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The spoilage and adhesion inhibitory effects of *Bacillus subtilis* against *Shewanella* and *Pseudomonas* in large yellow croaker (*Pseudosciaena crocea*)

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Abstract

Shewanella and *Pseudomonas* are considered to be the main spoilage bacteria for chilled fish. This work focused on the spoilage and adhesion ability of two spoilage bacteria (*Shewanella* spp. S-1 and *Pseudomonas* spp. P-1), and the adhesion inhibitory effects of *Bacillus subtilis* against the spoilage bacteria in large yellow croaker (*Pseudosciaena crocea*). We aimed to develop a feasible solution to prolong the shelf life of large yellow croaker. The spoilage and adhesion characteristics of *Shewanella* spp. and *Pseudomonas* spp. strains were compared. It was found that *Shewanella* spp. induced more significant spoilage of large yellow croaker than *Pseudomonas* spp. at low temperature in vitro. In addition, *Shewanella* spp. demonstrated stronger adhesion to the intestinal mucus than *Pseudomonas* spp. in vitro. The results of a one-week feeding experiment showed that *Shewanella* was more likely to adhere to large yellow croaker intestines than *Pseudomonas*. The supplementation of *B. subtilis* strain BS08 effectively inhibited the proliferation of *Shewanella* and slowed the spoilage process of large yellow croaker.

Keywords: large yellow croaker (*Pseudosciaena crocea*); spoilage and adhesion; *Shewanella*; *Pseudomonas*; *Bacillus subtilis*.

Practical Application: The results of this study may help to further our knowledge of large yellow croaker spoilage and provide potential probiotic solutions to maintain the freshness of large yellow croaker in low-temperature storage.

1 Introduction

Large yellow croaker (*Pseudosciaena crocea*) is one of the most important economically cultured fish species in southeast China. In fact, it is the most cultivated species in Chinese marine net-cage farming (Liu & Mitcheson, 2008). In order to maintain freshness, large yellow croaker is often stored at low temperature on ice. However, due to the high levels of moisture and nutrient content, air-exposed iced large yellow croaker can easily spoil within a few days.

During storage, the microflora changes, due to different abilities of microorganisms to tolerate the preservation conditions. Some psychrotolerant Gram-negative bacteria (such as *Pseudomonas* and *Shewanella*) grow on chilled fish and have been reported as specific spoilage organisms (SSOs) (Gram & Dalgaard, 2002). *Shewanella* and *Pseudomonas* are also considered potential spoilage bacteria in large yellow croaker (Ge et al., 2017). *Shewanella* is the dominant organism in large yellow croaker at low temperature (Quanyou et al., 2018). To date, studies have focused on quorum sensing (QS) signaling molecules in regulated biofilm formation and spoilage gene expression to clarify the causes of *Shewanella*-lead spoilage (Zhu et al., 2016; Fu et al., 2018a). Bio-preservatives such as nisin, green tea polyphenols, and bacteriocin, produced by *Bacillus coagulans*, have been implemented to preserve large yellow croaker through inhibition of quorum sensing and biofilm of *Shewanella* (Zhu et al., 2015; He et al., 2017; Fu et al., 2018b).

Bacterial adhesion to fish tissue surfaces is an important step in the initial stage of bacterial colonization (Vendrell et al., 2009). The characteristic s of pathogenic *Vibrio alginolyticus* adhesion to mucus show that the portal of entry for pathogenic *V. alginolyticus* into large yellow croaker is via the intestinal tract, rather than gill or skin, according to the kinetics of the bacterial adhesion to different mucus types (Chen et al., 2008). The use of probiotics may provide protection through competition for host extracellular matrix-binding sites, thereby blocking the adhesion and spread of pathogens (Reid et al., 2001), such as *Bacillus* (Etyemez & Balcazar, 2016; Kavitha et al., 2018) and lactic acid bacteria (Arellano-Ayala et al., 2020; Kos et al., 2003; Nwanna et al., 2014).

However, there have been few studies on the adhesion of spoilage bacteria (including *Pseudomonas* and *Shewanella*) to fish. Our previous research showed that *Shewanella* spp. had stronger surface hydrophobicity and auto-aggregation ability than *Pseudomonas* spp. which enhanced the adhesion ability to fish mucus and form biofilm (Zhang et al., 2019a). The adhesion ability of *Shewanella* spp.to gill and intestine were higher than which to skin,which was enhanced with the concentration of NaCl increased (Zhang et al., 2019b). In this work, we hypothesized that the adhesion and colonization ability of spoilage bacteria plays an important role in the spoilage potential of bacteria. Therefore, the adhesion and spoilage ability of spoilage bacteria (*Shewanella* and *Pseudomonas*) to large yellow croaker were investigated and one *Bacillus subtilis* strain, BS08, was employed to inhibit the adhesion of bacteria and to retain freshness of large yellow croaker during low-temperature storage.

