Effects of dietary probiotic supplementation on the growth, gut health and disease resistance of juvenile Nile tilapia (*Oreochromis niloticus*)

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**A B S T R A C T**

This study investigated the effects of the *Streptococcus agalactiae* antagonizing probiotics *Bacillus cereus* NY5 and *Bacillus subtilis* BS group supplemented with *B. subtilis* (1 × 10^8 CFU/g feed, BS group), *B. cereus* NY5 (1 × 10^8 CFU/g feed, BC group), and *B. subtilis* + *B. cereus* NY5 (0.5 × 10^8 CFU/g feed of each probiotic, BS + BC group) for 6 wk, and the probiotic supplementation groups were then fed the basal diet for 1 wk to investigate the gut microbial community. The results of this study showed that BS + BC and BC treatments significantly increased weight gain (WG), feed conversion ratio (FCR) and *S. agalactiae* resistance in Nile tilapia (P < 0.05). Gut microvilli length and density and c-type lysozyme (*lyzC*) gene expression were significantly increased by probiotic supplementation (P < 0.05). The results of high-throughput sequencing showed that the *B. cereus* NY5 and *B. subtilis* + *B. cereus* NY5-supplemented feed resulted in a significant improvement in tilapia autotrophic gut bacterial communities and had a stimulation effect on a variety of potential probiotics after 6 wk of feeding. After cessation of probiotic administration for 1 wk, the gut bacteria of the fish in the BS + BC and BC groups had minor changes and maintained a stable state. Consequently, it was inferred that, as a feed supplement, *B. cereus* NY5 and the mixture of *B. subtilis* and *B. cereus* NY5 at 1 × 10^8 CFU/g feed were able to promote growth and disease resistance, which may be associated with the supplement’s effects on gut immune status, intestinal morphology, and intestinal microbial community composition.

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1. Introduction

Tilapia is one of the most important species of farmed fish of freshwater aquaculture in China. In recent years, disease caused by *Streptococcus agalactiae* has become a major challenge for the culture of tilapia, resulting in massive losses for tilapia farmers all over the world (Amal and Saad, 2011). Probiotics (especially antagonistic probiotics) can reduce pathogenic bacteria by competitive exclusion, provide nutrients and enzymes to promote host growth, enhance the immune response by immune stimulation, and do not cause secondary pollution problems. In view of this, obtaining *S. agalactiae* antagonizing probiotics suitable for tilapia culture is of great practical significance for improving the resistance of tilapia and reducing the use of antibiotics.

Consumption of probiotics is an effective and attractive way to modulate the intestinal microbial composition and to maintain and promote host health (FAO/WHO, 2001). The major mechanisms of action of probiotics include enhancement of epithelial barrier function, improved adhesion to intestinal cells and pathogen inhibition by occupying adhesion sites, production of antibacterial substances, and regulation of the immune function (Rijkers et al., 2010). Through the above mechanisms, the purpose of regulating...
intestinal microbes and inhibiting the growth of pathogens is achieved (Almada et al., 2015). Recently, the application of beneficial bacteria in the form of probiotics has been demonstrated to be useful in aquaculture (Pérez-Sánchez et al., 2014). The main probiotic microorganisms used in aquaculture include species belonging to the lactic acid bacteria (LAB) (Beck et al., 2015; Liu et al., 2016a, 2017a) and Bacillus spp. (Chai et al., 2016; Giatisis et al., 2016; He et al., 2013; Sun et al., 2011).

According to the results of previous studies, due to the beneficial properties of enhancing the immune system, competitive exclusion, and producing antibacterial substances, some Bacillus spp. (including Bacillus subtilis) have been frequently used as probiotics in aquaculture (Aly et al., 2008a; Liu et al., 2010; Tseng et al., 2009). Bacillus subtilis probiotic candidates stimulated immune responses both locally and systemically in tilapia (Galagarza et al., 2018) and effectively enhanced the growth performance and disease resistance of Nile tilapia (Liu et al., 2017b). In addition, both B. subtilis and Bacillus cereus promote intestinal colonization and improve survival rate (SR) without negatively influencing feed intake, total biomass, gross revenue, partial operating costs and net revenue of tilapia (Nilton and Daniele, 2015). As a feed additive, the skin beneficial bacterial composition is promoted by the use of Bacillus spp., which can outcompete intestinal pathogenic bacteria (El-Rhman et al., 2009). Moreover, B. subtilis boosted the growth and vitality of beneficial LAB in the intestine of hosts (Hoa et al., 2000). Bacillus could prove to be effective for integrated prevention and control of streptococcus infections (Widanarni and Tanbiyaskur, 2015).

