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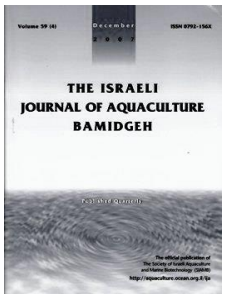

Survival Dynamics and Colonization of Exogenous Probiotic Bacteria *Bacillus subtilis* in Aquaculture Water and Intestine of Zebra Fish (*Danio rerio)*

Jiehao Xu[a], Song Li[a], Lin He[b], Sunjian Lv[a], Haisheng Xu[a]*, Yuyin Chen[a]

[a]*College of Animal Science, Zhejiang University, Hangzhou, China*
[b]*College of Biological and Environmental Science, Zhejiang Wanli University, Ningbo, China*

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Abstract

Adaptability of probiotic bacteria is an important trait for the survival and colonization in water or fish intestine and the performance of their bio-control function. *Bacillus* is a widely used genus of probiotic bacteria in aquaculture. However, its survival dynamics and effect on water or fish intestine is still unclear. In this study, we assessed the survival dynamics of exogenous *Bacillus subtilis* Bst51 and its effect on the microbial community structure in water and fish intestine by using green fluorescent protein (GFP) labeling and bacteriological methods. Results showed that GFP labeling was an efficient method for detection of the survival of *B. subtilis* in the water column and fish intestine. Our results showed that when administered only once, the concentration of Bst51 in water declined to one-tenth of the original concentration and reached a stable state after 24 h. This confirmed that Bst51 strain was able to survive and colonize in aquaculture water with concentrations higher than 10^3 CFU (Colony Forming Units)/mL. The concentration of Bst51 cells in zebra fish intestine decreased slightly and remained constant at around 5×10^6 CFU/g after only one treatment. The results confirmed that if Bst51 cells have a concentration of over 10^9 CFU/mL they can survive and colonize in zebra fish intestine.

* Corresponding author. hsxu@zju.edu.cn

Introduction

Aquaculture is an important food producing industry in the world. It has been reported that approximately 16% of the animal protein consumed worldwide comes from cultured fish and shellfish (Sihag and Sharma, 2012). However, diseases and deterioration of environmental conditions often occur, resulting in serious economic losses. Application of antibiotics is common practice for controlling diseases of fish and shellfish. However there are many reservations about the use of chemotherapeutants as they may eventually lead to the development of antibiotic-resistant bacteria and the inhibition of beneficial gut bacteria (Ekmaharaj, 2009). Aside from antibiotics, the use of probiotics is an alternative strategy to improve health, and these have been shown to be very promising. Several probiotic species such as *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Carnobacterium*, *Streptococcus*, *Bacillus*, *Vibrio*, *Enterobacter*, *Pseudomonas*, yeasts (Ganguly et al. 2010; Gatesoupe. 1999) and photosynthetic bacteria (Cui and Ding. 1997; Wang and Zhao. 1999) have been used in aquaculture. Among these probiotic species, *Bacillus* species have been the most widely used by farmers all over the world and have been proven to be effective probiotics and commercial products. *Bacillus* species efficiently improve water quality, fish and shellfish health. *Bacillus* species are commercially available and have been introduced to fish, shrimp, and molluscan aquaculture as a feed additive, or have been incorporated in pond water (Keysami et al., 2007; Wang et al., 2008; Tseng et al., 2009; Boonthai et al., 2011; Keysami et al., 2012; Zhao et al. 2012; Keysami and Mohammadpour. 2013; Cerezuela et al., 2013). Antibacterial peptide extracted from *Bacillus subtilis* has also enhanced physiological response and disease resistance of Megalobrama amblycephala (He et al., 2013). *Bacillus* species can be beneficial to the host animal by keeping a favorable balance of microflora in the gastrointestinal tract and improve water quality by decreasing the concentrations of ammonia and nitrites. Apart from the beneficial effects noted above, the survival of the bacteria in the aquatic environment and fish intestine remains unclear. Therefore, the objective of this study was to determine the survival of *B. subtilis* in aquaculture water and fish intestines by means of the green fluorescent protein (GFP).

