Supplementary Material

Functional and mechanistic studies of a phytogenic formulation, Shrimp Best, in growth performance and *Vibrio*sis in whiteleg shrimp

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A. SUPPLEMENTARY TABLES

Table S1. Growth performance of shrimp in the 4-week laboratory study.

	CTR	SB 0.04%	SB 0.2%	SB 1%
Initial weight (g)	3.26 ± 0.29	3.27 ± 0.32	3.29 ± 0.34	3.31 ± 0.28
Initial length (mm)	65.75 ± 2.67	65.85 ± 2.21	65.68 ± 2.57	66.11 ± 2.53
Final weight (g)	4.53 ± 0.03	4.63 ± 0.12[a]	5.09 ± 0.04[a,b]	5.41 ± 0.03[a,b,c]
Final length (mm)	71.04 ± 3.78	71.54 ± 3.72[a]	75.30 ± 2.59[a,b]	76.87 ± 3.71[a,b]
Body weight gain (g)	1.27 ± 0.04	1.36 ± 0.12[a]	1.81 ± 0.03[a,b]	2.10 ± 0.03[a,b,c]
Specific growth rate (%/day)	4.53 ± 0.15	4.87 ± 0.42[a]	6.46 ± 0.10[a,b]	7.49 ± 0.12[a,b,c]
Feed intake (g)	2.33 ± 0.05	2.47 ± 0.10[a]	2.83 ± 0.01[a,b]	3.11 ± 0.02[a,b,c]
Feed conversion ratio (FCR)	1.89 ± 0.15	1.79 ± 0.06[a]	1.57 ± 0.03[a,b]	1.48 ± 0.01[a,b,c]

Results are presented as Mean ± SD. One-way ANOVA was used to compare difference of mean among CTR, SB 0.04%, SB 0.2% and SB 1%. The letters a, b, and c indicate statistical significance ($P < 0.05$) between the CTR and the SB-treated groups; SB 0.04% and SB 0.2%; and SB 0.2% and SB 1%, respectively.

Table S2. Water quality parameters in a 4-week laboratory study.

Parameters	CTR	SB 0.04%	SB 0.2%	SB 1%	Range*
Temperature (°C)	28.15 ± 0.99	28.55 ± 1.08	28.33± 1.11	28.11 ± 0.75	25 - 32
Salinity (‰)	15.17 ± 1.11	15.35 ± 1.05	15.96± 1.09	15.72 ± 0.71	10 - 25
pH	7.96 ± 0.31	8.22 ± 0.14	8.56 ± 0.46	8.02 ± 0.22	7.5 - 8.5
Dissolved Oxygen (mg/ml)	9.67 ± 1.93	9.67 ± 0.57	9.36 ± 1.00	9.54 ± 1.33	>4
Nitrite(mg/ml)	0.43 ± 0.07	0.66 ± 0.16	0.35 ± 0.16	0.55 ± 0.18	<1
Total ammonia (mg/ml)	0.29 ± 0.26	0.19 ± 0.13	0.12 ± 0.02	0.22 ± 0.12	<1

Results are presented as Mean ± SD. One-way ANOVA was used to compare difference of mean among CTR, SB 0.04%, SB 0.2% and SB 1%. No statistical significance ($P < 0.05$) between the CTR and the SB-treated groups; SB 0.04% and SB 0.2%; and SB 0.2% and SB 1%, respectively, was observed. * The suitable range of water parameters for shrimps were checked with (Apud, 1989) and (Margabandu and Ramamurthy, 2015).

Table S3. Water quality parameters in the 135-day field trial study.

Parameters	CTR	SB 0.2%	SB 1%	Range*
Temperature (°C)	29.80 ± 0.69	30.10 ± 0.61	30.57 ± 1.27	25 - 32
Salinity (‰)	20.28 ± 0.5	20.39 ± 0.39	20.04 ± 1.06	10 - 25
pH	7.92 ± 0.20	7.89 ± 0.69	8.39 ± 0.49	7.5 - 8.5
Dissolved Oxygen (mg/ml)	9.32 ± 1.29	9.64 ± 0.94	9.77 ± 0.83	>4
Nitrite(mg/ml)	0.70 ± 0.19	0.80 ± 0.27	0.53 ± 0.19	<1
Total ammonia (mg/ml)	0.49 ± 0.29	0.49 ± 0.33	0.45 ± 0.25	<1

Results are presented as Mean ± SD. One-way ANOVA was used to compare difference of mean among CTR, SB 0.04%, SB 0.2% and SB 1%. No statistical significance ($P < 0.05$) between the CTR and the SB-treated groups; SB 0.04% and SB 0.2%; and SB 0.2% and SB 1%, respectively, was observed. *The suitable range of water parameters for shrimps were checked (Apud, 1989) and (Margabandu and Ramamurthy, 2015).