In this study, B. cereus was extracted from healthy Nile tilapia feces (Liu et al., 2016a), and B. subtilis was isolated from a mixed-species Bacillus spp. probiotic that was used for the regulation of aquaculture water quality in our lab (Liu et al., 2015). Our previous study indicated that both strains were effective at antagonizing S. agalactiae in vitro (Liu et al., 2015; Lu et al., 2016), but it is not clear whether they have a probiotic effect in vivo. This study assessed the effects of the potential probiotic strains with special antagonistic activity in Nile tilapia. Comprehensive evaluation of the effects on growth, survival, immunity, disease resistance and the intestinal microbiota provides a solid theoretical basis for subsequent commercialization and application of the potential probiotic strains.

2. Materials and methods

All animal work in this paper was conducted according to relevant national and international guidelines. All animal care and experimental procedures were approved by the Committee on Animal Care and Use and the Committee on the Ethics of Animal Experiments of Chinese Academy of Fishery Sciences.

2.1. Bacteria and feed preparation

The probiotic B. cereus NY5 and B. subtilis were identified based on morphological, physiological, and biochemical characteristics, as well as 16S rRNA gene sequencing. The bacteria were frozen in 50% glycerol and stored at −80 °C. The antagonistic experiments showed significant inhibition zones to S. agalactiae in vitro. Luria Broth (LB) liquid medium (OXOID), which had been centrifuged at 5,000 × g (Beckman Coulter, AK, USA) for 4 min, was used to culture Bacillus strains for 24 h at a temperature of 37 °C. Distilled water was used to wash the pellets twice, and these pellets were then lyophilized and suspended in phosphate-buffered saline (PBS) (1.8 mmol/L KH2PO4, 10.1 mmol/L NaH2PO4, 2.7 mmol/L KCl and 137 mmol/L NaCl, pH 7.4). A spread plate technique was used to determine viable cells according to the cell concentrations measured at OD600, which was linearly proportional to the number of viable cells in the suspension. All cell suspension OD600 values were adjusted to an adequate value (CFU/mL) for further experiments.

The formulation and the main ingredients of the basal diet are shown in Table 1. The experimental tilapia were given the probiotics, which were added in the basal diet at a dosage of 1 × 108 CFU/g, as described in a previously conducted experiment using Bacillus spp. in tilapia culture (Aly et al., 2008a; Wang et al., 2017). The tilapia fed a pure basal diet were considered the experimental control check (CK) group. There were 3 kinds of diets prepared for the experiment: the basal diet with 1 × 108 CFU/g of B. subtilis (BS group), the basal diet with 1 × 108 CFU/g of B. cereus NY5 (BC group), or the basal diet with both 0.5 × 108 CFU/g of B. subtilis and B. cereus NY5 (BS + BC group). The preparation of these experimental diets followed the same process described in our previous study (Xia et al., 2018). Briefly, powdered dietary ingredients were thoroughly mixed by hand, then blended with oil and water, and suitable Bacillus spp. cells were added to form a soft dough. To ensure the viability of the added probiotics, the diets were freshly prepared every day and kept sealed in plastic bags that were stored at 4 °C.

2.2. Fish and rearing conditions

All experimental juvenile tilapia were provided by the Gaoyao Fish Farm of the Pearl River Fisheries Research Institute (Guangzhou, China), and ethyl 3-aminobenzoate methanesulfonate (MS-222) was used to anaesthetize the fish when necessary. Healthy juvenile tilapia were selected and acclimated in 750-L tanks for 2 wk from 28 to 29 °C under laboratory conditions and were fed the basal diet. Thereafter, fish that were eating normally, disease-free, non-injured and smaller-sized (0.20 ± 0.05 g) were randomly distributed into twelve 50-L tanks on a random basis with 60 tilapia in each tank and 3 replicates for each treatment. The adopted feeding cycle was determined on the basis of previous reports.

