Research Article

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The fate of probiotic species applied in intensive grow-out ponds in rearing water and intestinal tracts of white shrimp, *Litopenaeus vannamei*

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Abstract

Introduction – Probiotics have been commonly practiced in commercial shrimp farms to increase pond production. However, these possibilities were based on the results of *in vitro* studies or laboratory *in vivo* trials. While studies on probiotic applications in commercial-scale farms are still rarely investigated, this study addresses the fate of probiotic species in ponds and the intestinal tract of white shrimps reared in an intensive aquaculture system.

Material and methods – Four commercial probiotic species (*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Bacillus subtilis*, and *Pseudomonas putida*) were applied to the commercial shrimp ponds (@800 m^2 area of high-density polyethene ponds) in the morning at a dose of 5 ppm once every 2 days in the first month, and once a week from second month onward. Then, the presence of the probiotic species was traced by collecting the rearing water and shrimp's intestines on day 47 of culture to monitor their composition and abundance using high-throughput sequencing.

Results – None of the commercial probiotic species could be detected from both rearing water and shrimp intestinal tracts. These results suggest that the probiotic species had low viability and adaptability in the rearing pond as well as the shrimp intestines when applied on commercial-scale farms. These facts may explain the high variation in the yield among shrimp ponds in spite of having similar treatments.

Conclusion – Probiotic strains had low viability and adaptability in commercial farms. Thus, methods and strategies in probiotic application to commercial-scale shrimp farms should be evaluated and further developed to increase probiotic efficacy.

Keywords: food production, GI tract, microbiome, NGS, probiotics, water

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1 Introduction

Probiotics have been considered an eco-friendly approach to increasing the yield of aquaculture production through several mechanisms including maintaining water quality, growth performance, or survival rate of aquatic organisms [1]. For example, studies have confirmed that probiotic application has enabled us to significantly reduce antibiotic use in aquaculture industries and avoid the occurrence of antibiotic resistance genes of microbes [2]. Some probiotics have been documented to produce digestive enzymes such as protease, amylase, lipase, alginate lyase, and cellulase which help animal hosts to digest ingested diets [3]. Probiotic strains were documented to produce antimicrobial compounds active against bacterial pathogens [4]. Also, some probiotic species have the capacity to degrade and prevent the accumulation of aquaculture

waste in culture ponds including solid organic waste or soluble toxic chemicals such as ammonia (NH_3) or nitrite (NO_2) [5–7].

Despite the benefits of the usage of probiotics in aquaculture, most of these studies were based on *in vivo* studies or *in vivo* laboratory trials and very small-scale rearing systems where environmental conditions were easily controlled. Some studies confirmed that the results of *in vitro* and *in vivo* studies are frequently uncorrelated. In a review by Toledo et al. [8], it was stated that many studies had inconsistent results concerning the efficacy of probiotic treatments on shrimp survival and growth performance by *in vitro* and *in vivo* studies. These inconsistent results were due to the fact that environmental conditions in commercial shrimp ponds could vary, fluctuate, and may be very difficult to control. According to a study by Huerta-Rábago et al. [9], it was reported that commercial probiotics consisting of *Bacillus* spp., *Lactobacillus* spp., and *Saccharomyces* spp. introduced to a commercial shrimp farm could not be detected in rearing water due to competition with native microflora in the rearing water, and different environmental conditions. The probiotics addition also had a significant effect on the specific growth rate or survival rate of white shrimp. Salinity for instance in marine aquaculture is very critical for the survival of some bacteria which were isolated from the terrestrial organism [10]. Do these various limitations pose a question of whether probiotic strains can survive and significantly contribute to the quality of rearing water, digestibility, or disease resistance as being reported by many *in vitro* or laboratory-scale studies?

To address this question, there is a need for a study that will trace the composition and abundance of commercial probiotic species applied in commercial shrimp farms (pond and the intestinal tract of white shrimps) in an intensive aquaculture system using a high-throughput sequencing.

2 Materials and methods

2.1 Sampling location, culture system, and probiotic application

A commercial shrimp farm located at the ordinate point, 113°01′14.7″ E and 6°52′59.3″ L were selected for the present study. White shrimp, *Litopenaeus vannamei*, was cultivated through an intensive system (275 indiv/m^2 and applied with commercial probiotics) in high-density polyethylene ponds (@800 m^2 area and a water depth of 120 cm). The pond consisted of three plots with an area of 800 m^2 and a stocking population of 220,000 individuals. The commercial probiotics were *Lactobacillus plantarum, L. fermentum, Bacillus subtilis*, and *Pseudomonas putida*. The probiotic consortia was applied in the morning at a dose of 5 ppm once every 2 days. Siphoning of solid wastes (feces and uneaten feed) was carried out 1 day before the stocking of the fry and during the rearing period for as much as 2–5 days to adjust the age of the shrimp. Feeding of shrimps was done manually 1–5 times a day according to the shrimp sizes.

