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# Field trial of beneficial effects of carotenoid-producing *Bacillus aquimaris* SH6 spores to whiteleg shrimps

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***Abstract:***

**This field study was performed to demonstrate that *Bacillus aquimaris* SH6 spores administered as a feed supplement in adequate amounts confer beneficial effects to the whiteleg shrimp. Shrimps were administered either *B. aquimaris* SH6 spores at $1\times10^6$ CFU $g^{-1}$ pellet (SH6 group) or a mixture of SH6 and *B. subtilis* SH23 spores at $5\times10^5$ CFU $g^{-1}$ pellet for each strain (SH6-SH23 group). After a 28-day feeding period, the number of SH6 spores in the gut of whiteleg shrimp in the SH6 group and the SH6-SH23 group were $5.7\times10^3$ CFU $gut^{-1}$ and $7.7\times10^3$ CFU $gut^{-1}$, respectively. The total bacterial population of these two experimental groups increased about 10-75% compared to that of the control group. The astaxanthin level and red colour score were the highest in the SH6-SH23 group (1.66 µg.$g^{-1}$ shrimp; 28-29), followed by the SH6 spore group (1.42 µg.$g^{-1}$ shrimp; 27-28), and were the lowest in the control group (0.61 µg.$g^{-1}$ shrimp; 26-27). Nevertheless, the growth rate of shrimps was similar (7.8-8%) among the three groups. In conclusion, feed supplements containing SH6 and SH23 spores co-effectively improved live counts and diversity of microbiota in shrimp guts, as well as improved astaxanthin level and red colour of whiteleg shrimps.**

***Keywords:*** **astaxanthin, *Bacillus aquimaris*, carotenoid, gut, spore.**

***Classification number:*** **3.4**

## Introduction

Probiotics play a fundamental role in health enhancement of the host. They have the ability to produce enzymes for food digestion and serve as a bio-competitor for inhibition of the growth of pathogenic microorganisms [1], increase in the immune response [2], improvement in water quality [3], and, subsequently, enhancement of the quantity and quality of shrimp aquaculture. Therefore, probiotics are considered the best choice for sustainable shrimp aquaculture development because of their safety characteristics [4].

In recent years, whiteleg shrimp (*Litopanaeus vannamei*) have become among the most valuable aqua-products based on their high nutrient and commercial value. This stems from their ability to provide a high level of astaxanthin [5], which is evident visually by their red body colour. Astaxanthin, a carotenoid pigment, is a natural antioxidant and is said to provide many human health benefits. However, fish, shrimps, and other crustaceans cannot produce astaxanthin themselves. Therefore, resources for carotenoids in general and for astaxanthin in particular attract great interest in the studies of environment and feed supplements. Natural algae in the shrimp's native habitat may be a source of astaxanthin. Regarding feed supplements, there are many reports on the screening non-pigmented effect of probiotics on shrimp aquaculture in both laboratory and field trials, mostly in *Bacillus* sp., such as *B. subtilis, B. lichenifromis, B. coagulans* [6], and *Lactobacillus* sp., such as *L. acidophilus, L. plantarum* [4, 7]… However, the research on effects of pigmented *Bacillus* strains producing carotenoids for shrimps remains limited. Recently, we have published a paper reporting beneficial effects of *B. aquimaris* SH6

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spores on whiteleg shrimps at laboratory scale, including red colour, astaxanthin level, and growth rate [8]. However, we have not yet performed field trials to confirm these beneficial effects, and we have not yet determined whether SH6 spores can be combined with any other *Bacillus* probiotic strains for use as feed supplements for shrimps. Thus, in this study, we performed a trial in ponds of whiteleg shrimps at Quang Ninh province for feeds supplemented with either *B. aquimaris* SH6 spores or a mixture of the SH6 spores with non-pigmented *B. subtilis* SH23, in comparison to the conventional feed used. In addition, we also analysed the persistence of SH6 spores and their effect on microbiota in shrimp guts to explain a possible mechanism behind the effects.