Table 1

| Item | Content, % |
|------|------------|
| Ingredients | | |
| Fish meal | 48 |
| Soybean meal | 22 |
| Wheat flour | 25 |
| Adhesives | 0.2 |
| Soybean oil | 2.0 |
| Ca(H2PO4)2 | 2.0 |
| Vitamin C phosphate ester | 0.1 |
| Choline chloride (50%) | 0.3 |
| Vitamin premix1 | 0.2 |
| Mineral premix2 | 0.2 |
|  |  |  |
| Calculated chemical compositions |  |  |
| Crude protein | 42.0 |
| Crude lipid | 7.3 |
| Ash | 9.5 |
| Crude fibre | 3.1 |
| N free extract | 27.9 |

1 One kilogram of vitamin premix contained the following: thiamine, 0.438 g; riboflavin, 0.632 g; pyridoxine HCl, 0.908 g; D-pantothenic acid, 1.724 g; nicotinic acid, 4.583 g; biotin, 0.211 g; folic acid, 0.549 g; vitamin B12, 0.001 g; inositol, 21.053 g; menadione sodium bisulfite, 0.889 g; retinyl acetate, 0.677 g; cholecalciferol, 0.116 g; DL-α-tocopherol-acetate, 12.632 g.

2 One kilogram of mineral premix contained the following: CoCl2·6H2O, 0.074 g; CuSO4·5H2O, 2.5 g; FeSO4·7H2O, 73.2 g; NaCl, 40.0 g; MgSO4·7H2O, 284.0 g; MnSO4·H2O, 6.50 g; KI, 0.68 g; Na2SeO3·5H2O, 0.10 g; ZnSO4·7H2O, 131.93 g; cellulose, 501.09 g.
The probiotic supply to assess the persistence of the intestinal microbiota was conducted with SPSS 17.0 (SPSS, Inc.), and differences at 
P < 0.05 were considered significant.

3. Results

3.1. Growth performance and survival

After the 6-wk feeding trial, there were greater WG values in the BS + BC and BC groups compared with the CK group (P < 0.05) (Table 2). The FCR in the BC and BS + BC groups were lower compared to the CK group (P < 0.05). No significant differences were identified for WG and FCR between the CK and BS groups (P > 0.05). In addition, the tilapia in each group barely showed any significant differences in SR (P > 0.05).

3.2. Intestinal histology

Compared with the CK group, the tilapia fed probiotic-supplemented feed had longer and denser intestinal microvilli (P < 0.05) (Fig. 1 and Table 3).

3.3. Expression of intestinal c-type lysozyme gene

As shown in Fig. 2, the expression of intestinal lyzc in the probiotic-fed groups was higher compared with the CK group (P < 0.05), and the BC group had the highest level of lyzc gene expression.

3.4. Challenge test

Fig. 3 presents the effects of probiotics on WC1535 resistance in tilapia. According to the results, each group had the highest daily mortality rate on the first day. Cumulative mortality was significantly lower in the BC (48%) and BS + BC (43%) groups compared to the CK (81.33%) and BS (85.33%) groups (P < 0.05).
Table 2
Growth performance of tilapia fed diets supplemented with *Bacillus subtilis* and/or *Bacillus cereus* for 6 wk.1

| Item   | Groups2 | CK       | BS       | BS + BC  | BC       |
|--------|---------|----------|----------|----------|----------|
| IBW, g |         | 0.20 ± 0.02 | 0.20 ± 0.02 | 0.19 ± 0.02 | 0.20 ± 0.02 |
| WG, %  |         | 2,595.69 ± 229.91a | 2,772.65 ± 229.21a | 3,527.77 ± 359.08b | 3,532.20 ± 403.43b |
| FCR    |         | 1.39 ± 0.05b | 1.33 ± 0.07b | 1.07 ± 0.04a | 1.04 ± 0.02b |
| SR, %  |         | 94.07 ± 6.12 | 91.11 ± 2.22 | 89.63 ± 2.31 | 93.33 ± 2.94 |

IBW = initial body weight; WG = weight gain; FCR = feed conversion ratio; SR = survival rate.

1 a, bWithin a row, means with different letter superscripts are significantly different (P < 0.05).
2 Data represent means ± standard deviation (n = 6 fish).