Table S4. The number of sequences, operational taxonomic units (OTUs), and diversity indices in shrimp gut digesta.

Sample	Number of sequences	Observed OTUs	Shannon diversity	Simpson diversity	ACE	Chao1 richness	Coverage
CTR	38170	332	3.443	0.737	371.016	381.763	0.998
SB 1%	38170	311	2.738	0.564	341.331	351.182	0.999

Table S5. The composition of shrimp microbiota in phylum, class, order, family and genus level fed with SB for 28 days during **a 4-week laboratory study**.

Taxonomy	Name	Proportion in total sequences (%)	
		CTR	SB 1%
Phylum	*Proteobacteria*	51.708	67.692
	Bacteroidetes	3.591	2.166
	Firmicutes	0.788	1.205
	Cyanobacteria	0.168	0.361
	Actinobacteria	0.267	0.456
	Verrucomicrobia	0.024	0.008
Class	*Alphaproteobacteria*	15.672	16.317
	Gammaproteobacteria	25.440	10.051
	Verrucomicrobiae	0.267	0.456
	Deltaproteobacteria	0.189	0.068
	Acidimicrobiia	0.757	0.652
	Planctomycetacia	3.510	2.103

	Mollicutes	0.788	1.205
	Bacilli	0.168	0.123
Order	*Clostridiales*	0.002	0.001
	Alteromonadales	0.042	0.081
	Lactobacillales	0.047	0.045
	Bacillales	0.121	0.079
	Bifidobacteriales	0.005	0.024
	Verrucomicrobiales	0.246	0.435
	Bacteroidales	0.168	0.231
	Vibrionales	23.452	7.348
	Flavobacteriales	51.354	67.330
	Mycoplasmatales	0.778	1.205
	Pseudomonadales	0.144	0.149
Family	*Bacteroidaceae*	0.031	0.052
	Lactobacillaceae	0.005	0.031
	Enterobacteriaceae	0.003	0.066
	Ruminococcaceae	0.065	0.024
	Prevotellaceae	0.029	0.058
	Bifidobacteriaceae	0.005	0.024
	Bacillaceae	0.076	0.042
	Clostridiaceae_1	0.018	0.013
	Flavobacteriaceae	36.303	59.883
	Rhodobacteraceae	0.081	0.330
	Vibrionaceae	50.084	21.658
	Mycoplasmataceae	0.778	1.205
	Pseudomonadaceae	0.079	0.100
	Enterococcaceae	0.000	0.000
	Pseudoalteromonadaceae	0.000	0.000
	Shewanellaceae	0.000	0.000
Genus	*Lactobacillus*	0.005	0.031
	Megamonas	0.008	0.003
	Ruminococcus	0.000	0.003
	Megasphaera	0.000	0.011
	Alistipes	0.000	0.000
	Butyricicoccus	0.000	0.000
	Bifidobacterium	0.005	0.024
	Prevotella	0.050	0.115
	Collinsella	0.005	0.018
	Photobacterium	0.049	0.000
	Pseudoalteromonas	0.008	0.005
	Planctomicrobium	1.153	0.097

Tenacibaculum	0.000	0.000
Pirellula	0.018	0.084
Corynebacterium_1	0.031	0.010
Bythopirellula	0.045	0.045
Rubripirellula	0.000	0.026
Blastopirellula	0.186	0.220
Vibrio	18.512	7.308

Table S6. Level of antimicrobial metabolites in the supernatant of *L. johnsonii in vitro*.

Metabolite (µg/ml)	CTR	SB
AA	7,760.00 ± 22.24	8,191.70 ± 216.98***
LA	2,278.27 ± 44.30	2,633.16 ± 223.75***
PA	5.90 ± 0.34	8.66 ± 2.23***
BA	12.68 ± 0.59	14.46 ± 1.20**
3-HPA	3,718.91 ± 185.99	4,466.83 ± 263.44**

The supernatant of *L. johnsonii* was grown in MRS containing 0.00025 µg/ml methanol (CTR) or SB at 2 µg/ml at the indicated times, then LC-ESI-MS was used for analysis. Data, n=3 (mean ± standard deviation). One-way ANOVA: *P* (*) < 0.05, *P* (**) < 0.01, and *P* (***) < 0.001 were considered statistically significant.