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2 Materials and methods

2.1 Bacterial strains

Shewanella spp. (S-1) and *Pseudomonas* spp. (P-1) isolated from iced large yellow croaker and *Bacillus subtilis* BS08 isolated from bee gut (Zhang et al., 2017) were kept in our laboratory.

2.2 Spoilage potential evaluation

Large yellow croakers (approx. 500 g) were transported to the laboratory on ice from the aquatic products market in Fuzhou, China, within 6 h of fishing. Subsequently, they were scaled; the gills and guts were removed. The fish were cleaned and filleted (weigh 2.0 ± 0.1 g each). Then, the fillets were washed thoroughly with sterile water, placed in a sterile petri dish, and irradiated with ultraviolet light for 20 min. Sterile fish fillets were soaked in a bacterial suspension of *Shewanella* spp. or *Pseudomonas* spp. (approx. 10^8 CFU mL^{-1}) for 20 s, drained, and placed in a 50 mL sterile centrifugal tube. The tube was stored at 4 °C. Sterile fish fillets without inoculated bacteria were used as a blank control.

A fish sample (2 g) was homogenized with 18 mL of sterile saline solution for 2 min. Then, 10-fold serial dilutions were made and inoculated on beef extract peptone medium. Total viable counts (TVC) were determined by counting the number of colony-forming units after incubation at 28 °C for 24 h.

The fish fillets were homogenized. Appropriate quantities of homogenized fish were used for the determination of the following chemical parameters. Total volatile basic nitrogen (TVB-N) was determined according to a previously published method (Malle & Poumeyrol, 1989). Trimethylamine (TMA) was determined according to the method of Association of Official Analytical Chemists (1990), with slight modifications. Samples (5 g) were homogenized with 20 mL sterile distilled water and mixed with 10 mL of 20% trichloroacetic acid. After centrifugation at 10,000 g for 10 min, 5 mL supernatant were mixed with 1 mL of formaldehyde (10%), 10 mL of anhydrous toluene and 3 mL of K_2CO_3 in a tube. The tube was covered immediately and shaken vigorously about 60 times. After standing for 20 min, the 5 mL toluene layer was transferred to another tube containing 5 mL picric acid solution (0.02%). The absorbance of the mixed solution was measured at 410 nm. The TMA value was calculated by standard curve of TMA (purity>98%). The results of TVB-N and TMA are expressed as mean mg-N 100 g^{-1} ± standard deviation of three replicates from different batches.

The K value was estimated according to a previously published method (Yu et al., 2018). Nucleotide standards, including adenosine triphosphate (ATP ≥ 98%) nosine diphosphate (ADP ≥ 98%), adenosine monophosphate (AMP ≥ 98%), inosine monophosphate (IMP ≥ 98%), hypoxanthine riboside (HxR ≥ 98%), and hypoxanthine (Hx ≥ 98%), were purchased from Lanji Co. (Shanghai, China). Samples (2 g) were homogenized with 7.5 mL for 3 min and centrifuged at 10,000 *g* for 5 min at 4 °C. The extraction process was repeated once, and the combined supernatants were neutralized immediately to pH 6.0-6.4 with 1 mol L^{-1} NaOH solutions. The precipitate in neutralized solution was removed by centrifugation (3,000 *g*, 5 min and 4 °C), and the supernatant was diluted to 50 mL with cold distilled water. The final solution was filtered through a nominal 0.22 μm membrane filter, and ATP-related compounds were determined using an L-2000 HPLC (HITACHI, Japan) equipped with a HITACHI L-2455 detector and an Agilent Zorbax SB-C18 column (4.6×250 mm, 5 μm). The mobile phases were 0.02 M phosphate buffer (KH_2PO_4 and K_2HPO_4, pH 6.0) and methanol (98:2, v/v), with a flow rate of 1 mL min^{-1}. The detection wavelength and column temperature were 254 nm and 30 °C, respectively. The K value was calculated using the following Equation 1:

$$K\ value(\%) = \frac{M_{HxR} + M_{Hx}}{M_{ATP} + M_{ADP} + M_{AMP} + M_{IMP} + M_{HxR} + M_{Hx}} \times 100 \quad (1)$$