## Materials and methods

### *Preparation of spores*

Orange-pigmented *B. aquimaris* SH6 (Accession No. KF443807) [8] and non-pigmented *B. subtilis* SH23 (Accession No. KP735610; a strain of shrimp intestinal origin belonging to the GreenBio S1 product of ANABIO R&D JSC) are strains isolated from the whiteleg shrimp gut (*L. vannamei*). These strains have been isolated and identified based on the previous research project KLEPT.12.03 funded by the Ministry of Science and Technology for Key Laboratory of Enzyme and Protein technology. The two strains were used to produce spores in Difco Sporulation medium (DSM, Oxoid, England), following the methods described by Nicholson, et al. (1990) for the SH23 strain [9] and Ngo, et al. (2016) for the SH6 strain [8]. The spore shapes of SH6 and SH23 in the cultures were observed under a conventional microscope and shown in Fig. 1A. The sprayed powder contained condensed spores at concentrations of about $2\times10^{10}$ CFU $g^{-1}$ pellet for SH6 and $1\times10^{10}$ CFU $g^{-1}$ pellet for each strain (ratio 1:1) in the mixture (Fig. 1B).

### *Preparation of the feed pellets*

Commercial feed pellets (Grobest Industrial Vietnam) were assigned as the 'control' feed. The commercial feed was further coated with either condensed SH6 spores