Materials and Methods

Bacterial Strains. B. subtilis was isolated from intestines of grass carp (*Ctenopharyngodon idella*) and bream (*Parabramis pekinensis*), and identified by biochemical tests according to Bergey's manual (Holt et al., 1984), and polymerase chain reaction (PCR)-based 16S rRNA gene variable V6-V8 region with the universal primers F968 (5'-AACGCGAAGCTTAC-3') and L1401 (5'-CGGTGTGTACAAGACCC-3').

The *B. subtilis* cells were prepared according to Spizizen (1958). In order to measure the survival dynamics of exogenous *B. subtilis* in the water column and intestine of zebra fish, the plasmid pGFP315 (Shangran biological company, Hangzhou, China) which carried a GFP gene was transferred into *B. subtilis.* A single colony of *B. subtilis* strain was inoculated in 5 mL lysogeny broth (LB) and cultured overnight. A 160 μL aliquot of the culture was transferred to 8 mL SPI medium (0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.02% $MgSO_4 \bullet 7H_2O$, 0.5% sugar and 1% 100 ×CAYE) and cultured at 37°C and 250 rpm until its OD600 reached approximately 0.8-1.2. Then, 200 μL of this culture was transferred to 2 mL SPII medium (SPI medium supplemented with 1% 50 mM $CaCl_2$ and 1% 250 mM $MgCl_2$) and cultured at 100 rpm for 90 min. 20 μL EGTA (10 mmol/L) was added into this culture, and incubated for an additional 10 min at 100 rpm. The cells were divided into 500 μL aliquots and placed in 1.5 mL Eppendorf tubes. The plasmid pGFP315 (5 μg) was added to 500 μL of competent cells and incubated for 60 min at 200 rpm. All the cells were then plated onto LB agar plates supplemented with Erythromycin (100 mg/mL) and incubated at 37°C for 24 hours. The *B. subtilis* strain named Bst51 with the strongest green fluorescence were selected and cultured in a poor nutrient medium (80%-60%-40%-20%-10% LB medium). After that, the surviving Bst51 that was able to stabilize in the water with fluorescence after generations was selected and used for further experiments.

Experimental Fish. Zebra fish *Danio rerio,* (3-4 cm long) were purchased from a local aquarium shop, and acclimatized for 5 days with feed containing Crude protein > 40, Moisture < 10, Crude fiber 2-5, Ca 0.9-1.6, P 0.8-1 (Sanyou Chuangmei Feed Technology Co., Ltd. Peking, China). Healthy, active fish were selected for the experiments. 280 uniform individuals were randomly divided into 4 groups and each group was held in a 12 L capacity tank each with 7 L water, under natural photoperiod conditions. The culture water for acclimation was taken from a local aquaculture pond. The temperature and dissolved oxygen, for acclimatization and experiments were set at 24±1°C and 7±0.5 mg/L, and pH was 7.3± 0.2. All of the experiments were replicated three times.

Analysis of Survival Dynamics of Exogenous B. subtilis in Water Column. Culture water of each tank was inoculated with probiotic Bst51 at three concentrations of 10^1 CFU/ml (Group A_1), 10^3 CFU/ml (Group A_2), 10^5 CFU/ml (Group A_3), respectively. The control without the probiotic bacteria was designated as Group A_0. Water samples were taken from each group at 1.5 h, 6 h, 12 h, 24 h, 48 h, 96 h, 120 h post inoculation, respectively. The planktonic bacterial counts were determined from 1 mL water.

Water samples were diluted 10 fold (10^0-10^{-3}) and counted repeatedly in triplicate by LB plate method. 100 µL water samples with the LB agar medium containing ampicillin (100 g/L) were taken to count the number of Bst51 under UV 366 nm light. All the plates were incubated at 25°C for 24 h and triplicate samples were counted.