2.2 Collection of water and shrimps' intestinal samples

Water sampling was carried out according to the protocol previously described by Gomes et al. [11] with a slight modification. Water samples were collected from six ponds using a long pole sampling device and a 20 mL sterile plastic cup. The collected water was stored in a 50 mL falcon tube which was previously filled with 30 mL of absolute ethanol for DNA preservation. Samples were kept on ice until processed in the laboratory within the next 8 h. The shrimp intestine was sampled as previously described by Amin et al. [12]. A total of 30 healthy shrimps showing no symptoms of the disease were collected from 3 shrimp ponds (10 shrimps per pond) on day 47. The length and weight of shrimps were measured individually using a ruler and balance. Thereafter, each shrimp was washed with sterile distilled water, followed by 76% alcohol and rewashed with sterile distilled water to remove exogenous bacterial contamination. Then, the intestinal tract of each shrimp was dissected aseptically and placed into a sterile 1.5 mL microcentrifuge tube containing RNAlater™ (R0901, SIGMA) and stored at −20°C until DNA extraction.

Ethics approval: The conducted research is not related to either human or animal use.

2.3 Extraction and amplification of bacterial DNA

DNA from pond water and the intestinal tract of white shrimp was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In brief, falcon tubes were decontaminated with

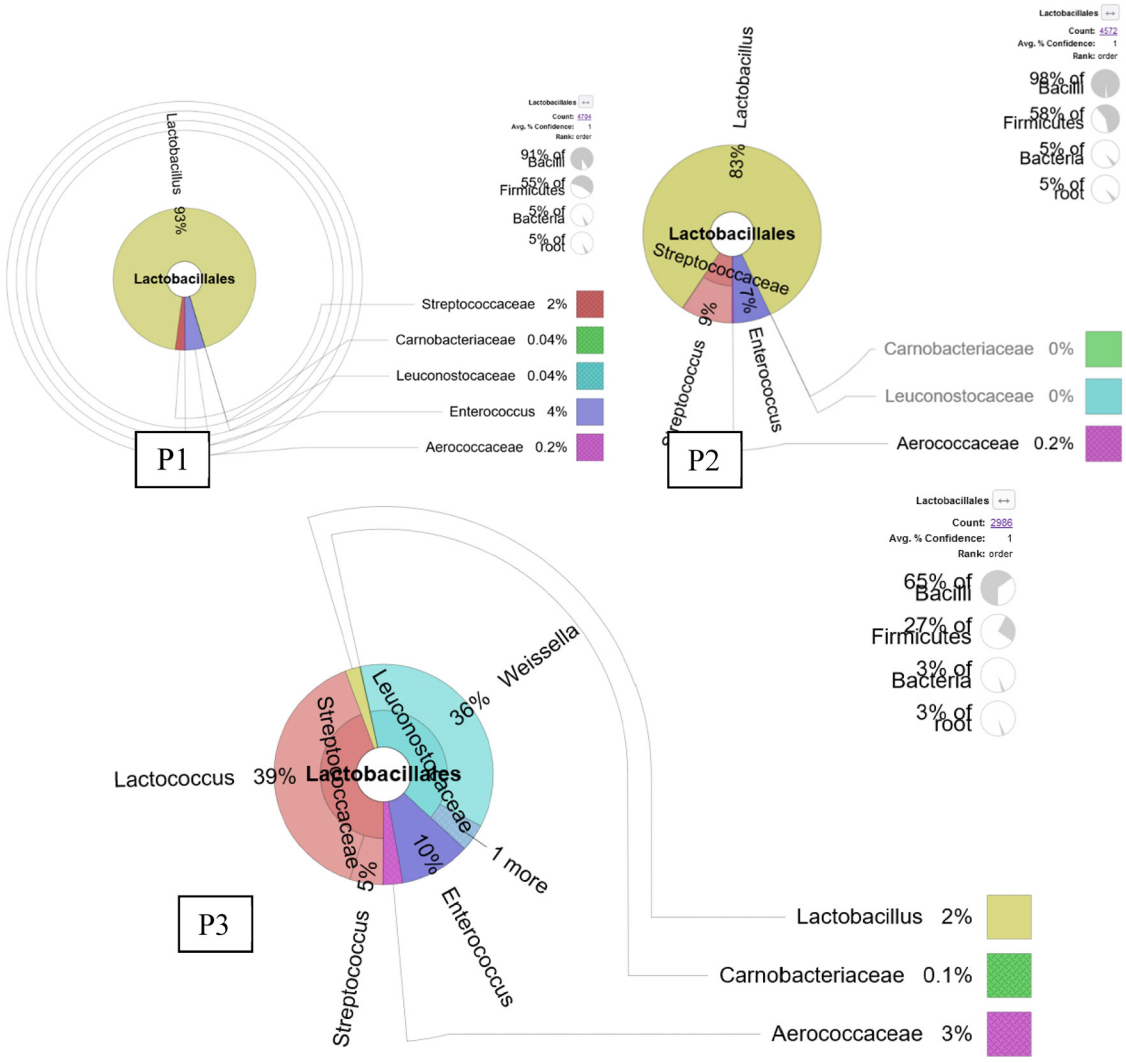

Figure 1: KRONA visually displays the analysis result of taxonomic annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of the sector means a respective proportion of different OTU annotation results. The abundance of *Lactobacillus* spp. Was detected from the rearing water of commercial shrimp ponds on the day of culture (DOC) 47. P1 is pond 1, P2 is pond 2, and P3 is pond 3.

76% ethanol and washed with sterile distilled water. Thereafter, all the falcon tubes were centrifuged (3,220 × *g*, 10 min, 6°C) for DNA precipitation and the supernatant was discarded [11]. The precipitated pellet was mixed with a buffer contained in the PowerBead Tube (Qiagen, Hilden, Germany). Other steps were carried out based on the manufacturer's protocol [13]. The DNA amplification process was carried out through several stages, namely, denaturation, annealing, and extension which are carried out in as many cycles as needed using a thermocycler. The stages started with predenaturation at a temperature of 94°C for 5 min, then the sample was heated in the annealing stage at 56°C for 1 min, and continued heating at the extension stage at 72°C for 1 min. For the extension stage, the sample was heated at 72°C for 7 min for 40 repetitions [14].

2.4 Sequencing and bioinformatic analysis