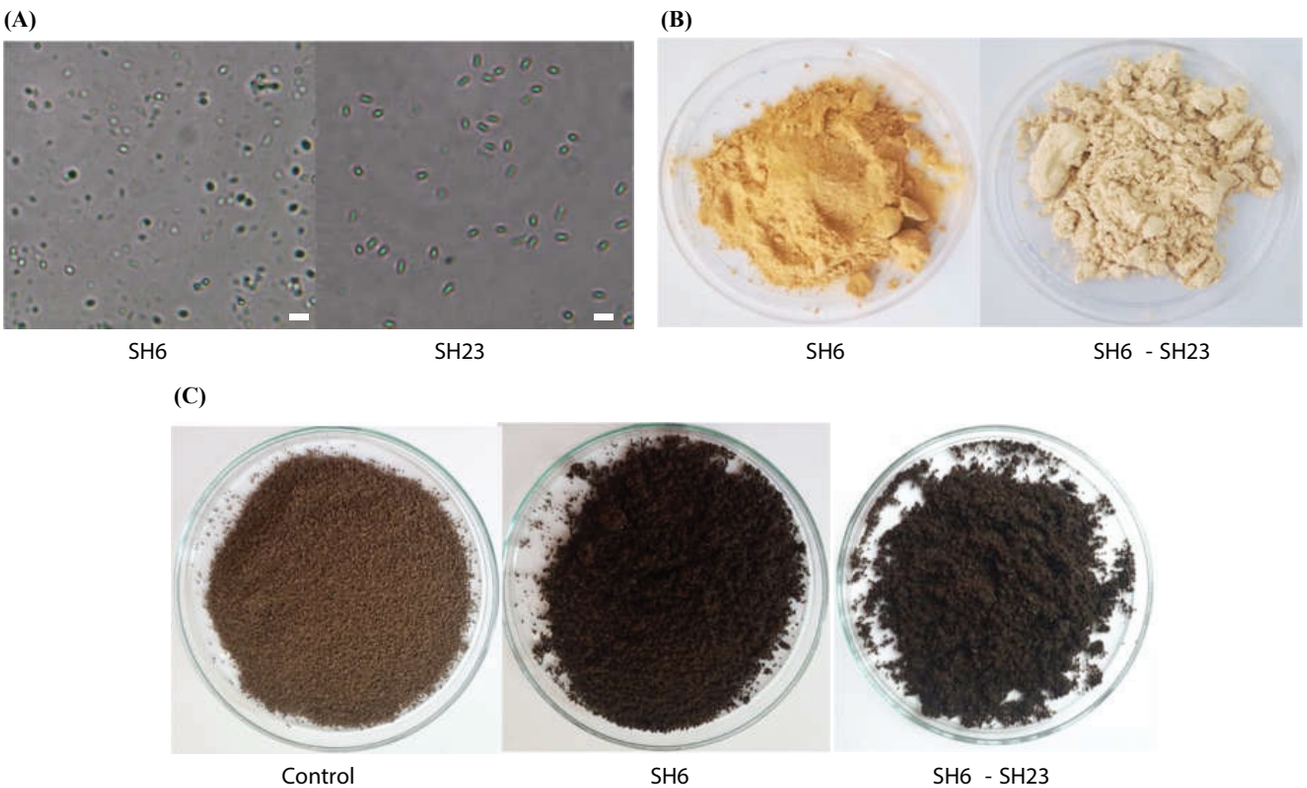


**Fig. 1. Spore shapes and spore powders of *B. aquimaris* SH6 and *B. subtilis* SH23; non-coated and spore-coated feed pellets.** Panel (**A**) Images of spore shapes of SH6 and SH23 under microscope. Scale bars are 2 µm. Panel (**B**) The condense spores of SH6 at $2\times10^{10}$ CFU $g^{-1}$ pellet and mixture of SH6 and SH23 spores at ratio 1:1 of $1\times10^{10}$ CFU $g^{-1}$ pellet for each strain. Panel (**C**) Control non-coated feed and feeds coated with SH6 spores at final concentration of $1\times10^{6}$ CFU $g^{-1}$ pellet or mixture of SH6 and SH23 spores $5\times10^{5}$ CFU $g^{-1}$ pellet.

to have the final concentration of $1\times10^6$ CFU $g^{-1}$ pellet or a mixture of condensed SH6 and SH23 spores at ratio 1:1 to have the final concentration of each strain of $5\times10^5$ CFU $g^{-1}$ pellet. These control and two supplemented feeds, as presented in Fig. 1C, were then coded randomly for blind field trial in ponds culturing whiteleg shrimps.

***Experimental design for ponds, shrimps and feed supplements***

Three ponds ($n$=3) with surface areas of about 0.8 ha were selected at Hai Dong village, Mong Cai city, Quang Ninh province, Vietnam. Twenty-five-day-old whiteleg shrimps (*L. vannamei*) which weighed about 2.05±0.05 grams, were divided into three ponds and fed with different feed types: control group (control feed), SH6 group (*B. aquimaris* SH6 spores $1\times10^6$ CFU $g^{-1}$ pellet), and SH6-SH23 group (each *B. aquimaris* SH6 and *B. subtilis* SH23 spores at $5\times10^5$ CFU $g^{-1}$ pellet). Water in the ponds was changed every week and monitored regularly. Shrimps were maintained under the following conditions: temperature 26-28°C, pH = 7.5-8.5, DO ≥ 4 mg $l^{-1}$, 12 ppt salinity. These ponds were cultured at a density of (80 shrimps/$m^2$). Shrimps were fed three times per day continuously for 28 days. After 28 days of the experiment, live counts of SH6 spores and major bacteria in shrimp gut, astaxanthin concentration in shrimp muscle, and growth rate of shrimps were evaluated.

***Counts of SH6 and total bacteria in shrimp gut***

On the 28th day, three shrimps in each group were sacrificed and their guts were collected for live counts of SH6 and total bacteria on MRS agar (for *Lactobacili* bacteria) and LB agar (for other bacteria). Each shrimp gut was prepared in 0.9% NaCl and then vigorously suspended by vortex until a homogenous suspension was obtained. Serial dilutions with 0.9% NaCl were made before plating on LB or MRS agar for live counts of total bacteria. Colony-forming units (CFU) were determined after a 24-hour incubation period for LB agar and after 48 hours for MRS agar plates. The single colony was collected based on their morphology and their species was identified using the 16S rRNA sequencing method. In brief, the extracted genomic DNA from each colony was used as template for PCR and partial 16S rRNA sequencing of a PCR-amplified 1,500 bp fragment using primers 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1527R: 5′-AAAGGAGGTGATCCAGCC-3′, following the method described by Ngo, et al. (2016) [8]. Three colonies with the same morphology and colour were used to repeat the species identification three times. Then, the number of colonies with the same morphology and colour grown on the plates were taken from each group we counted.