Fig. 1. Electron microscope images of the gut microvilli. (A) Scanning electron microscopy (SEM) images for microvilli density; (B) transmission electron microscope (TEM) images of the microvilli length. CK group: the basal diet; BS group: the basal diet with 1 × 10^8 CFU/g of *B. subtilis*; BS + BC group: the basal diet with both 0.5 × 10^8 CFU/g of *B. subtilis* and *B. cereus* NY5; BC group: the basal diet with 1 × 10^8 CFU/g of *B. cereus* NY5.
Analyses were conducted on the indices of bacterial diversity and richness from the proportion of the OTU for the calculation of each experimental group’s bacterial diversity (Table 4). The BC group had a lower species richness (Chao1 and ace) in comparison with the CK group (P < 0.05). Upon cessation of probiotics for 1 wk, compared with the BS group, the BS group after cessation of probiotic consumption for 1 wk (BS-7D group) had higher Shannon and Simpson indices (P < 0.05). In comparison with the BS + BC group, the BS + BC group after cessation of probiotic consumption for 1 wk (BS + BC-7D group) had lower ace and Chao1 indices (P < 0.05). However, there was no significant difference in bacterial richness or diversity between the BC group and the BC group after cessation of probiotic consumption for 1 wk (BC-7D group) (P > 0.05). The replicates in the BS and CK groups were closely clustered, while clearly separated from those in the other groups, which all clustered closely in the principal component analysis (PCoA) plot and non-metric multidimensional scaling (NMDS) diagram (Fig. 4).

The composition of major bacteria at the phylum level in the intestines of fish fed different diets is shown in Fig. 5. In general, the most abundant phylum in the BS + BC and BC groups (accounting for 83.78% and 99.02% of 16S reads, respectively) was Proteobacteria, whereas the CK and BS groups were dominated by Fusobacteria (82.35% and 73.59%, respectively). The average amount of Proteobacteria and Bacteroidetes in the CK group (12.37% and 3.92%, respectively), Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes and verrucomicrobia in the BS group (17.05%, 1.73%, 2.16%, 4.05% and 1.78%, respectively), and Fusobacteria, Actinobacteria and Chloroflexi in the BS + BC group (5.18%, 3.21% and 6.40%, respectively) were all above 1%. Sacccharibacteria and Cyanobacteria were also present with relative abundances below 1% in the samples. Seven days after the probiotic supply was ceased, the Proteobacteria reads in BS group increased from 17.05% to 65.61%, while Fusobacteria reads dropped from 73.59% to 21.22%. The Fusobacteria and Proteobacteria reads in the BS + BC-7D (90.52% and 6.71%) and BC-7D groups (99.1% and 0.21%) were similar to those in the BS + BC and BC groups, which were 83.78% and 5.18% in the BS + BC group and 99.02% and 0.32% in the BC group, respectively.

**Rhizobium** was found at a significant greater proportion (58.78% and 82.26%, respectively) in the BS + BC and BC groups and was also detected in the intestines of tilapia of BS and CK groups, yet it was higher in the former 2 groups (Fig. 6). **Cetobacterium** was the predominant genus (accounting for 82.35% and 73.53% of reads) in CK and BS groups; however, it was found much less frequently in the BS + BC and BC groups (4.18% in BS + BC group and 0.32% in BC group). Reads of Phyllobacterium, one of the major genera that accounted for 3.52% in BS + BC group and 2.61% in BC group, yet there was no Phyllobacterium detected in the 3 replicates of either BS or CK group. **Plesiomonas** (accounting for 6.08% and 2.72% of the reads in CK and BS groups) was present in the CK and BS groups; however, this genus was found at lower levels in the BS + BC and BC groups (0.4% in BS + BC group and...
Table 4
Richness and diversity statistics of the samples.

| Group | Ace       | Chao1     | Shannon | Simpson | Good’s coverage |
|-------|-----------|-----------|---------|---------|-----------------|
| CK    | 54.89 ± 5.26<sup>a</sup> | 57.29 ± 3.71<sup>b</sup> | 1.18 ± 0.16<sup>c</sup> | 0.32 ± 0.08<sup>d</sup> | 0.95 ± 0.02 |
| BS    | 59.61 ± 5.84<sup>c</sup> | 58.83 ± 5.71<sup>d</sup> | 1.85 ± 0.47<sup>c</sup> | 0.45 ± 0.11<sup>d</sup> | 0.96 ± 0.01 |
| BS + BC | 67.58 ± 8.85<sup>c</sup> | 69.17 ± 6.93<sup>d</sup> | 1.94 ± 0.27<sup>c</sup> | 0.50 ± 0.08<sup>d</sup> | 0.95 ± 0.03 |
| BC    | 37.06 ± 3.74<sup>a</sup> | 36.44 ± 3.09<sup>b</sup> | 1.16 ± 0.18<sup>a</sup> | 0.31 ± 0.07<sup>a</sup> | 0.98 ± 0.02 |
| BS-7D | 63.13 ± 5.94<sup>c</sup> | 62.78 ± 4.88<sup>d</sup> | 2.85 ± 0.29<sup>c</sup> | 0.74 ± 0.07<sup>c</sup> | 0.95 ± 0.05 |
| BS + BC-7D | 48.29 ± 3.46<sup>b</sup> | 47.72 ± 4.44<sup>d</sup> | 1.74 ± 0.30<sup>b</sup> | 0.46 ± 0.06<sup>b</sup> | 0.97 ± 0.01 |
| BC-7D | 29.29 ± 2.64<sup>a</sup> | 27.67 ± 3.88<sup>a</sup> | 1.47 ± 0.13<sup>a</sup> | 0.40 ± 0.03<sup>a</sup> | 0.95 ± 0.05 |