Analysis of Survival Dynamics of Exogenous B. subtilis in Fish Intestine. The Bst51 was mixed into the basal diet at different dose levels, then spread out and dried for 1-2 h at 30°C. Zebra fish were randomly assigned into 4 groups : Group B_0, Group B_1, Group B_2 and Group B_3, containing different the following different dose levels of Bst51: 0, 10^3, 10^5, 10^7 CFU/g, respectively. Oral administration was given after 24h starvation. Immediately after that 6 fish were randomly taken from each group and dissected aseptically at the same time point with Group A. The intestines were removed from the different groups, three were used for bacterial counts, and the others were frozen and sliced for the detection and count of Bst51.

Every intestine sample was divided into two parts: foregut and hindgut. Each part was homogenized and washed with PBS buffer (0.3% PVPP included), centrifuged at 2000 rpm for 6 min, and the resulting supernatant was further centrifuged at 12000 rpm for 5 min to separate the bacteria. These were washed with PBS twice and diluted ten fold (10^0-10^{-3}). 100 µL of the diluted liquid was plated on the LB agar medium to determinate the number of total bacteria in water, and the other 100 µL liquid was plated on the LB agar medium containing 100 mg/mL ampicillin to count the number of Bst51 under the UV 366 nm light. Triplicate plates were incubated at 25°C for 12 h.

Frozen sections were prepared for fluorescent microscopy with the method described by Kawamoto (2003). The embedded tissues were sectioned at 8 µm and mounted on a slide. The slices were serially dehydrated in ethanol, sealed with neutral resins, and observed by fluorescent micrograph (Olympus, Japan).

Results

Preparation of Fluorescent microscopy of B. bacillus. Strong green fluorescence under UV 366 nm, Bst51 was easily seen under fluorescent microscopy. These were easily distinguished from other native bacteria in the experimental water column and in the intestines (Fig.1) even though the colony morphology of the GFP-tagged and the untagged *B. subtilis* in LB medium seemed similar under the naked eye.

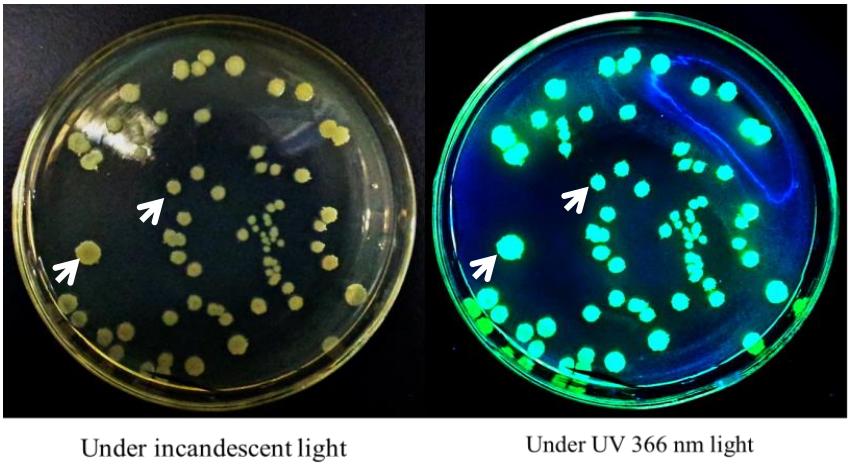

Fig. 1. The characteristic of the colonies of strain Bst51 (arrows) under UV light.