***Measurement of body colours and astaxanthin levels in shrimps***

For comparing body colour, eight shrimps ($n$=8) in each group were taken after 28 days, boiled to observe red colour level using the Roche index, SalmoFan™ standard colour [8]. For measuring astaxanthin concentration, eight shrimps ($n$=8) in each group were also taken at day 28. The astaxanthin level was extracted and identified spectrophotometrically at $A_{480}$, following the method described by Ngo, et al. and Khaneja, et al. [8, 10].

***Weight gain measurement for shrimps***

For determination of weight gain in shrimps, 100 shrimps at day 28 were taken from five positions of shrimp pond for each group in order to measure the average weight and compare it to that of day 0 following the equation: GR (growth rate) = $\frac{final\ weight - initial\ weight}{days\ of\ feeding}$ x 100%. The survival rate (%) of shrimps was calculated by the following formula: SR = 100 × ($n_t/n_o$), where SR is the survival rate, $n_t$ is the number of shrimps at the time t, and $n_o$ is the number of shrimps at the initial.

***Statistical analysis***

Data of astaxanthin concentration, live counts, and growth rate among three treatment groups were compared using a Student's t-test, with the accepted significance level at $p < 0.01, 0.05, 0.001$. Statistical analyses were performed using the Analysis ToolPak in MICR Microsoft Excel Software (Microsoft, Redmond, WA). Experimental data were expressed as mean ± SD.

## Results and discussion

***Persistence of B. aquimaris SH6 spores in the shrimp gut and the cooperative effect of SH6 and SH23 to live counts and diversity of shrimp gut microbiota***

For 28 days, three groups of shrimps were fed with pellets coated with or without either SH6 spores ($1\times10^6$ CFU $g^{-1}$ pellet) and a mixture of SH6 and SH23 spores ($5\times10^5$ CFU $g^{-1}$ pellet for each strain). The counts of *B. aquimaris* SH6 and other bacterial strains recovered in the shrimp intestines were determined by plating samples of excised GI-tracts onto selective agar (see Methods). As shown in Fig. 2A, our data revealed that SH6 colonies were present in the SH6 and SH6-SH23 groups; meanwhile, the SH6 colonies were not detectable in the control group. Surprisingly, the number of SH6 colonies in the SH6-SH23 groups ($7.7\times10^3$ CFU $gut^{-1}$) was even higher than that in the SH6 group ($5.7\times10^3$ CFU $gut^{-1}$) although the input dose of SH6 spores in the SH6-SH23 group was just half of that in the SH6 group. Our data in the field trial once again confirm that SH6 spores can colonize well into the intestinal epithelium of whiteleg shrimps and provide new information that feeding SH6 together with SH23 allows more effective residence of SH6 spores in the shrimp gut.