<sup>a</sup>,<sup>b</sup> Within a column, means with different letter superscripts are significantly different (P < 0.05).

1 Data represent means ± standard deviation (n = 3 fish).

2 CK group: the basal diet; BS group: the basal diet with both 0.5 × 10⁸ CFU/g of Bacillus subtilis and Bacillus cereus NY5; BC group: the basal diet with 1 × 10⁹ CFU/g of B. cereus NY5; BS-7D group: the BS group after cessation of probiotic consumption for 1 wk; BS + BC-7D group: the BS + BC group after cessation of probiotic consumption for 1 wk; BC-7D group: the BC group after cessation of probiotic consumption for 1 wk.

Fig. 4. Comparison of autochthonous intestinal microbiota composition between fish fed different diets. (A) Principal component (PC) analysis; (B) Non-metric multidimensional scaling (NMDS) diagram. CK group: the basal diet; BS group: the basal diet with both 1 × 10⁹ CFU/g of Bacillus subtilis and Bacillus cereus NY5; BC group: the basal diet with 1 × 10⁹ CFU/g of B. cereus NY5; BS-7D group: the BS group after cessation of probiotic consumption for 1 wk; BS + BC-7D group: the BS + BC group after cessation of probiotic consumption for 1 wk; BC-7D group: the BC group after cessation of probiotic consumption for 1 wk.

0.14% in BC group). Escherichia-Shigella (accounting for 0.16% of the reads) was detected in the CK group, and with probiotic treatment a larger amount of Escherichia-Shigella was detected (7.54% in BS group, 2.37% in BS + BC group and 4.22% in BC group). However, there were no significant differences in Phyllobacterium, Plesiomonas and Escherichia-Shigella after ANOVA of every group, due to high individual variability (Appendix Fig. 1). After the cessation of probiotic supplementation plus 7 d of basal diet feeding, reads of Rhizobium in the BS-7D group increased on average from 0.32% (BS) to 43.57%, but Cetobacterium dropped from 73.53% (BS) to 21.13%. However, Rhizobium and Cetobacterium in the BS + BC and BC groups both had smaller changes after the cessation of probiotic supplementation.

At the species level, Bacillus licheniformis, Bacteroides sp., Rhizobium radiobacter, Ambiguous taxa, and Unclassified were the main components. Further, a larger fraction of R. radiobacter, which belong to the α-Proteobacteria, was detected in the BC and BS + BC groups than in the CK group (Appendix Fig. 2).

3.6. Statistical analysis of metagenomic profiles

The linear discriminate analysis (LDA) effect size (LEfSe), which is a statistical instrument developed for locating biomarkers in the
metagenome, was used with default parameters for identifying possible discriminating taxa among groups. In this research, statistical analysis was only conducted on a phylum-to-genus level. In total, 14 distinguishing taxa were detected between the groups of BS and CK with LDA scores both over 3 (Fig. 7A). One phylum (Firmicutes), 1 class (Bacilli), 1 order (Lactobacillales), 1 family and 2 genera were statistically higher in *B. subtilis*-treated fish. However, in the CK group, there was 1 phylum (Fusobacteria), 2 classes (Fusobacteria and Bacteroidia), 2 orders (Fusobacteriales and Bacteroidales), 2 families and 1 genus that were enriched. The cladogram shows the 34 distinguishing taxa from the CK and BS + BC groups (LDA score > 3) (Fig. 7B). Compared to the CK group, 1 phylum (Proteobacteria), 2 classes (α- and β-Proteobacteria), 5 orders (Frankliales, Rickettsiales, Sphingomonadales Burkholderiales and Rhizobiales), 6 families and 8 genera all had larger amounts in the BS + BC group. However, the amounts of 2 phyla (Fusobacteria and Bacteroidetes), 2 classes (Bacteroidia and Fusobacteria), 2 orders (Bacteroidales and Fusobacteriales), 3 families and 3 genera were greater in the CK group. In total, there were 42 obviously distinguishing taxa between the CK and BC groups (Fig. 7C). On the phylum level, the BC group clearly had a greater number of Proteobacteria compared to the CK group. In addition, in comparison, the CK group had enriched reads of Fusobacteria, Actinobacteria and Bacteroidetes.