Table S7. The contents of AM in the intestine of *L. vannamei* that were fed the control and three SB diets for 28 days (µg/g) *in vivo*.

Metabolite (µg/g)	CTR	SB 0.04%	SB 0.2%	SB 1%
AA	17.13 ± 4.50	16.70 ± 0.20	35.61 ± 0.31[a, b]	46.29 ± 0.64[a, b, c]
LA	18.06 ± 0.33	22.46 ± 7.13	31.87 ± 1.11[a, b]	34.53 ± 0.87[a, b, c]
PA	0.20 ± 0.01	0.22 ± 0.02	0.25 ± 0.00[a, b]	0.28 ± 0.00[a, b, c]
BA	0.06 ± 0.00	0.07 ± 0.01	0.08 ± 0.01[a, b]	0.18 ± 0.00[a, b, c]
3HPA	3.14 ± 0.62	5.37 ± 1.18	7.98 ± 1.21[a, b]	11.85 ± 1.52[a, b, c]

Results are presented as Mean ± SD. One-way ANOVA was used to compare difference of mean among CTR, SB 0.04%, SB 0.2% and SB 1%. The letters a, b, and c indicate statistical significance ($P < 0.05$) between the CTR and the SB-treated groups; SB 0.04% and SB 0.2%; and SB 0.2% and SB 1%, respectively.

B. **SUPPLEMENTARY FIGURES**

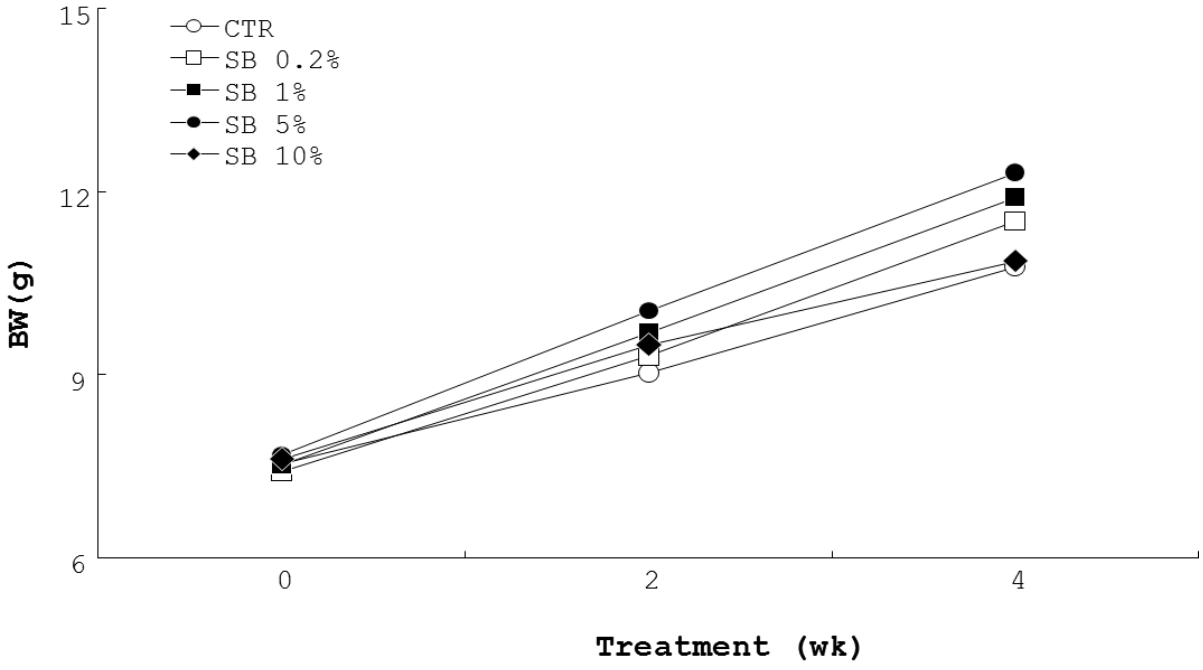

Fig. S1. Toxicity of SB in shrimp. Five groups of 135-day-old shrimp, 30 animals a group, were fed with standard diet (CTR) or the diet containing SB at 0.2%, 1%, 5% and 10% for 4 weeks. Their body weight (BW) was measured for toxicity assessment.