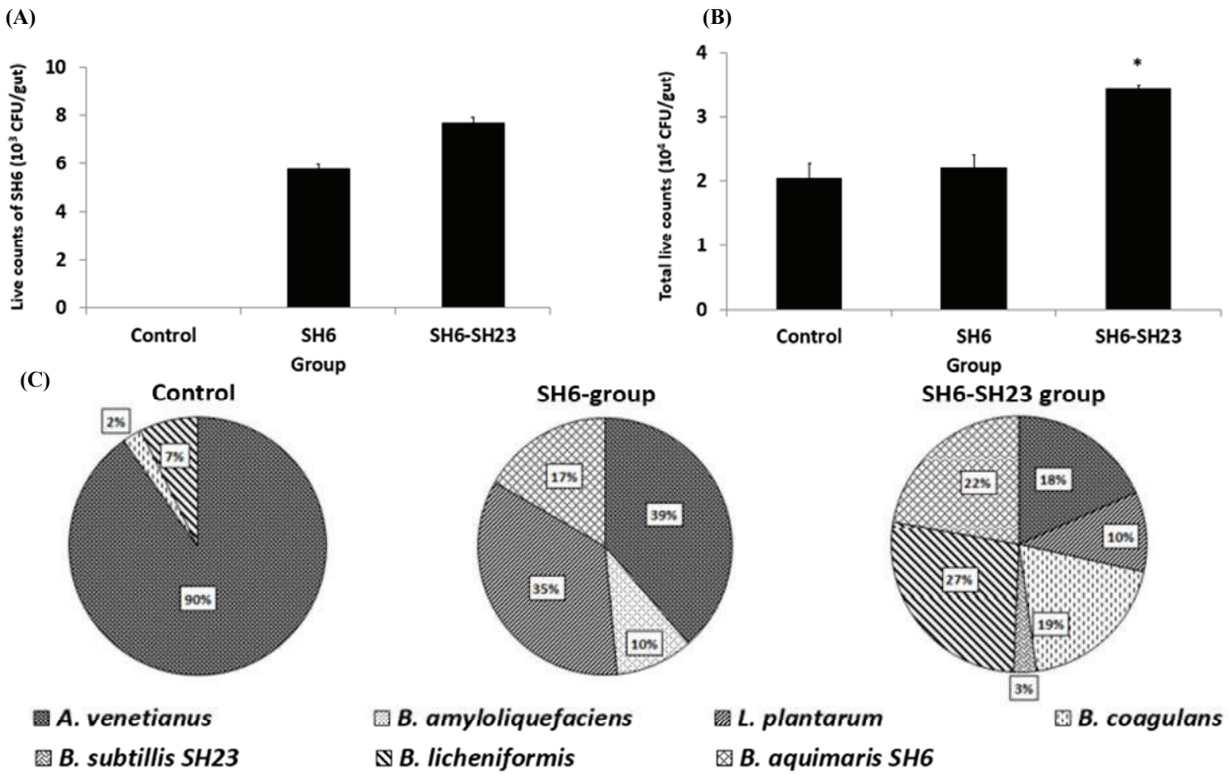


**Fig. 2. Live counts of *B. aquimaris* SH6 and total bacteria in shrimp gut during the 28-day feeding.** Experiment groups include SH6 spores ($1x10^6$ CFU $g^{-1}$ pellet), SH6-SH23 spores ($5x10^5$ CFU $g^{-1}$ pellet for each) and control (without supplements). Panel (**A**) Live counts of SH6 in shrimp gut. Panel (**B**) Live counts of total bacteria in shrimp gut. P values were generated by ANOVA using the Student's t-test for multiple comparisons to the control (*$p<0.05$). Panel (**C**) Pie chart of useful bacterial population in shrimp gut.

Regarding the bacterial microbiota of shrimp gut among three groups, we found significant differences in both live counts and diversity of the morphology of colonies. In detail, the live counts in the control, SH6, and SH6-SH23 groups were $2.0\times10^4$, $2.2\times10^4$, and $3.5\times10^4$ CFU $gut^{-1}$ in 28$^{th}$ day, respectively (Fig. 2B). However, no significant difference was noted between the SH6 group and the control group, whereas the 75% increase in live counts in the SH6-SH23 group compared to that in the control group ($p<0.05$). Our data also revealed that the SH6 and SH6-SH23 groups exhibited more diversified useful bacterial population than the control group. As shown in Fig. 2C, there were six major useful species, including the *B. aquimaris* SH6 (22% population), and five other strains which closely related (>98% identity of 16S rRNA sequence) to the following species *B. licheniformis* (27% population), *B. coagulans* (19% population), *A. venetianus* (18% population), *L. plantarum* (10% population), and *B. subtilis* (3% population) in the shrimp gut of the SH6-SH23 group. These species have been either commonly used as probiotic bacteria or are naturally found in the intestines of many other aquatic species [11, 12]. For example, *B. subtillis, B. coagulans,* and *B. licheniformis* have been reported to play roles in increasing the survival and net production of channel catfish and shrimps [13], increasing the survival of shrimps [14, 15], as well as decreasing luminous *Vibrio* densities [16]. *L. plantarum* is effective on the growth rate and microflora in the shrimp gut [17] while *A. venetianus* is naturally found useful in shrimp GI-tracts [11, 12]. In the group fed with SH6 spores only, we found only four major bacterial species, including *A. venetianus, L. plantarum, B. aquimaris* SH6, and *B. subtilis,* with respective population of 39, 35, 17, and 10% in the shrimp gut. When shrimps were fed with the control feed pellets, only three major species were found, including the naturally found species *A. venetianus* with 90% population, *B. licheniformis* with 7% population, and *B. coagulans* with 3% population. In conclusion, our data indicate that SH6 and SH23 strains cooperatively improve the live counts and diversity of bacterial microbiota in the whiteleg shrimp gut.