2.3 Bacterial adhesion ability

Mucus was prepared using the method published by Chen et al. (2008), with a slight modification. The intestinal mucus was harvested by scraping off the inner surface of the intestines with a spatula to remove the mucous gel layer covering the intestine. Finally, the mucus was homogenized in sterile 0.01 mol L^1 PBS centrifuged at 4 °C and centrifuged twice at 10,000 g for 30 min to remove particulate matter. The supernatant were filtered through 0.22 μm filters. The mucus samples were adjusted to 0.5 mg mL^{-1} with sterile 0.01 mol L^{-1} PBS and stored at -20 °C until use. The protein concentration was determined by the Lowry method (Hartree, 1972).

FITC staining was performed according to a previously published method (Vinderola et al., 2004). Fresh cultures of each strain were cultured overnight at 28 °C. Then, bacteria were harvested by centrifugation at 10,000 *g* for 10 min at 4 °C and washed twice with 0.01 mol L^{-1} PBS (pH 7.4). Cells were labelled with fluorescein isothiocyanate (FITC; Sigma, St Louis, MO, USA, 0.2 mg mL^{-1}) in PBS and incubated for 1.5 h at 30 °C in the dark. Labeled bacteria were washed a few times with PBS solution to remove unincorporated FITC. The final pellet was resuspended in PBS to a concentration of 1×10^8 CFU mL^{-1} and stored at -20 °C in the dark.

To black 96-well plates, 150 μL of mucus was added and stored overnight at 4 °C. The residual mucus was washed twice with 200 μL of sterile 0.01 mol L^{-1} PBS. To the wells, 150 μL of FITC-labeled bacterial suspension was added and incubated at 4 °C for 90 min. The non-adhered bacteria were flushed twice with sterile physiological saline. Adhered bacteria were released and lysed with 150 μL of a 1% SDS (0.1 mol L^{-1} NaOH) solution at 60 °C for 1 h. Fluorescence was measured in a multiscan fluorometer (SpectraMax i3+MiniMax, Molecular Devices, USA) at λex 495 nm and λem 525 nm. Negative controls of labeled bacteria were used to calculate the percentage of adhesion. This percentage was expressed as the percentage of fluorescence recovered after attachment to mucus, relative to the initial fluorescence of the bacterial suspension added to the wells (Equation 2).

$$Adhesion\ rate\ \% = \frac{FI_2}{FI_1} \times 100 \quad (2)$$

where FI_2 and FI_1 are the fluorescence intensities of the experimental group and the FITC-labeled pure bacterial suspension, respectively.

2.4 Adhesion inhibition

Competition inhibition

FITC-labeled spoilage bacteria were mixed in equal volume with *Bacillus subtilis* BS08. To the 96-well plates coated with mucus, 150 μL of the bacterial suspension was added and incubated at 28 °C for 2 h.

Displacement inhibition

To the 96-well plates coated with mucus, 150 μL of FITC-labeled bacterial suspension was added and incubated at 28 °C for 2 h. The non-adhered bacterial cells were discarded by washing twice with 200 μL of sterile 0.01 mol L^{-1} PBS. Next, 150 μL of *Bacillus subtilis* BS08 suspension was added and incubated at 28 °C for 2 h.

Exclusion inhibition

To the 96-well plates coated with mucus, 150 μL of *Bacillus subtilis* BS08 suspension was added and incubated at 28 °C for 2 h. The non-adhered cells were discarded by washing twice with 200 μL of sterile 0.01 mol L^{-1} PBS. Next, 150 μL of FITC-labeled bacterial suspension was added and incubated at 28 °C for 2 h.

After incubation for 2 h, the cells were washed twice with 200 μL of sterile 0.01 mol L^{-1} PBS. The adhered cells were released and lysed with 150 μL of a 1% SDS (0.1 mol L^{-1} NaOH) solution at 60 °C for 1 h. Fluorescence was measured in a multiscan fluorometer (Equation 3).

$$Inhibition\ rate(\%) = 1 - A_2 / A_1 \times 100\% \quad (3)$$

where A_1 is the fluorescence intensity of the FITC-labeled spoilage bacterial adhered on mucus, and A_2 is the fluorescence intensity of the FITC-labeled spoilage bacterial adhered on mucus inhibited by *Bacillus subtilis*.