DNA samples with a concentration of 50 ng/μL were sent to Novogene Biological Information Technology Co. (Singapore) for sequencing and community analysis of microbiota in the digestive tract of white shrimps using Next Generation Sequencing (NGS, Illumina platform) based on 16S rRNA gene. Prior to sequencing, the hypervariable V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction with primer pairs 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) [14]. The results of the bacterial sequences that have been obtained were then analyzed using the UPARSE software. Sequences with a similarity of 97% were designated as the same operational taxonomic unit

(OTU). The taxonomic classification of each OTU representative sequence was carried out using the MOTHUR program through the SILVA database with a confidence level of 95%.

3 Results

3.1 Profiles of probiotic species in grow-out ponds

The results showed that the number of bacteria classified as Ordo *Lactobacillales* was quite abundance in the three ponds (Figure 1). A total of 4,704 bacterial sequences or 5% of total bacteria detected in pond 1 were assigned to Ordo *Lactobacillales*, of which 4,375 sequences (93%) were identified as genus *Lactobacillus* and belonged to 12 bacterial species Table 1. From pond 2, a total of 4,572 bacterial sequences (5% of the total identified bacteria in pond 2) were assigned to Ordo Lactobacillales, of which 3,795 sequences (83%) were classified as genus Lactobacillus and belonged to 12 bacterial species, Table 1. Both pond 1 and pond 2 appeared to be very similar in terms of *Lactobacillales* proportions (5%) and the number of *Lactobacillus* species (12 species). Five most dominant species in both ponds 1 and 2 were *Lactobacillus aviarius* followed by *Lactobacillus* sp. (OUT_39), *Lactobacillus* sp. (OUT_97), *Lactobacillus* sp. (OUT_157), and *Lactobacillus salivarius* (OUT_165). The only difference between the 2 ponds was that *Lactobacillus iners* (OTU_228) in pond 2 was more abundant than in pond 1, 64 and 4 for ponds 2 and 1, respectively. While the other 11 species were higher in pond 1.

Table 1: Bacterial species identified from rearing water of commercial shrimp ponds on the day of culture (DOC) 47

Consensus lineage	Sequence numbers			OTU ID
	P1	P2	P3	
1. *Lactobacillus aviarius*	1,938	1,584	—	OTU_19
1. *Lactobacillus* sp.	1,116	914	2	OTU_39
1. *Lactobacillus* sp.	687	591	—	OTU_97
1. *Lactobacillus* sp.	386	456	—	OTU_157
1. *Lactobacillus salivarius*	112	95	60	OTU_165
1. *Lactobacillus* sp.	91	86	—	OTU_226
1. *Lactobacillus iners*	4	64	—	OTU_288
1. *Lactobacillus ruminis*	6	4	3	OTU_363
1. *Lactobacillus saerimneri*	15	9	—	OTU_514
1. *Lactobacillus agilis*	13	4	—	OTU_535
1. *Lactobacillus mali*	6	4	—	OTU_548
1. *Lactobacillus coleohominis*	8	2	—	OTU_590

P1, P2, and P3 are shrimp pond 1, shrimp pond 2, and shrimp pond 3. OUT is operational taxonomic unit. "–" means not detected.