Survival Dynamics of Exogenous B. subtilis in Water Column. Similar trends of total bacteria in the water column were observed in all three bacterial treatments and control group. The total bacterial counts in water column among different groups slightly increased from 2×10^4 CFU/mL to 6×10^4 CFU/mL after 1.5 h, followed by a gradual decline for the next 6h. After 24 h the total bacterial counts returned to the original level and remained stable (Fig. 2 A). The strain Bst51 in group A_1 was not detected after 1.5 h, whereas the concentration of Bst51 in A_2 and A_3 declined to the one-tenth of the original concentration and kept stable after 24 h (Fig.2 B). The concentration of Bst51 in group A_2 fluctuated within the first 12 h, which decreased sharply in the first 1.5 hours then increased slightly until 12 h.

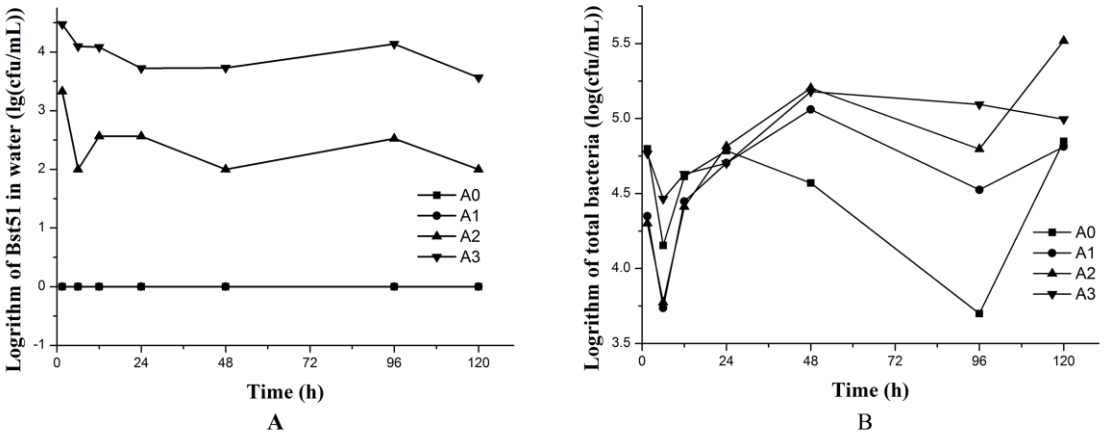

Fig. 2. Survival dynamics of exogenous Bst51 and its effect on total bacteria counts in water column.

Survival Dynamics of B. subtilis in Intestine of Zebra Fish. The total bacterial counts in both foregut and hindgut increased after feeding the diet with Bst51 (Fig.3). The concentration of total bacteria in group B_3 was much higher than the others after 1.5 h and remained stable after 24 h to 120 h, while that in other 3 groups decreased sharply after 96 h and then increased after 120 h. The concentration fluctuation of these three groups was much more unstable than that of group B_3. At 120 h, the concentration of each group reached 10^8-10^9 CFU/mL.

No Bst51 cells were observed in group B_0 of both foregut and hindgut. The concentration of Bst51 cells in group B_3 decreased slightly after feeding and finally stabilized after reaching the density of 5×10^6 CFU/g, while that of group B_2 declined sharply and became undetectable after 12 h in foregut and after 24 h in hindgut. In B_1, the Bst51 cells could not be detected after 12 h in both foregut and hindgut. (Fig.3 C; Fig.3 D).