2.5 Feeding experiment

Basal fish feed was purchased from Fuzhou Haima Feed Co., Ltd. Its main components included fishmeal, flour, soybean meal, fish oil, vitamins, and minerals. *Bacillus subtilis* strain BS08 was grown in MNB at 37 °C in a rotary shaker for 24 h. Bacterial cells were harvested by centrifugation (10,000 g for 10 min) and washed and re-suspended in normal saline solution (NSS). Subsequently, gum arabic was added into the bacterial suspension with a final concentration of 15% (w/v). Then, it was spray dried to obtain a bacterial powder with approx. 5×10^{10} CFU g^{-1}. Afterward, 10 g of the bacterial powder was resuspended in NSS and sprayed on the surface of the basal fish feed (1 kg), forming a probiotic supplemented fish feed (SF) with *Bacillus subtilis* BS08.

A total of 80 juvenile large yellow croakers, with an average body weight of 20 g, were purchased and kept in a fish farm (Ningde Fufa Aquatic Co., Ltd.). The fish were randomly divided into 2 groups (n=40), including one control group and one treatment group. The fish in the same group were kept in two separated plastic tanks (20 fish per tank, capacity, 100 L). The fish in the control group (Con) were fed with basal feed, while the treatment group (SFG) was fed with supplemented fish feed (SF). Feed was supplied twice a day (09:00 and 17:00 h). The cultivation water was marine water with a temperature of 22 °C to 24 °C. During the experiment, approximately 30% of cultivation water was changed daily, along with the purge of the unconsumed feed and fish feces. After one week of adaptation with basal feed, the feeding experiment lasted one week.

After 24 h of fasting at the end of experiment, all fish were caught and stored at 4 °C immediately. At storage for 1 d and 3 d, fish were washed with sterile water to reduce contamination by commensal bacteria. Digestive tracts were aseptically removed and homogenized in a sterile homogenization bag. The bags were stored at -20 °C for DNA extraction. The fish were scaled; the gills and guts were removed. The fish were cleaned, filleted (weigh 2.0 ± 0.1 g each), and stored at -20 °C for the TVB-N test.

2.6 DNA extraction, qPCR reaction, and high-throughput sequencing analysis

Total DNA was extracted from the intestines using a fecal genomic DNA Extraction Kit and bacterial genomic DNA extraction kit (Tiangen, China), respectively. The qPCR reaction consisted of 2.0 μL of 10-fold diluted DNA, 10 μL of SYBR Green, and 0.2 μL of each primer (10 μM) in a total volume of 20 μL. The PCR program comprised 45 cycles at 94 °C (35 s), 60 °C (30 s) and 72 °C (30 s), followed by melt curve generation. Melt curves were analyzed to check the specificity of amplification. Gene-specific primers were designed as:

- *Pseudomonas*, Pse-F, GCAACGCGAAGAACCTTACC, Pse-R, CATCTCACGACACGAGCTGA;
- *Shewanella*, She-F, CTTGGGGCGGCTACACAT, She-R, GTGTCTCAGTCCCAGTGTGG;
- *Bacillus subtilis*, Bac-F, AAAGTCTGACGGAGCAACGC, Bac-R, ACCGCCCTATTCGAACGGTA.

Bacterial primers 341-F (50-CCT AYG GGR BGC ASC AG-30) and 806-R (50-GGA CTA CNN GGG TAT CTA AT-30) were used to amplify the V3-V4 region of bacterial 16SrRNA genes. The sequencing library of bacterial 16S rRNA genes was generated for high-throughput sequencing, employing the TruSeqfi DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA). Next, the library was sequenced on an Illumina HiSeq2500 platform by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

2.7 Statistical analysis

The results are expressed as the mean of three to five independent replicates. Data were analyzed using ANOVA, and the means were compared with Tukey's test (5% probability), using SPSS 24.0 software. The graphs were prepared with GraphPad Prism 8.0 software.

3 Results

3.1 Analysis of spoilage ability of Shewanella spp. and Pseudomonas spp.

Microbiological and biochemical changes are associated with the deterioration of fish quality. The total number of viable bacteria (TVC), total volatile basic nitrogen (TVB-N), trimethylamine (TMA), and K value are commonly used as indicators of fish spoilage (Chytiri et al., 2004).

Changes in TVC of large yellow croaker fillets inoculated with spoilage bacteria during storage in 4 °C are shown in Figure 1A. The TVC was 4.10 log CFU g^{-1} on day 1 in the control group and increased to 6.64 log CFU g^{-1} three days later. The spoilage limit of 7.00 log CFU g^{-1} (Ojagh et al., 2010) was reached after four days of storage. TVC increased rapidly and reached the spoilage limit after three days of storage for the groups inoculated with spoilage bacteria, particularly with *Shewanella* spp.(P<0.05).