From pond 3, a total of 2,986 sequences or 3% of the total identified bacteria in pond 3 were assigned to Ordo Lactobacillales, of which 65 sequences (2% of Lactobacillales) were identified as genus Lactobacillus and belonged to three bacterial species which are *Lactobacillus* sp. (2 sequences), *L. salivarius* (60 sequences), and *L. ruminis* (3 sequences). However, none of the *Lactobacillus* species identified in the three ponds showed to be the introduced probiotic species which were *L. plantarum* and *L. fermentum*.

Member of genus *Bacillus* was not found in ponds 2 and 3, but was found only in pond 1, Figure 2. A total of 441 bacterial sequences or 0.4% of the total detected bacterial sequences were assigned to Ordo *Bacillales*. Of which 395 sequences or 99% were classified as *Bacillus* sp. (OTU_160).

Other NGS results showed that *Pseudomonas* spp. were detected only from two ponds with very low abundance (Figure 3). A total of 39 bacterial sequences or 0.04% of total bacterial sequences detected from rearing water of pond 1 were assigned to Ordo *Pseudomonadales*, but none of them belonged to *Pseudomonas* spp. While in pond 2, 35 bacterial sequences were assigned to Ordo *Pseudomonadales*, and only one sequence was identified as *Pseudomonas azotoformans*. The highest abundance sequences of Ordo *Pseudomonadales* were detected from pond 3 which were 6,325 bacterial sequences, of which 303 sequences belonged to the genus *Pseudomonas* and were assigned to 3 species which were *Pseudomonas psychrotolerans* (213 sequences), *Pseudomonas azotoformans* (81 sequences), and *Pseudomonas* sp. (9 sequences) (Table 2). These results indicated that *Pseudomonas putida* which came from commercial probiotics had difficulties adapting and proliferating in the rearing water of shrimp ponds. Based on NGS results, the most abundant species was *P. Psychrotolerans* (213 sequences) followed by *Pseudomonas azotoformans* (81 sequences) and *Pseudomonas* sp. with 9 sequences.

3.2 Profiles of probiotic strains in intestinal tracts

3.2.1 *Lactobacillus* in shrimp intestines

From the shrimp intestines collected in pond 1, a total of 172 bacterial sequences or 0.2% of the total identified

Figure 2: KRONA visually displays the analysis result of taxonomic annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of the sector means a respective proportion of different OTU annotation results. The abundance of *Bacillus* spp. Were detected from the rearing water of commercial shrimp ponds. P1, P2, and P3 are ponds 1, 2, and 3, respectively.

bacteria were assigned to Ordo *Lactobacillales*. Of these sequences, 90 sequences (52% of *Lactobacillales*) belonged to genus *Streptococcus*, 33 sequences (19% of *Lactobacillales*) belonged to genus *Enterococcus*, 17 sequences (10% of *Lactobacillales*) belonged to genus *Lactobacillus*, 9% (16 OTUs) belonged to genus *Weisella*, 5% (9 sequences) belonged to genus *Lactococcus*, and 4% (7 sequences) belonged to genus *Leuconostoc* (Figure 4). The 17 sequences of genus *Lactobacillus* were identified as 3 species which were *L. ruminis* (12 sequences), *L. aviaries* (4 sequences), and *Lactobacillus* sp. (1 sequence) (Table 3).

From the shrimp intestines collected in pond 2, a total of 1,669 bacterial sequences or 2% of the total identified bacteria were assigned to Ordo *Lactobacillales*. 1,569 sequences (94% of *Lactobacillales*) belonged to genus *Lactobacillus*, 84 sequences (5% of *Lactobacillales*) belonged to *Streptococcus*, 11 sequences (0.7% of *Lactobacillales*) belonged to *Enterococcus*, and one sequence belonged to *Weisella* (Figure 4). 1,569 *Lactobacillus* were identified as 12 species and the 3 top most abundant species were *Lactobacillus* sp. (469 sequences), followed by *L. pentosus* (339 sequences), and *L. reuteri* (287 sequences). While the lowest abundance species were *L. agilis* and *L. acidipiscis* with a single sequence each (Table 3).