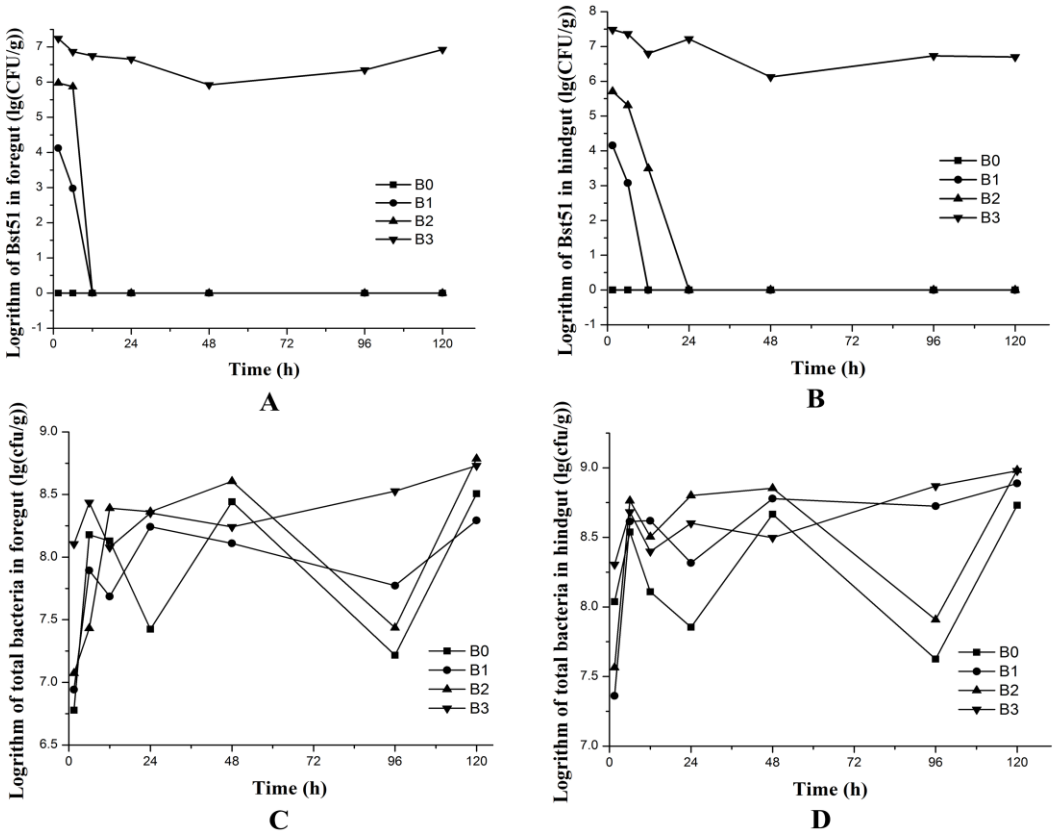

Fig.3. Survival dynamics of exogenous Bst51 and its effect on total bacteria counts in gut.

Under UV microscopy, fluorescent cells could be observed in the fish digestive tract, including stomach, foregut and hindgut (Fig. 4). Few fluorescent cells could be observed in the digestive tract of group B_0, B_1, B_2 at 24 h after feeding. However, the fluorescent cells were clearly observed in the hindgut of group B_3 (Fig. 4). In group B_3, 1.5 h after oral administration, the strain Bst51 was already detected in the stomach and foregut, and even in the hindgut (Fig. 5). In the stomach, foregut and hindgut of group B_3, the concentration of Bst51 cells was identified but changed with time (Fig. 5).

In the stomach, the concentration of *Bst51* cells reduced gradually and few fluorescent cells could be found after 12 h. However in foregut and hindgut, the concentration of Bst51 cells increased gradually with time so that a few fluorescent cells were observed in hindgut after 1.5 h. The fluorescence micrographs followed a concentration trend after feeding as follows: stomach > foregut >hindgut at 1.5 h and hindgut > foregut > stomach at 12 h.