***Colour and astaxanthin concentration in L. vannamei after 28 days of feeding***

After the 28-day feeding, the body colour of shrimps in the different groups was measured, and the image of boiled shrimps is shown in Fig. 3A. Shrimps in the SH6-

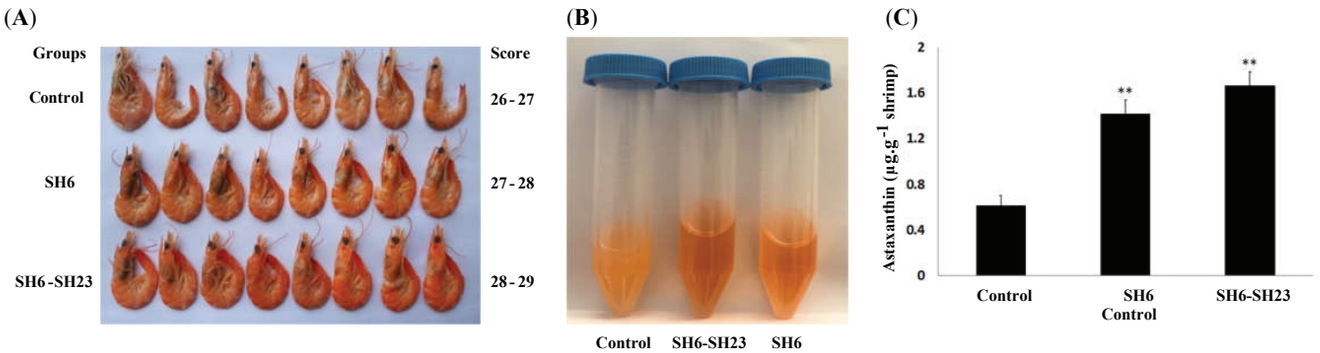


**Fig. 3. Colour and astaxanthin level of shrimp after the 28-day feeding with and without SH6 spores.** Experiment groups include SH6 ($1x10^6$ CFU $g^{-1}$ pellet), SH6-SH23 ($5x10^5$ CFU $g^{-1}$ pellet for each strain), and control (without supplements). Panel (**A**) Image of boiled shrimps and their colour variable scores indicating the levels of red pigmentation ($n$=8). Panel (**B**) Astaxanthin concentrations in extracts from shrimp muscle. P value was generated using Student's t-test for comparisons with the control (**p<0.001). Panel (**C**) Image of astaxanthin extraction from shrimp muscle. Data are presented as arithmetic means and error bars are standard deviations.

SH23 group had the highest red scores of 28-29, followed by the SH6 group (red scores of 27-28), and the control group (red scores of 26-27). It has been reported that carotenoid-supplemented feed enhances the red or orange colour of shrimp when they are cooked [10, 18]. Moreover, carotenoids may provide enhanced immune function and disease resistance in intracellular oxygen supply for shrimp, allowing survival under the hypoxic conditions in the pond bottom [19]. Furthermore, carotenoid-producing bacteria have potential as a feed supplement for shrimp because of their nature-friendliness. In order to correlate the relationship between the shrimp colour and the presence of astaxanthin in shrimp tissue, we determined the astaxanthin levels in shrimp of each group and present the data in Fig. 3B and 3C. It is clear that astaxanthin level in the SH6-SH23 and SH6 groups were 2.72-fold and 2.3-fold higher, respectively, than in the control group ($p$<0.001). Fig. 3C also confirms the significant different level of redness in extracts from shrimp muscle between the two experimental groups and the control group.