Fig. 6. Heatmap of the 30 most predominant genus among all gut samples. CK group: the basal diet; BS group: the basal diet with \(1 \times 10^8\) CFU/g of *Bacillus subtilis*; BS + BC group: the basal diet with both \(0.5 \times 10^8\) CFU/g of *B. subtilis* and *Bacillus cereus* NY5; BC group: the basal diet with \(1 \times 10^8\) CFU/g of *B. cereus* NY5; BS-7D group: the BS group after cessation of probiotic consumption for 1 wk; BS + BC-7D group: the BS + BC group after cessation of probiotic consumption for 1 wk; BC-7D group: the BC group after cessation of probiotic consumption for 1 wk.

Fig. 7. Cladograms indicating the polygenetic distribution of bacterial lineages associated with different groups of samples. CK group: the basal diet; BS group: the basal diet with \(1 \times 10^8\) CFU/g of *Bacillus subtilis*; BS + BC group: the basal diet with both \(0.5 \times 10^8\) CFU/g of *B. subtilis* and *Bacillus cereus* NY5; BC group: the basal diet with \(1 \times 10^8\) CFU/g of *B. cereus* NY5. Indicators between the CK and probiotic-fed groups with an linear discriminate analysis (LDA) score larger than 3, with red circles representing those bacteria richer in CK group samples, and green circles representing those richer in samples of BS (A), BS + BC (B) and BC (C) groups, respectively.
The BS group had richer *Escherichia-Shigella* and *Weissella* reads compared to the CK group, while reads from *Cetobacterium* were obviously richer in the CK group in comparison with the BS group at the genus level. The reads of *Methyllobacterium*, *Phyllobacterium*, *Rhizobium* and *Escherichia-Shigella* were enriched in the BS + BC and BC groups compared to the CK group. However, the CK group had more enriched *Plesiomonas* and *Cetobacterium* reads compared to the BS + BC and BC groups. In comparison with the CK group, the BC group had more prevalent *Bacillus* sp., and the most enriched *Bacillus* OTU (54 OTU) shared a sequence identity of 100% with *B. licheniformis*.

As shown in Appendix Fig. 3, after cessation of probiotic administration for 1 wk, *Weissella*, *Cetobacterium* and *Bosea* were enriched in the BS group compared to the BS–7D group, but *Phyllobacterium*, *Rhizobium* and *Achromobacter* were significantly enriched in the BS–7D group compared to the BS group, which was under successive probiotic feeding. Reads of *Achromobacter* were enriched in the BS + BC-7D and BC-7D groups compared to the BS + BC and BC groups, respectively. However, *Naumurella* was enriched in the BC + BC group compared to the BS + BC–7D group.

### 4. Discussion

The growth and feed utilization of juvenile Nile tilapia was improved by supplementation of combined *B. subtilis* and *B. cereus* or *B. cereus* by itself. Similar to the results of this study, tilapia growth performance has been improved by the use of *Lactobacillus plantarum* and *B. subtilis* or a mixture of *Saccharomyces cerevisiae*, *L. plantarum* and *B. subtilis* (Essa et al., 2010). After feeding for 4 to 8 wk, the weight gain of Nile tilapia was significantly increased in the *Bacillus pumilus* and the commercial probiotic product Organic Green (Hangpoong Industry Co. Ltd, Korea) supplementation groups in comparison to the CK group (Aly et al., 2008b). In addition, when the fish feed, which contained 40% or 27% crude protein, was supplemented with combined or single probiotics, it was more conducive to improving WG and FCR in comparison to the basal diet (Lara-Flores et al., 2010). After a 10-wk feeding trial, growth rather than FCR of tilapia was promoted by dietary *B. licheniformis* (Han et al., 2015). However, there were no positive effects of some probiotic strains on Nile tilapia growth performance in other studies. After a 21-d growth trial, the growth performance of tilapia in a *B. subtilis* strain-amended diet group was similar to that of the control group (Addo et al., 2017a). According to an observation of tilapia fed with *B. subtilis*– and Previa-supplemented feed for 8 wk, the growth performance of these fish did not significantly change (Addo et al., 2017b). *Bacillus amyloliquefaciens* at a concentration of $1 \times 10^4$ CFU/g was not significantly effective in improving tilapia growth performance after 30 d of feeding, but it promoted the growth of fish at the end of 60 d (Reda and Selim, 2015). The different antibiotic activities of probiotics, together with the different interactions among the intestinal beneficial bacteria, probiotics, feed, host and research conditions have led to the observed distinguishing effects of probiotics on the growth performance of Nile tilapia. These differences can consequently influence probiotic effects on growth performance in other studies. In this study, the single *B. subtilis* diet had no effect on tilapia WG and FCR. However, BS + BC and BC treatments positively affected the WG and FCR of these fish.