Furthermore, a total of 1,265 bacterial sequences were assigned to Ordo *Lactobacillales* from the shrimp intestines collected from pond 3. Of these sequences, 945 sequences (75% of *Lactobacillales*) were identified as genus *Lactobacillus*. Lower taxonomic annotation

Figure 3: KRONA visually displays the analysis result of taxonomic annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of the sector means a respective proportion of different OTU annotation results. The abundance of *Pseudomonas* spp. was detected from the rearing water of commercial shrimp ponds. P1 is pond 1, P2 is pond 2, and P3 is pond 3.

Table 2: Bacterial species identified from rearing water of commercial shrimp ponds on DOC 47

Consensus Lineage	Sequence numbers			#OTU ID
	P1	P2	P3	
Pseudomonas psychrotolerans	—	—	213	OTU_145
Pseudomonas azotoformans	—	1	81	OTU_266
Pseudomonas sp.	—	—	9	OTU_600

P1, P2, and P3 are shrimp pond 1, shrimp pond 2, and shrimp pond 3. OTU is operational taxonomic unit. "–" means not detected.

indicated that the sequences were classified into 12 bacterial species. The 3 top most abundant species were *Lactobacillus* sp. (216 sequences), followed by *L. pentosus* (209 sequences) and *L. reuteri* (101 sequences) (Table 3).

3.2.2 *Bacillus* in shrimp intestines

From the shrimp intestines collected in pond 1, 48 sequences or 0.05% of the total identified bacteria were classified as Family Bacillaceae. Of the sequence, 18 sequences were identified as *Bacillus badius*, 24 sequences as *Bacillus* sp., and 6 sequences were identified as *B. thermoamylovorans* (Figure 5). While from the shrimp intestines collected in pond 2, 43 sequences or 0.05% of the total identified bacteria were assigned to Family Bacillaceae (Figure 5). Of these, 36 sequences (84% of Bacillaceae) belonged to genus *Oceanobacillus*. Six sequences (14% of Bacillaceae) belonged to genus Bacillus, and were identified as four species which were *B. thermoamylovorans* (2 sequences), *B. badius* (2 sequences), *Bacillus coagulans* (1 sequence), and *Bacillus* sp. (1 sequence) (Table 4).

In addition, from the shrimp intestines collected in pond 3, 12 bacterial sequences or 0.01% of the total identified

Figure 4: KRONA visually displays the analysis result of taxonomic annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of the sector means a respective proportion of different OTU annotation results. Number and proportion of *Lactobacillus* spp. in the GI tract of white shrimps are depicted in the figure. Each figure represents sampling locations (P1 is pond 1, P2 is pond 2, and P3 is pond 3). Each figure consists of ten pooled shrimp intestines.

bacterial sequences were assigned into Family Bacillaceae (Figure 5). Of these, 7 sequences (58%) were identified as *B. thermoamylovorans*, while the other 5 sequences (42% of Bacillaceae) were "*unclassified*."

3.2.3 *Pseudomonas* in shrimp intestines

Pseudomonas spp. also appeared to be in very low abundance in the intestinal tract of white shrimp reared in commercial ponds (Table 5 and Figure 6).

In pond 1, a total of 106 sequences or 0.1% of the total identified bacterial sequences were assigned into Ordo *Psedomonadales*, of which only 4 sequences (4% of *Pseudomonadales*) were identified as *Pseduomonas* sp. While in pond 2, 28 sequences or 0.03% of total bacterial sequences were assigned to Ordo *Pseudomonadales*. Seven sequences (25% of *Pseudomonadales*) belonged to genus *Pseudomonas*, 5 sequences of *P. geniculata*, and 2 sequences of *Pseudomonas* sp. Furthermore, 13 sequences were assigned to Ordo *Psedomonadales* but none belonged to *Pseudomonas* spp. in pond 3.

4 Discussion

The application of probiotics has been considered the most eco-friendly method to boost aquaculture production through several mechanisms including maintaining

Table 3: *Lactobacillus* identified from the gastrointestinal (GI) tracts of white shrimps reared in commercial shrimp ponds

Consensus lineage	P1	P2	P3	OTU ID
Lactobacillus aviarius	4	60	685	OTU_29
Lactobacillus sp.	—	97	—	OTU_36
Lactobacillus pentosus	—	339	—	OTU_51
Lactobacillus reuteri	—	287	132	OTU_87
Lactobacillus sp.	1	157	22	OTU_96
Lactobacillus futsaii	—	79	2	OTU_172
Lactobacillus salivarius	—	46	9	OTU_174
Lactobacillus sp.	—	—	84	OTU_187
Lactobacillus ruminis	12	20	18	OTU_216
Lactobacillus saerimneri	—	10	—	OTU_532
Lactobacillus agilis	—	4	1	OTU_534
Lactobacillus acidipiscis	—	5	—	OTU_610
Lactobacillus sp.	—	469	1	OTU_793