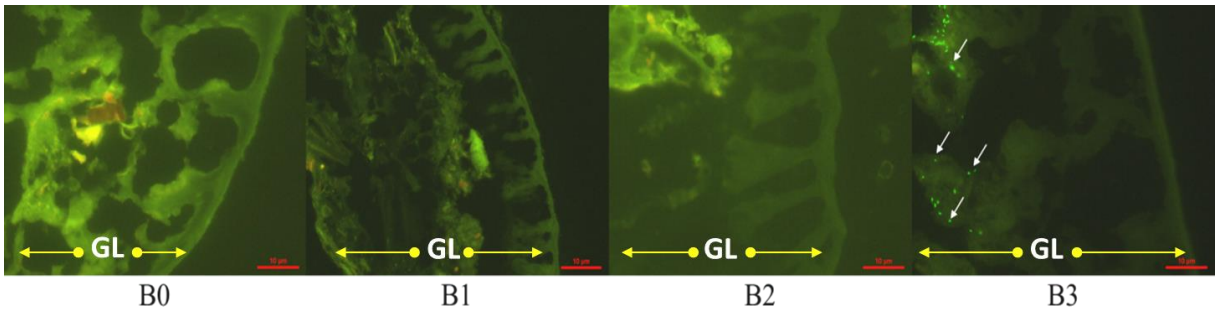

Fig.4. Fluorescent Bst51 cells (arrow) observed in group B3 by the fluorescence micrographs of frozen sections in hindgut at 24 h after diet.

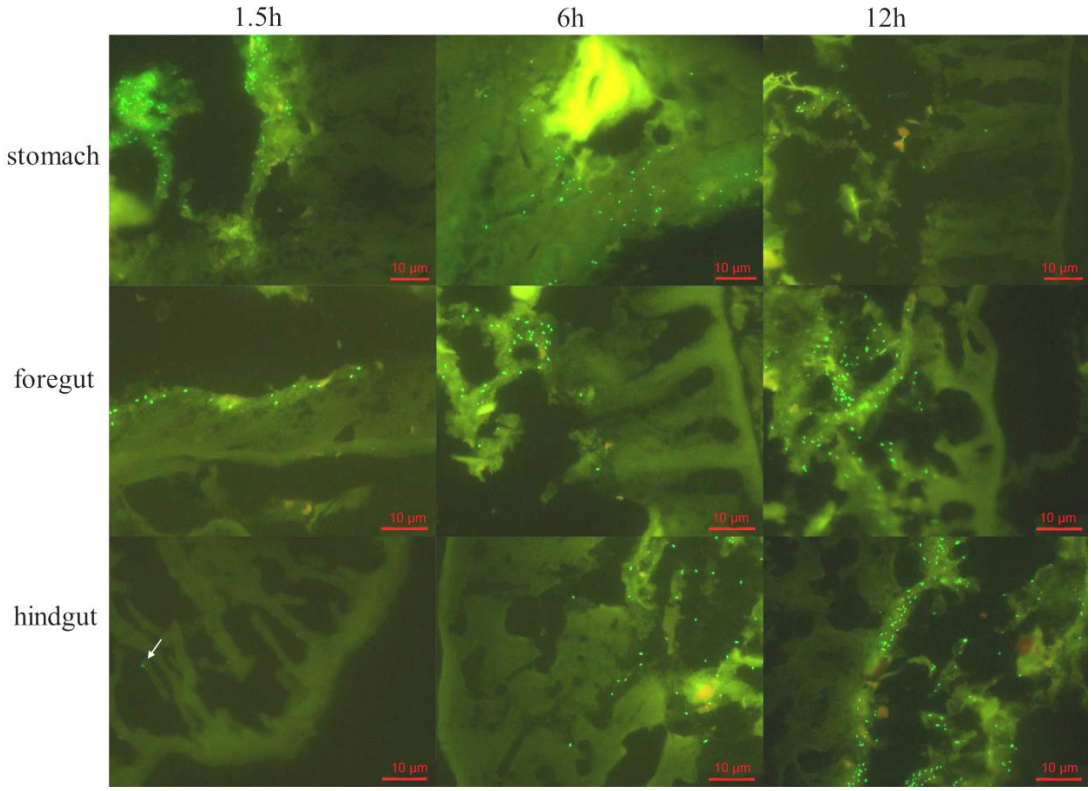

Fig.5. Fluorescent Bst51 cells (arrow) in digestive tract and the change in counts with time in group B_3.

Discussion

In the present study, we used a GFP protein to trace the bacterial growth and precisely determine the growth dynamics in the gastrointestinal tract and in water.