TVB-N values in the control group increased slightly during storage at 4 °C (Figure 1B). TVB-N on day 1 and day 5 was 16.06 mg-N 100 g^{-1} and 21.54 mg-N 100 g^{-1}, respectively. The TVB-N of fillets inoculated with spoilage bacteria increased rapidly during storage, especially for the fillets inoculated with *Shewanella* spp.. TVB-N values approached the upper acceptable level of 25 mg-N 100 g^{-1} (Gimenez et al., 2002) after one day of storage in the *Shewanella* spp. inoculated group and after two days of storage in the *Pseudomonas* spp. group. The TVB-N values of fillets inoculated with *Shewanella* spp. were significantly higher than those of fillets inoculated with *Pseudomonas* spp. after five days of storage, and the TVB-N values reached about 70 mg-N 100 g^{-1} and 40 mg-N 100 g^{-1}, respectively.

The TMA values of samples inoculated with *Shewanella* spp. reached 45 mg-N 100 g^{-1} at the end of the storage period (Figure 1C). However, the TMA values of samples inoculated with *Pseudomonas* spp. were near 10 mg-N 100 g^{-1}. Conversely, the values of the control samples were 4.24 mg-N 100 g^{-1}, which was lower than the upper acceptable limit of 10 mg-N 100 g^{-1} (Teskeredzic & Pfeifer, 1987). TMA is produced by the decomposition of TMAO due to bacterial spoilage and enzymatic activity. It has been reported that two *Pseudomonas* spp. strains isolated from large yellow croaker are unable to produce TMA. Additionally, the TVB-N value of *S. baltica* was significantly higher than that of *Pseudomonas* spp. in sterile fish juice, although its growth was slower than that of *Pseudomonas* spp. (Ge et al., 2017).

The K value, the ratio of ATP-related compounds, is widely utilized to assess the freshness of refrigerated fish. K values <20% are very fresh, those of 20–60% are moderately fresh, and >60% should be rejected (Ehira, 1976). As shown in Figure 1D, the initial K values were near 20%. The K values of fillets inoculated with *Shewanella* spp. exceeded the recommended limit value (60%) after four days. However, the K values of fillets inoculated with *Pseudomonas* spp. were still acceptable on day 4.

3.2 In vitro adhesion of spoilage bacteria and inhibition by Bacillus subtilis

As shown in Figure 2A, adhesion of *Shewanella* spp. S-1 to the intestinal mucus of large yellow croaker was significantly higher than *Pseudomonas* spp. P-1. However, adhesion of spoilage bacteria was inhibited by *Bacillus subtilis* BS08 (Figure 2B).