It has been reported that a multispecies (*Pediooccus acidilactici, Enterococcus faecium, B. subtilis* and *Lactococcus reuteri*) probiotic-amended diet significantly increased tilapia mid-intestinal microvilli density in 8 wk (Pirarat et al., 2011). The numerical increases of microvilli length and perimeter ratio (PR, internal perimeter of the intestine lumen to external perimeter of the intestine ratio) suggest that intestinal morphology is improved by probiotic administration (Pirarat et al., 2011). The improved length and density of the mid-intestinal microvilli may indicate the increased intestinal absorptive surface area from the application of probiotics (Standen et al., 2016). Further, the higher microvilli density may be more conducive to the enhancement of tilapia resistance to possible pathogens by reducing the extent to which the interenterocyte junctions are exposed. In this research, supplementation with probiotics significantly increased microvillus length and density in the mid-intestine, which was consistent with previously obtained results regarding tilapia (Pirarat et al., 2011). These results indicate the improvement effect of *B. subtilis* and *B. cereus* on the health condition of the intestines. The increased length and density of mid-intestinal microvilli may be one of the reasons for enhanced resistance to disease, utilization of feed and performance in growth of tilapia within the BS + BC and BC groups in this study.

This study showed a higher lycZ gene expression in the mid-intestines of tilapia fed a probiotic-supplemented diet than those in the control group. Previous studies showed that the c-type lysozymes of tilapia were effective in lysing both Gram-negative and Gram-positive strains (Gao et al., 2012). The tilapia could recognize the bacterial challenges, generate an acute stress, and enhance body immunity by secreting specific proteins such as lysozymes (Gao et al., 2012). Lysozymes can break down the cell wall of bacteria by hydrolyzing the chemical bonds between N-acetylgalactosamine and N-acetylmuramic acid. Therefore, lysozymes are capable of lysing specific Gram-positive and even several Gram-negative strains (Alexander and Ingram, 1992). Previously, a study demonstrated the significant effect of dietary *Bacillus* spp. on increasing lysozyme levels in Nile tilapia, which resulted in a high rate of survival after challenge with *Edwardsiella tarda* (Taoka et al., 2006). Changes in the leukocyte population in the process of immune response development can be reflected in variations of lysozyme levels. A higher level of lysozymes detected in the probiotic-fed tilapia may serve as an indicator showing their improved immunity (Saurabh and Sahoo, 2008). In a previous study, enhanced nonspecific defense systems and enhanced resistance ability to *Streptococcus iniae* by feeding dietary *B. licheniformis*-supplemented feed were reported in Nile tilapia (Han et al., 2015). In this research, similar results were observed in tilapia fed with combined *B. subtilis* and *B. cereus* alone when challenged with *S. agalactiae*.