P1, P2, and P3 are shrimp pond 1, shrimp pond 2, and shrimp pond 3. OTU is operational taxonomic unit. "–" means not detected.

water quality, improving growth rate, and enhancing disease resistance [1]. However, positive results of probiotic applications are mostly based on *in vitro* studies or small-scale *in vivo* trials in which all environmental conditions are easily managed and controlled. Meanwhile, the application of probiotics on large scales such as on commercial shrimp farms is still less investigated. Thus, questions such as does introduced probiotics could cope or compete with native bacteria and contribute to the culture of organisms in commercial farms remained to be answered. The present study traced and identified four commercial probiotic species (*L. plantarum*, *L. fermentum*, *B. subtilis*, and *P. putida*) which were applied in three commercial shrimp ponds. *Lactobacillus* and *Bacillus* are among the most frequently used microorganisms as probiotics in both terrestrial and aquatic cultured species [8]. *Bacillus*-based probiotics generally improved specific

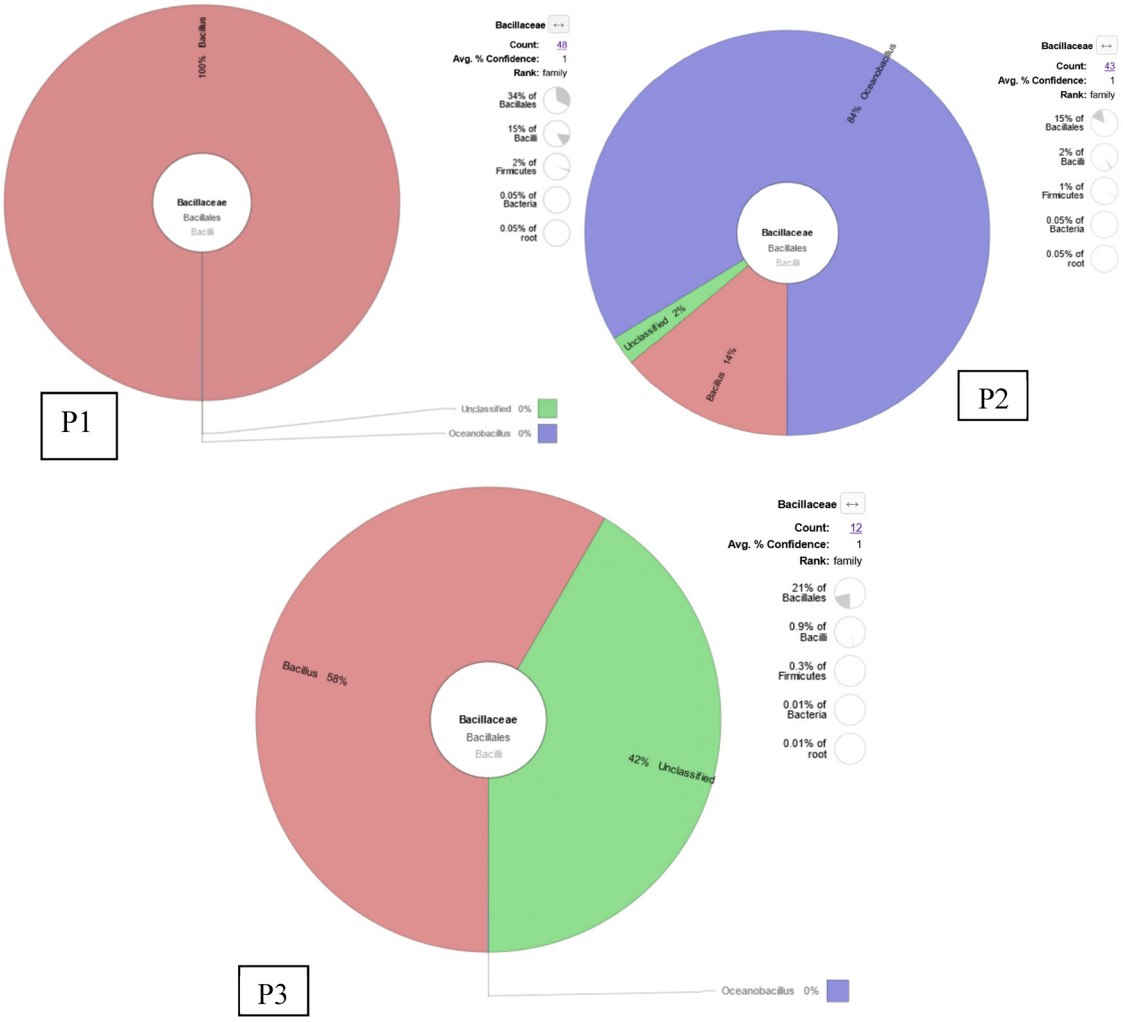

Figure 5: KRONA visually displays the analysis result of taxonomic annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of the sector means the respective proportion of different OTU annotation results. Each figure represents sampling locations (P1 is pond 1, P2 is pond 2, and P3 is pond 3). Each figure consists of ten pooled shrimp intestines.