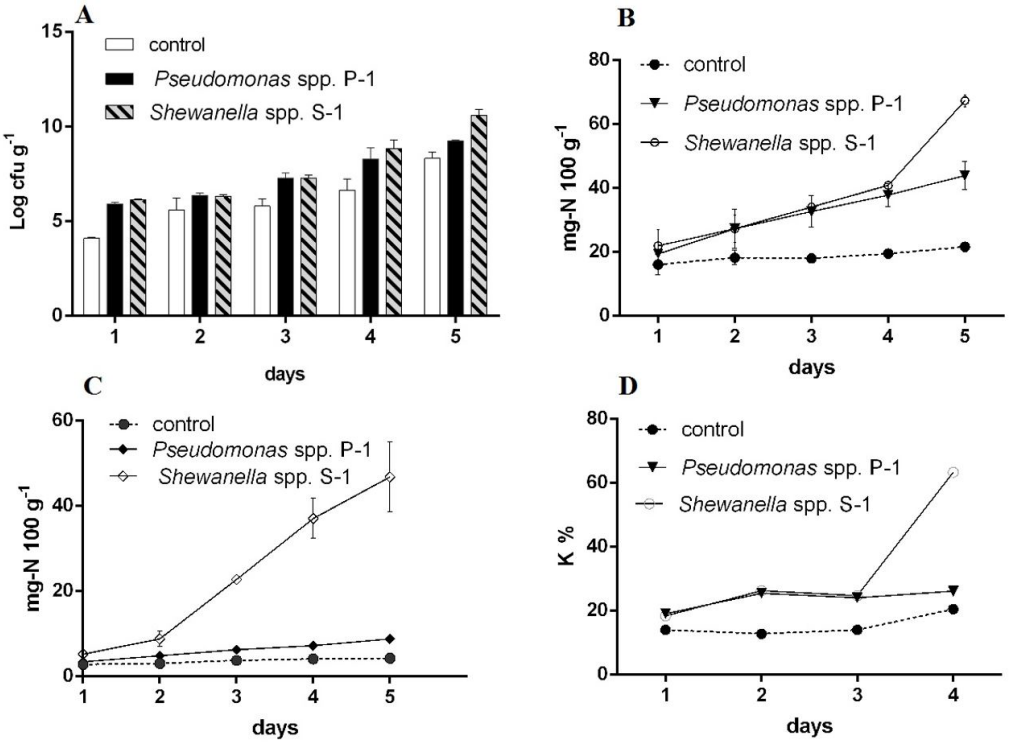

Figure 1. In vitro evaluation of spoilage potential of *Shewanella* spp. S-1 and *Pseudomonas* spp. P-1 by inoculating cultures on Large yellow croaker fillets at 4 °C storage. (A) Total viable counts; (B) TVB-N value; (C) TMA value; (D) K values.

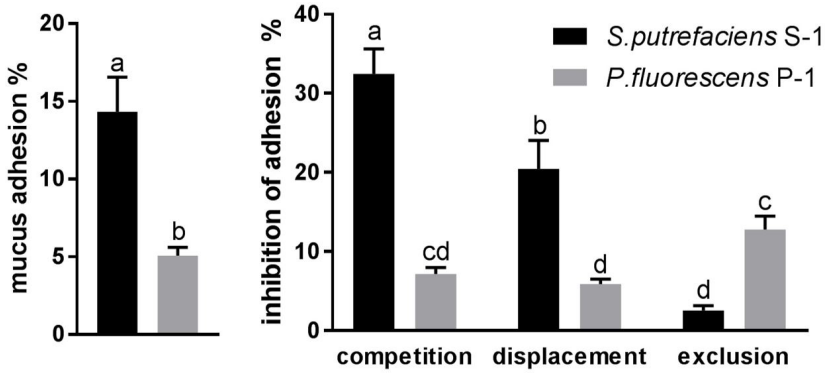

Figure 2. Adhesion of *Shewanella* spp. S-1 and *Pseudomonas* spp. P-1 to intestinal mucus of large yellow croaker and inhibition by *Bacillus Subtilis* (BS08) in vitro.

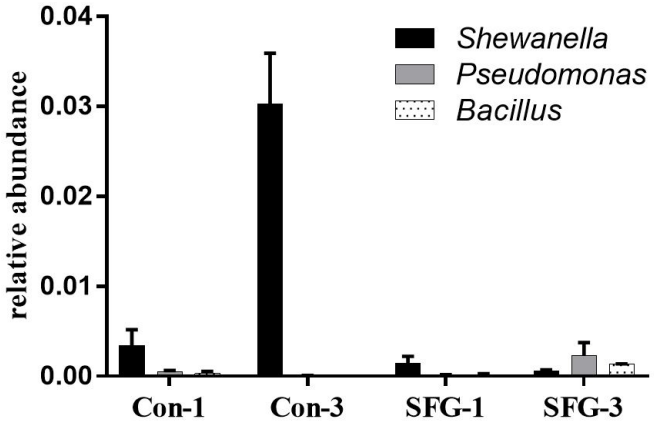

Figure 3. Relative abundance of *Shewanella*, *Pseudomonas*, and *Bacillus* analyzed by high-throughput sequencing analysis.

The adhesion of *Shewanella* spp. to intestinal mucus was significantly inhibited by competition and displacement of *Bacillus subtilis*, while adhesion of *Pseudomonas* spp. was significantly inhibited by exclusion.

3.3 In vivo inhibition of adhesion by Bacillus subtilis

In vivo experiments were carried out to evaluate the inhibitory effects of *Bacillus subtilis* BS08 against *Shewanella and Pseudomonas* in the intestines of large yellow croaker.

As shown in Figure 3, high-throughput sequencing of intestinal flora of large yellow croaker revealed that the relative abundance of *Shewanella* were higher than *Pseudomonas* in the control group on day 1 and increased sharply 3 days later (Con_3). Conversely, the relative abundance of *Shewanella* decreased in the supplement feed group (SFG). The results indicate *Shewanella* could adhere and grow better than *Pseudomona* in the intestines of large yellow croaker under natural water condition, but *Shewanella* were significantly inhibited by *Bacillus subtilis*.