Our data suggest that SH6 combined with SH23 might have a stronger effect in producing a higher astaxanthin level in shrimp muscle, subsequently inducing a redder colour of shrimps. These data are also consistent with better colonization of SH6 spores and improvement of microbiota in the shrimp gut found in the SH6-SH23 group, in comparison to those found in the SH6 group. Taken together, our data in the field trial once again confirm that carotenoids produced by *B. aquimaris* SH6 were successfully converted to astaxanthin by whiteleg shrimps. These data also provide new information that the SH23 strain may play an effective role in improving the SH6 counts in the gut, thereby enhancing carotenoid production of SH6 and resulting in a higher astaxanthin level and redder colour in shrimps.

### *Growth performance*

We then chose to further analyse the effects of the *B. aquimaris* SH6 and *B. subtilis* SH23 supplement on whiteleg shrimp growth performance. As shown in Fig. 4, the growth rate (%) of all three groups demonstrated positive values: 8.0±0.6%, 7.7±1.8%, and 7.8±1.1% in the control, SH6, and SH6-SH23 groups, respectively. After the 28 days of experimental feeding, there was no statistically significant difference in growth rate between shrimps in the SH6 and SH6-SH23 groups in comparison to the control group. These data from the field trial are different from what we have observed at laboratory scale, where SH6 has demonstrated significant effect on the growth performance of shrimps [8]. We do not have a reasonable explanation for this field trial data but suppose that the size of shrimp samples we collected

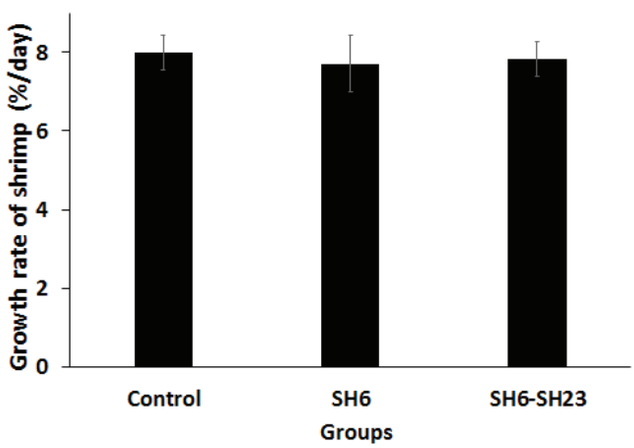


**Fig. 4. The growth rate of shrimp after 4-week feeding with SH6 spores.** Experiment groups include SH6 (fed with spores at $1x10^6$ CFU $g^{-1}$ pellet), SH6-SH23 (fed with SH6 spores at $5x10^5$ CFU $g^{-1}$ pellet and fed with SH23 spores at $5x10^5$ CFU $g^{-1}$ pellet), and control (without supplements). Data are presented as arithmetic means and error bars are standard deviations.

might be not representative for all shrimps in ponds. In the future, we need to collect more shrimps at various positions in the shrimp pond for weighing, perform field studies with a larger number of ponds, and include the control group of only *B. subtilis* SH23 in order to have better statistical data concerning growth rate.

## Conclusions

This field study demonstrated that when *B. aquimaris* SH6 spores were administered as a single-strain feed supplement at a concentration of $1\times10^6$ CFU $g^{-1}$ pellet or as a double-strain probiotic together with *B. subtilis* SH23 at a concentration of $5\times10^5$ CFU $g^{-1}$ pellet, the following benefits were conferred to the whiteleg shrimps: 10-75% increased live counts and more diversity of bacterial population in the intestine, as well as significantly increased astaxanthin levels (2.3-2.7-fold) in the muscle of shrimps and higher red colour scores (27-29 vs. 26-27). Although more field studies should be performed in the future, the SH6 and SH23 spores exhibit to be potential co-effective strains for further development of spore-based mixture probiotics to improve astaxanthin level and red colour in whiteleg shrimps.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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