Strong adherence to the inner facets of the intestines (mucus and mucosa), which has been shown to be of great importance for beneficial activities, is one criterion for probiotic strain selection (Boyle et al., 2006; Martinez et al., 2015). As suggested in other studies, probiotics that combined unstably with the gut mucosal zone may release rapidly when suspended, bringing about dysbiosis of intestinal strains as well as ensuing disorders, and finally leading to an increased chance of infection (Liu et al., 2016b). In this research, during probiotic administration, the probiotics were not located in the gastrointestinal mucosal zone of tilapia, which indicated that these probiotics could not adhere to the intestinal mucosa and proliferate. This finding showed the challenge of successfully colonizing the fish gut with a probiotic strain. The persistence of probiotics in the gut is species-specific (Standen et al., 2015), and the dosage and duration of supplementation and the selection of probiotic strain(s) might influence colonization success, while the persistence of the probiotic might also depend on the developmental stage of the animal (Perez et al., 2010; Ramos et al., 2013; Gerritsen et al., 2011). However, the gut microbiota developed significant differences between treatments (except between the BS and CK groups), and the composition of the gut microbiota remained stable 1 wk after cessation of the administration of the probiotics, which means that discontinuation of probiotic administration (except
for the BS group) did not cause significant fluctuations in gut microbes. In this study, potential probiotics, such as *Rhizobium* sp., which belong to the phylum Proteobacteria, were the major strains detected in tilapia intestines from the BS + BC and BC groups but were rarely found (<1%) in the CK and BS groups. Generally, many enzymes with pectolytic and cellulolytic activities were produced by *Rhizobium* bacteria and have the ability to hydrolyze the glycosidic skeleton in the cytoderm of plant cells (Huang et al., 2012; Robledo et al., 2008). *Rhizobium* live as endosymbionts in some phytophagous insect intestines and are helpful with the synthesis of nitrogen-containing substances that are generally insufficient in the food of the host insects (Russell et al., 2009). In this study, the coenzyme Q10 producing *B. radiobacter* was the most common *Rhizobium* species and may function to improve immunity (Al-Hasso, 2001; Wu et al., 2005). Changes in the autochthonous gut bacterial community and the increase in some beneficial bacteria in the intestines may be related to the improved resistance to diseases, utilization of feed and performance in growth of tilapia within the BS + BC and BC groups.

The use of combined *B. subtilis* and *B. cereus* or *B. cereus* by itself decreased the distribution of *Plesiomonas* and *Cetobacterium* and increased the distribution of *Phyllobacterium* and *Escherichia-Shigella* in the tilapia intestine. Operational taxonomic units of *Plesiomonas* and *Cetobacterium* were found in the intestinal tract of tilapia, forming the core microbiome, which were quite stable and resistant in response to dietary treatments (Adeoye et al., 2016). The genus *Plesiomonas* was also found within the intestines of tilapia that were cultured in earthen ponds (Pakingking et al., 2015), and it is considered to be a conditional pathogen in aquaculture systems. *Plesiomonas* is a pathogen for goldfish (Zhang et al., 2015) and grass carp (Hu et al., 2014). *Plesiomonas* is more prevalent in sick gibel carp than in healthy gibel carp, acting as a conditional pathogen in gibel carp (She et al., 2017). One possible way to use probiotics and prebiotics to reduce infection of hosts is by restoring intestinal microbial diversity (She et al., 2017; van Nood et al., 2013). *Cetobacterium* was the most abundant genus (82.15%) in control fish intestines, and this genus has previously been isolated from other fish intestines (including tilapia) (Adeoye et al., 2016; Li et al., 2015; Standen et al., 2015). *Cetobacterium* was the most dominant species in both sick fish that were infected by *Cyprinid herpesvirus 2* (CyHV-2) and in healthy fish (Pakingking et al., 2015). *Phyllobacterium* sp. is a slow-growing, N-2-fixing bacterium (Rojas et al., 2001) that has the ability to degrade organic matter and remove nitrogen and phosphorus from sludge particles (Zuo et al., 2015). *Phyllobacterium* bacteria were specific to the intestines of both the BS + BC and BC groups in this study. *Escherichia-Shigella* is a common pathogen of aquatic animals and can cause disease, such as diarrhoea, ascites and sepsis, in aquatic animals (Sun et al., 2012). Although there is no significant difference between the groups due to the existence of individual differences, the impact of *Escherichia-Shigella* in this study needs to be further explored. The metagenomics and metatranscriptomics of the intestinal bacteria should be investigated in future studies to reveal the functionality and contribution to improved immunity and growth performance of the tilapia fed the combination of *B. subtilis* and *B. cereus* or *B. cereus* alone.

5. Conclusions

The *B. subtilis* and *B. cereus* or *B. cereus*-supplemented fish feeds at 1 × 10⁸ CFU/g are capable of promoting growth, improving feed utilization and morphology of the intestine, enhancing intestinal lyz expression and disease resistance, and altering the intestinal microbiota composition of tilapia. In future studies, further investigations are required to determine the effects of the probiotic-induced gastrointestinal microbiota on immune responses and growth performance in tilapia. Interesting new lines of research may someday arise from gnotobiotic fish, microbial engineering and metabolomics.

Conflict of interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2019.07.002.

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