The qPCR results in Figure 4 show that adhesion and colonization of *Shewanella* and *Pseudomonas* in the intestine of large yellow croaker were better than *Bacillus subtilis*. However, the growth of *Shewanella* in large yellow croaker intestinal tract and proliferation to the flesh were significantly inhibited by *Bacillus subtilis*. The inhibition of *Pseudomonas* was slightly weaker than that of *Shewanella*, which was consistent with the inhibition of adhesion in vitro.

As shown in Figure 5A, TVB-N values in the control and supplement feed groups were near 20 mg-N 100 g^{-1} at the initial stage of storage. The values in the control group were higher (27.87 mg-N 100 g^{-1}, near the upper acceptable limit 30 mg/100 g) than those in the supplement feed group (SFG_3) on day 3. K values are shown in Figure 5B. The freshness of large yellow croaker was significantly different after three days of storage. The results show that treatment with *Bacillus subtilis* could significantly delay the spoilage of large yellow croaker.

4 Discussion

Shewanella and *Pseudomonas* are widely known to be responsible for the spoilage of fish. In this study, the TVC, TVB-N, TMA, and K values of *Shewanella* spp. were higher than those of *Pseudomonas* spp.. *Shewanella* is considered a spoilage bacterium due to its ability to induce TMAO reduction to trimethylamine (TMA), which is associated with the typical strong fishy, urine, and ammonia-like off-odors (Gram & Huss, 1996; López-Caballero et al., 2001). The TVB-N value of *Shewanella* spp. inoculated in sterile fish juice of large yellow croaker was previously found to be significantly higher than that of *Pseudomonas* (Ge et al., 2017), which was consistent with our research.

As exogenous microorganisms of large yellow croaker, the adhesion of *Shewanella* and *Pseudomonas* is important for colonization. In this study, the adhesion ability of *Shewanella* spp. S-1 and *Pseudomonas* spp. P-1 were determined in vitro. The results show that the adhesion of *Shewanella* spp. to intestinal mucus was higher than that of *Pseudomonas* spp.. Furthermore, in vivo tests showed that *Shewanella* had better adhesion than *Pseudomonas. Shewanella* then spread and proliferated to the flesh, and this result was in accordance with adhesion in vitro. Although there are few adhesion studies regarding mucus adhesion of spoilage bacteria, pathogenesis studies indicate that the adherence and invasive capacities of bacteria are important in early pathogenesis. It has been suggested that pathogenic bacteria in the gill adhere to the gill mucus and thereafter invade

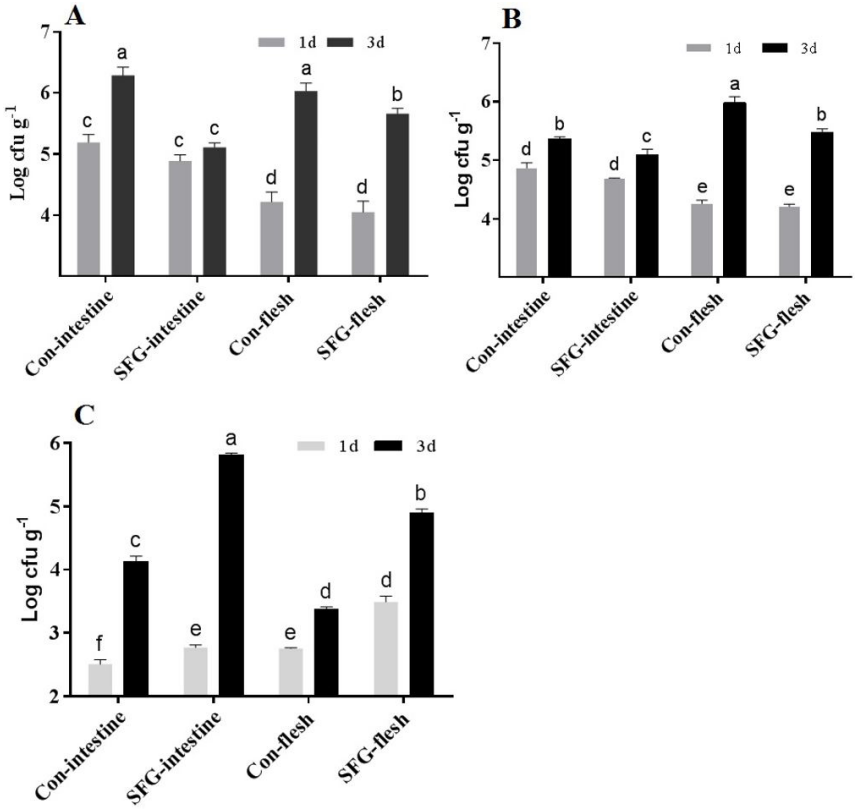

Figure 4. qPCR analysis of bacterial growth in intestine and flesh of large yellow croaker during storage at 4 °C. (A) *Shewanella*; (B) *Pseudomonas*; (C) *Bacillus*.