Table 4: Bacterial species identified from the GI tracts of white shrimps reared in commercial shrimp ponds

Consensus lineage	P1	P2	P3	OTU ID
Bacillus badius	18	2	—	OTU_335
Bacillus sp.	24	1	—	OTU_358
Bacillus thermoamylovorans	6	2	1	OTU_365
Bacillus coagulans	—	1	2	OTU_473

P1, P2, and P3 are shrimp pond 1, shrimp pond 2, and shrimp pond 3. OTU is operational taxonomic unit. "–" means not detected.

Table 5: Genus *Pseudomonas* were identified from the GI tracts of white shrimps reared in commercial shrimp ponds

Consensus lineage	P1	P2	P3	OTU ID
Pseudomonas geniculata	—	5	—	OTU_623
Pseudomonas sp.	4	2	—	OTU_395

P1, P2, and P3 are shrimp pond 1, shrimp pond 2, and shrimp pond 3. OTU is operational taxonomic unit. "–" means not detected.

growth rate and feed conversion ratio through several mechanisms such as digestive enzyme secretion and production of many supplemental nutrients such as biotin, vitamin B12, fatty acids, essential amino acids, and other necessary growth factors [3]. Meanwhile, *Lactobacillus*-based probiotics have been reported to produce antimicrobial compounds to suppress bacterial pathogens [4].

The result of this study indicated that none of the four commercial probiotics was able to be detected in the shrimp ponds nor the intestinal tracts of white shrimps sampled on DOC 47. Each shrimp pond appeared to develop specific microbial communities in both rearing water and the shrimp intestines. Ponds 1 and 2, for instance, had 12 *Lactobacillus* species and the most dominant species was *L. aviarius*, but pond 3 had only two species of *Lactobacillus* and was dominated by *L. salivarius*. Similarly, the genus

Figure 6: KRONA visually displays the analysis result of taxonomic annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of the sector means the respective proportion of different OTU annotation results. The image depicts the number and proportion of *Pseudomonas* in the GI tract of white shrimps. Each figure represents sampling locations (P1 is pond 1, P2 is pond 2, and P3 is pond 3). Each figure consists of 10 pooled shrimp intestines.

Bacillus which developed in rearing water was different from commercial bacillus. In addition, bacillus was only detected in pond 1, and none was detected in ponds 2 and 3. While three *Pseudomonas* were detected in pond 3 (*P. psychrotolerans*, *P. azotoformans,* and *Pseudomonas* sp.), only one species in pond 2 (*P. azotoformans*) and none were detected in pond 1. A similar result was reported by Huerta-Rábago et al. [9], where three commercial probiotics (*Bacillus* sp., *Lactobacillus* sp., and *Saccharomyces* sp.) introduced into white shrimp ponds at nursery stages could not be detected on DOC 7, 21, and 42. These results may suggest that the introduced probiotics were unable to cope with their new environments and failed to proliferate and grow in the target sites (the intestinal tracts of white shrimps or rearing water). There were several possibilities as to why the commercial bacteria were unable to survive based on previous studies. First, the probiotic species were isolated from significantly different environmental conditions and therefore had difficulty in adapting to the environmental condition in the shrimp ponds or intestines of shrimps. A large loss of viability has been frequently attributed to the high acid and bile salt concentrations in the stomach and intestines [15]. Conditions of rearing water that are different from conditions in culture including dissolved oxygen, pH, salinity, temperature, and nutrient sources will affect the growth rate of probiotic bacteria and total cell yields [1]. Another possibility is that native bacteria out-compete the introduced probiotics for the same organic substrate such as carbon [1]. This result might explain the inconsistent results concerning the efficacy of probiotic treatments on the survival and growth performance of white shrimps [8].

Since the introduced probiotics were not viable in the target sites, a question to be answered is "are these commercial probiotics able to contribute to the aquaculture species? According to Chauhan and Singh [16], probiotic viability is a very important factor in aquaculture species and serves as one of the prerequisites in screening probiotics for aquaculture. Less viable probiotics may not contribute well because the commercial probiotics are not viable in the target sites; thus, they may not contribute to shrimp farms. This might be the reason why studies reported that the probiotic application does not have a significant effect on the production yields. A study by Huerta-Rábago et al. [9] reported that the addition of commercial probiotics did not affect the dominant bacteria in both phyla and genus levels in rearing ponds. Similarly, a study by Arias-Moscoso et al. [17] also reported that the addition of commercial organic and ammonia-oxidizing bacteria does not have any significant effect on water quality or waste degradation in shrimp farms cultured with biofloc technology. All these facts suggest that methods and strategies in applying probiotics in aquaculture species should still be carefully restudied in order to increase their efficacy.