In our study, *B. subtilis* was labeled with GFP to trace the colonization ability of probiotics in water and aquatic organisms. The stability of fluorescence of GFP-tagged *B. subtilis* strain Bst51 after generations corresponded to the stable expression of GFP protein in the strains and the compatibility between the indigenous plasmids and the commercially introduced plasmids.

The exogenous Bst51 strain was found to colonize in water and in the intestine of zebra fish. According to the study of Tam N. K. et al. (2006) and Baril E, et al (2012), the *B. subtilis* strain can sporulate in water and the gastrointestinal tract (GIT). During the first 12 h, some of the Bst51 strain became spores due to changes in the environment. To colonize in a new habitat, the Bst51 needed to compete with other *bacterial species* in the water and GIT for space and nutrition, and at the same time had to overcome damage from digestive enzymes in GIT. Surviving Bst51 could germinate and then resporulate in water and GIT, then its numbers increased slightly and stabilized. The same phenomenon of spore production and the cycle of germination-growth-sporulation was observed in the intestinal tracts of mice. (Tam N. K. et al. 2006).

The addition of different concentrations of Bst51 in water (A_0, A_1, A_2, and A_3) did not affect the concentration of total bacteria ($P > 0.05$). Similarly, no significant difference was detected among the four groups with different Bst51 concentrations in diets (B_0, B_1, B_2 and B_3) ($P > 0.05$). When the probiotic was only fed once, it was stable in water at the minimal dose of 10^3 CFU/mL. The final concentration of live Bst51 was directly proportional to the supplemented dosage. Under the same conditions, the oral probiotic experiment showed that the minimum concentration of bio-addition of Bst51 required to colonize bacteria in the gut of zebra fish was 10^9 CFU/g. In a previous study, Zhang et al. (2013) found that consecutive addition of *Bacillus* strain SC02 (10^3 CFU/mL) at 7-day intervals led to significant improvement of microbial diversity in

water. Diets containing *B. subtilis* E20 at the level of 10^5 CFU/g led to a significant increase in the immune response of shrimp after a 98-day feed (Tseng, Ho et al. 2009). However, there is no evidence showing whether the added probiotic would colonize and remain stable in the water, or in the gut.

After oral administration of fluorescent probiotics, the dynamics of Bst51 was monitored by fluorescent microscopy of frozen sections. In group B_3, due to the relatively high concentration of the bacteria, we observed that 1.5 h was enough for the Bst51 strain to go through the entire gastrointestinal tract. It was demonstrated that the density of Bst51 changed with the passage of feeds, and that Bst51 was widely distributed in the digestive tract from the stomach to the hindgut. Some bacteria were present in gut lumen, and others in the epithelial cells or even between the epithelial cells. According to date from plate counting, the Bst51 strain can persist at a high level (about 10^6 CFU/g) in the foregut and hindgut after 12 h and up to 120 h. Similarly, previous research revealed that the EGFP-*Vibro fluvialis* could be clearly detected in *Einephelus awoara* foregut and midgut after 12 h (Qin et al., 2012). Six hours after ingestion of feeds supplemented with GFP labeled *lactobacilli,* the bacteria were detectable in all luminal contents of chicken (stomach, jejunum, ileum and caecum). After 24 h of administration, the microorganism was present at maximum concentration throughout the intestine (Yu et al., 2007). However, our results indicated that no Bst51 entered the gastrointestinal system when the concentration was lower than 10^9 CFU/g. We found that the period of time for successful colonization of the probiotic depended on the concentration of supplemented bacteria.

In conclusion, labeling *B.subtilis* with GFP is very efficient for detecting its survival dynamics and colonization in water or fish intestines. The strain Bst51 is able to survive in aquaculture water at a concentration higher than 10^3 CFU/mL and colonize zebra fish intestine at concentrations higher than 10^9 CFU/g, even when administrated only once. Our results provided some insight into the life cycle of exogenous probiotic bacteria in the open ponds.

Acknowledgements

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