not significantly influence the growth of Nile tilapia larvae, which might be due to the restricted feeding masking the probiotic effect on fish growth. *Bacillus* spp. were reported to increase the disease resistance of fish (Kuebutornye, 2019). In this study, we found that RASB had a numerically higher survival than RAS, although the difference was not significant. Still, the effect of dietary probiotic supplementation on fish performance in FTS needs further research.

Bacillus spp. have been widely used as probiotics in aquaculture (Kuebutornye, 2019). In several studies, dietary supplementation of *B. subtilis* in tilapia was reported to increase the immune response and resistance to pathogen infection (Liu et al., 2017; Won et al., 2020; Zhu et al., 2019; Aly et al., 2008). In studies with different fish species, dietary supplementation of *B. subtilis* decreased the gut bacterial diversity in gilthead sea bream and the bacterial counts in rainbow trout (Cerezuela et al., 2013; Newaj-Fyzul et al., 2007). However, *Bacillus* species did not affect the gut microbiota alpha diversity in Nile tilapia and grass carp (Maas et al., 2021; Shi et al., 2020; Tachibana et al., 2021). Dietary supplementation of *B. subtilis* to Nile tilapia could modulate the gut microbiota profiles according to DGGE analysis (Guimarães et al., 2021; He et al., 2013; Tachibana et al., 2021). In our study, the effect of *B. subtilis* on gut microbial communities was investigated by next generation sequencing which confirmed the modulatory effect of *B. subtilis* on tilapia gut microbiota.

4.3. Microbial functionality influenced by rearing system and probiotic supplementation

Both the rearing system and the probiotic supplementation in the current study modulated the microbial composition in the gut of tilapia (Fig. 5). Although the alpha diversity was similar between the treatments (Table 3), we show a trend of FTS < RAS < RASB, with RASB having the highest diversity and richness. The addition of probiotics to tilapia feeds was previously shown to increase the gut bacterial diversity andimprove fish recovery from stress (Tang et al., 2020), which implies that RAS and probiotic supplementation may contribute to healthier gut microbiota and further result in better survival of tilapia larvae.

According to previous studies, the gut microbiota of Nile tilapia is dominant with taxa belonging to the phyla Proteobacteria, Actinobacteriota, Firmicutes and Fusobacteria (Kathia et al., 2018; Liu et al., 2019). Similar to previous studies, tilapia fed with *B. subtilis* showed a lower relative abundance of Proteobacteria and a higher abundance of Verrucomicrobiota and Firmicutes (Tachibana et al., 2021; Tang et al., 2020). At the genus level, *Plesiomonas*, commonly detected in freshwater fish hypothesized to be an opportunistic pathogen, showed high abundance in previous experiments (Behera et al., 2018; Yilmaz, 2019). In this study, although RAS showed a higher survival rate than FTS, the relative abundance of *Plesiomonas* in the gut of FTS (RA = 24%) and RAS (RA = 23%) were similar. Therefore, we can hardly attribute the mortality detected in this experiment to the prevalence of *Plesiomonas*. Still, to be noticed, probiotic supplementation reduced the relative abundance of this taxon in RASB (RA = 15%).

In the present study, FTS treatment group was significantly enriched with *Shinella* and *Hyphomicrobium*. Both *Shinella* and *Hyphomicrobium* were previously reported to be present in high abundance in RAS (Schneider et al., 2007; Sugita et al., 2005), however, the role of these genera in the fish gut is still not clear. Besides, the fish gut microbiota from RAS and RASB treatments were dominant with *Cetobacterium,* while it was detected in low abundance in FTS (RA = 0.02%). *C. somerae* is an anaerobic microbe which produces vitamin B12 in the freshwater fish intestine (Sugita et al., 1991) and is related to fermentative metabolism of peptides and amino acids (Finegold et al., 2003). *C. somerae* was commonly detected as core species in freshwater fish species, including tilapia (Roeselers et al., 2011; Tarnecki et al., 2017). In a study from frogs, a low abundance of *Cetobacterium* in the gut during juvenile stages was connected to a reduced host resistance to disease infection

Table 3
The alpha-diversity indices of tilapia fry gut microbial community.

	FTS	RAS	RASB	SEM	*P* value
Richness	115	143	146	10.3	ns
Chao1	127	156	161	12.0	ns
ACE	126	157	164	11.8	ns
Shannon	2.97	3.15	3.24	0.15	ns
Simpson	0.86	0.83	0.88	0.03	ns
Fisher	22.8	29.2	29.6	2.42	ns

SEM, standard error of mean; ns, not significant ($P > 0.05$). FTS, flow-through system; RAS, recirculating aquaculture system; RASB, RAS + *B. subtilis* diet.

later in life, during the adult stages (Knutie et al., 2017). Moreover, a decrease in abundance of *Cetobacterium* in zebrafish gut by antibiotic treatment was shown to increase the susceptibility of fish to pathogen infection (He et al., 2017). In our study, the high mortality of fish larvae in FTS could be related to the low occurrence of *C. somerae* in the fish gut. In addition, RAS was enriched with *Mycobacterium* (RA = 8.7%). Some species belonging to *Mycobacterium* genus, such as *M. marinum*, were reported as pathogens and cause mycobacteriosis in fishes (Gauthier and Rhodes, 2009). However, whether *Mycobacterium* causes pathology depends on the species and the host's susceptibility.

In several studies, B. subtilis has been isolated from tilapia intestine (Del'Duca et al., 2013; Etyemez and Balcazar, 2016; Ridha and Azad, 2016; Sookchaiyaporn et al., 2020; Tang et al., 2020). In this study, *Bacillus* spp. occupied 0.29% and 1.78% in the intestine from FTS and RAS, respectively. Dietary supplementation of *B. subtilis* spores enriched the *Bacillus* spp. in the gut of RABS treatment (RA = 4.0%), which implied its colonization in the tilapia gut. Besides, dietary probiotic supplementation increased the abundance of *Gemmobacter* in our study. *Gemmobacter* was shown to be a dominant genus in the gut of zebrafish larvae (Siriyappagouder et al., 2018), thus confirming the presence of this taxa in freshwater fish gut. A study showed that *Gemmobacter tilapiae* was isolated from a tilapia pond, which could accumulate poly-β-hydroxybutyrate that is considered beneficial to fish growth and health (Sheu et al., 2013; Siriyappagouder et al., 2018). To summarize, recirculating system and probiotic administration may benefit the gut microbial colonization of tilapia larvae as evidenced by the observed positive correlation between the gut microbiota distribution and the standard body length as well as survival in RAS and RASB treatments.

5. Conclusions

This study demonstrated the feasibility of modulating the gut microbiota of tilapia larvae through different rearing systems (i.e. FTS and RAS) and dietary probiotic supplementation (RASB). Though FTS had similar or even superior water quality compared to RAS, RAS showed better survival of larvae than FTS. This result could be partly explained by the alterations in the gut bacterial colonization, for instance, the absence of *Cetobacterium* in FTS. Dietary *B. subtilis* supplementation in RAS increased the abundance of potentially beneficial *Bacillus* and *Gemmobacter* in the fish gut. Our study indicated that RAS is superior to FTS for fish larvae culture concerning survival, while dietary probiotic supplementation may further improve gut health with potential implications during later life stages.

Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737297.

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