Other authors explained that probiotics may modify the balance of microbial communities in the target sites [18,19]. A study by Torpee et al. [20], for instance, reported that the introduction of probiotics suppresses opportunistic and/or pathogenic bacteria in the intestinal tract of white shrimp. Similarly, Vargas-Albores et al. [21] documented that probiotic strains keep the microbial balance of beneficial bacteria by suppressing the growth of vibrio. Then, what is the effect of probiotics in the present study on the microbial composition in general? The results of the present study showed that probiotic supplementation appeared not to change the structure of microbial compositions in the GITs of shrimps, indicated by no significant different in the top threbacterial phyla in both probiotic-treatment and the controls, which were *Proteobacteria*, *Bacteroidetes* and *Planctomycetes*. At the genus level, *Rhodopirellula*, *Ketogulonicigenium*, *Ruegeria*, and *Sulfurimonas* were the most dominant phyla regardless of probiotic treatment. The bacterial diversity (phyla and genera) in probiotic treatments was also very similar to the control. microbial compositions in the rearing water of the shrimp ponds largely varied in both rearing water and the intestine of white shrimps even though all sampling ponds had similar treatments. The large variation in the microbial composition of water ponds as well as intestinal tracts of white shrimps has been previously reported in many studies [12,22]. The present study indicated that the application of probiotics in commercial outdoor farms where environmental conditions are difficult to control seems not effective yet. This hypothesis has been supported by many studies which reported no probiotic effect of the productions [9,17]. Thus, more studies and investigations are still required to apply probiotics on commercial outdoor farms.

Acknowledging these issues, the probiotic application in commercial outdoor shrimp farms should be evaluated. More studies are still required in order to develop more effective strategies, especially in the commercial outdoor system. Applying probiotics directly as practiced in the present study should be avoided. Some factors such as time and frequency of administration, probiotic species, administration (encapsulation) method [23], and the supplementation of prebiotics to support the nutrient requirements for probiotic species should be considered. In terms of introducing time, probiotics may exert a better effect when introduced during early life [24]. Previous studies also explained stable gut microflora in the early

life stages of white shrimp have not yet been established therefore a perfect time to introduce, stir and manipulate its microbial species. In addition, shrimp at larval and early post-larval stages have less developed immune systems which may exclude the introduced probiotics species [25]. Furthermore, probiotic species may also determine the viability of probiotics in outdoor commercial farms. Some studies revealed that several commercial probiotics were isolated from terrestrial which have very different environmental conditions such as salinity, nutrient availability, pH, or dissolved oxygen. These differences made such probiotic species difficult to adapt, grow, and proliferate in aquatic environments. Thus, it is highly recommended to isolate and develop native/indigenous probiotic strains from surrounding environments. The native/indigenous probiotics may more easily adapt and contribute to aquaculture production.

The concept of indigenous probiotics has been documented to be more effective in enhancing aquaculture productions and viability is the keyword behind the success. In addition, based on the present study results, *Lactobacillus* appeared to be good candidates in general both in rearing water and intestinal tracts since its availability seems better than both *Bacillus* and *Pseudomonas*. Our observation that probiotic treatment is less effective in earthen containers could be related to the difficulty of exerting control over variables (probiotic access, temperature, dose, farm hygiene, etc.). *Lactobacillu*s is also a member of lactic acid bacteria whose members have generally regarded as safe status for probiotics [26]. The other approach is synbiotic, which is the application of probiotics and prebiotics at the same time. Prebiotics is a nutrient which is required by probiotics to grow and proliferate in target sites [27]. Few studies have recently reported the application of synbiotics in white shrimps [28]. These approaches should be investigated more to increase the effectiveness and efficacy of probiotics in commercial outdoor farms of white shrimps.

5 Conclusion

Four commercial probiotic species applied in the commercial grow-out shrimp ponds could not be detected from the rearing water or intestinal tracts of the white shrimps. These facts might answer why commercial ponds applying probiotics had high yield variations. The characteristics of probiotic species and environmental conditions in commercial outdoor farms may explain these results. Thus, more studies on selecting proper probiotic strains having good tolerance in a wide range of environmental conditions or strategies on how probiotics are applied in commercial outdoor farms should be done in future in order to increase the probiotic efficacy in white shrimp production.

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