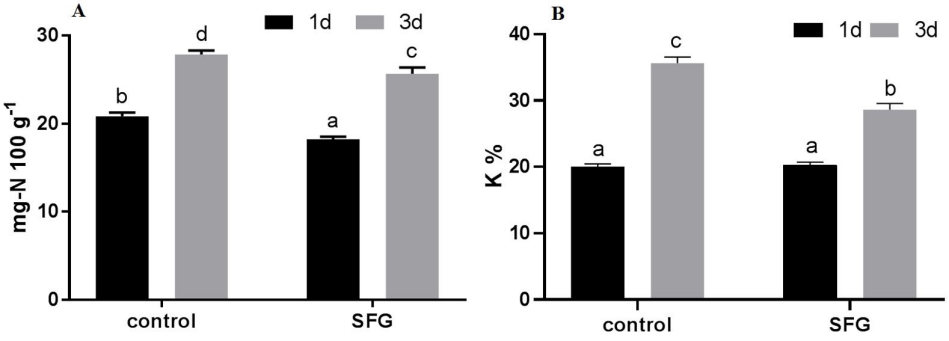

Figure 5. Effect of *Bacillus subtilis* (BS08) on freshness of large yellow croaker stored at 4 °C. (A) TVB-N value of large yellow croaker; (B) K value of large yellow croaker.

the branchial vasculature, leading to septicemia and colonization of the internal organs (Tobback et al., 2009).

The results in vitro show that *Bacillus subtilis* inhibited the adhesion of *Shewanella* spp. and *Pseudomonas* spp. to intestinal mucus of large yellow croaker through competition, displacement, and exclusion. The adhesion of *Shewanella* spp. to intestinal mucus was significantly inhibited by competition and replacement inhibition of *B. subtilis*, while *Pseudomonas* spp. was significantly inhibited by exclusion. The results of in vivo experiments showed that *B. subtilis* could effectively inhibit the growth of *Shewanella* in the intestinal tract and the proliferation to the flesh of large yellow croaker during storage, but *B. subtilis* had no significant effect on *Pseudomonas*. Our findings indicated that competitive inhibition and displacement inhibition of *B. subtilis* might play important roles in the intestinal tract. The TVB-N and K values of samples mediated with *Bacillus subtilis* changed slowly during storage. Thus, we concluded that *Bacillus subtilis* delayed spoilage of fish through adhesion inhibition. Since the infections of spoilage bacteria usually originate in former colonization, decolonization should be a possible means of preventing the infection of spoilage bacteria. Inhibitory and adhesive abilities are regarded as indictors for the screening of potential probiotic, such as lactic acid bacteria and *Bacillus* species (Etyemez & Balcazar, 2016), against pathogenic bacteria. This study showed *B. subtilis* could be used as a probiotic in aquaculture against spoilage bacteria. Studies have found a widespread mechanism exerted by *Bacillus subtilis* species that inhibits adhesion, colonization, and growth of spoilage bacteria. For example, *Bacillus* inhibited *S. aureus* intestinal colonization through an Agr quorum sensing regulatory system (Piewngam et al., 2018). Our findings suggest

a probiotic-based method for spoilage bacteria decolonization and new ways to fight spoilage in aquatic foods.

In conclusion, the spoilage ability of *Shewanella* spp. in the flesh of large yellow croaker was higher than that of *Pseudomonas* spp.. *Shewanella* spp. adhesion to fish intestinal mucus was significantly greater than that of *Pseudomonas* spp. in vitro. Further fish feeding experiments showed that *Shewanella* more easily adhered to fish intestines than *Pseudomonas* and formed specific spoilage bacteria without the interference of exogenous microorganisms. Subsequently, *Shewanella* proliferated to the flesh and produced a strong spoilage effect. *Bacillus subtilis* strain BS08 inhibited the adhesion of *Shewanella* to fish intestinal mucus in vitro and inhibited the adhesion and growth of *Shewanella* in the intestines of large yellow croaker, delaying spoilage. The results of this study suggest that the usage of *Bacillus subtilis* in fish might become a new way of preservation.

Acknowledgements

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