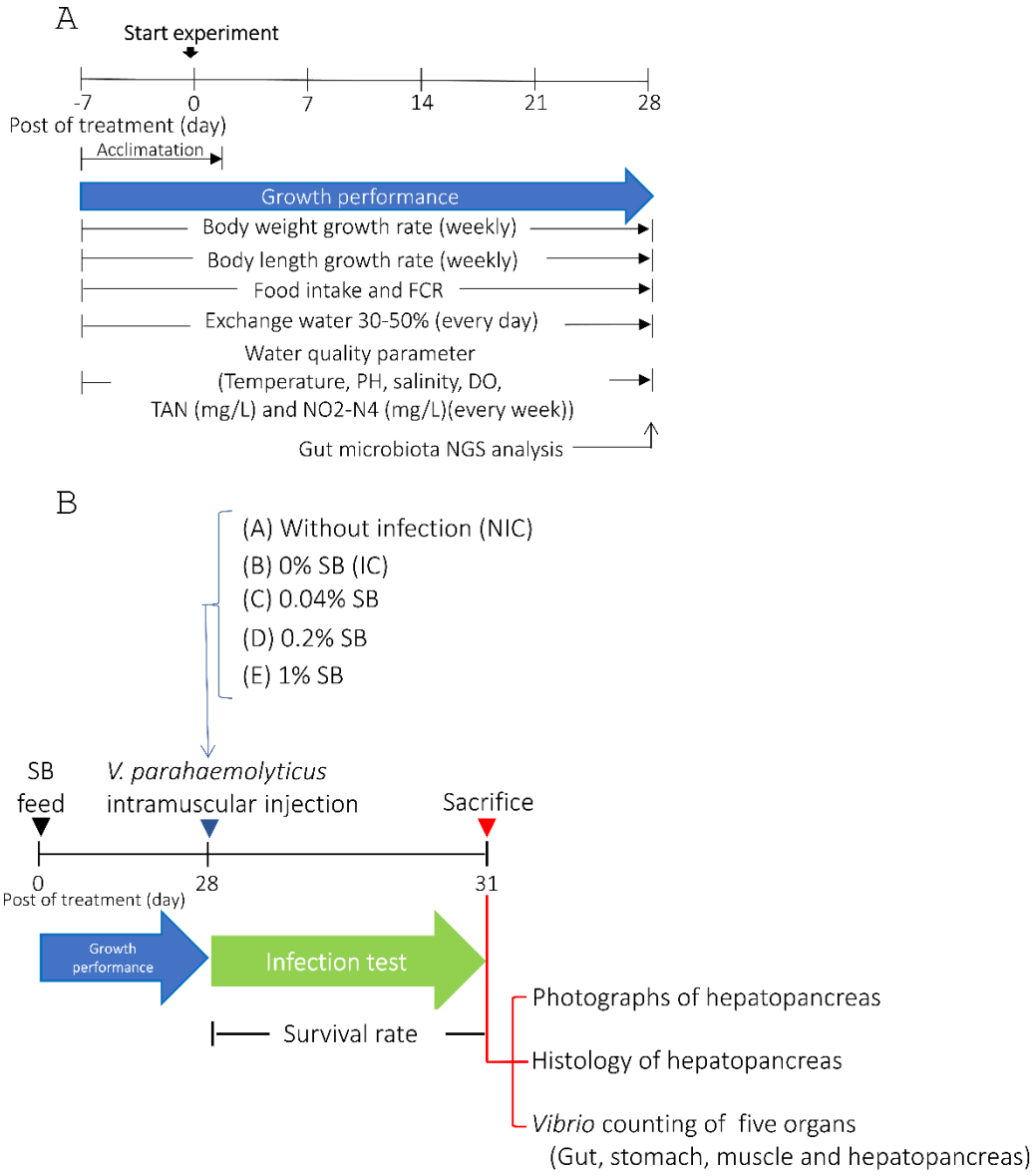

Fig. S2. Experimental design used for growth performance, analysis of gut microbiota, and *Vibrio* infection in whiteleg shrimp. (A) A flow chart of the experimental design used in the laboratory study. (B) A flow chart of the experimental design used in the challenge study.

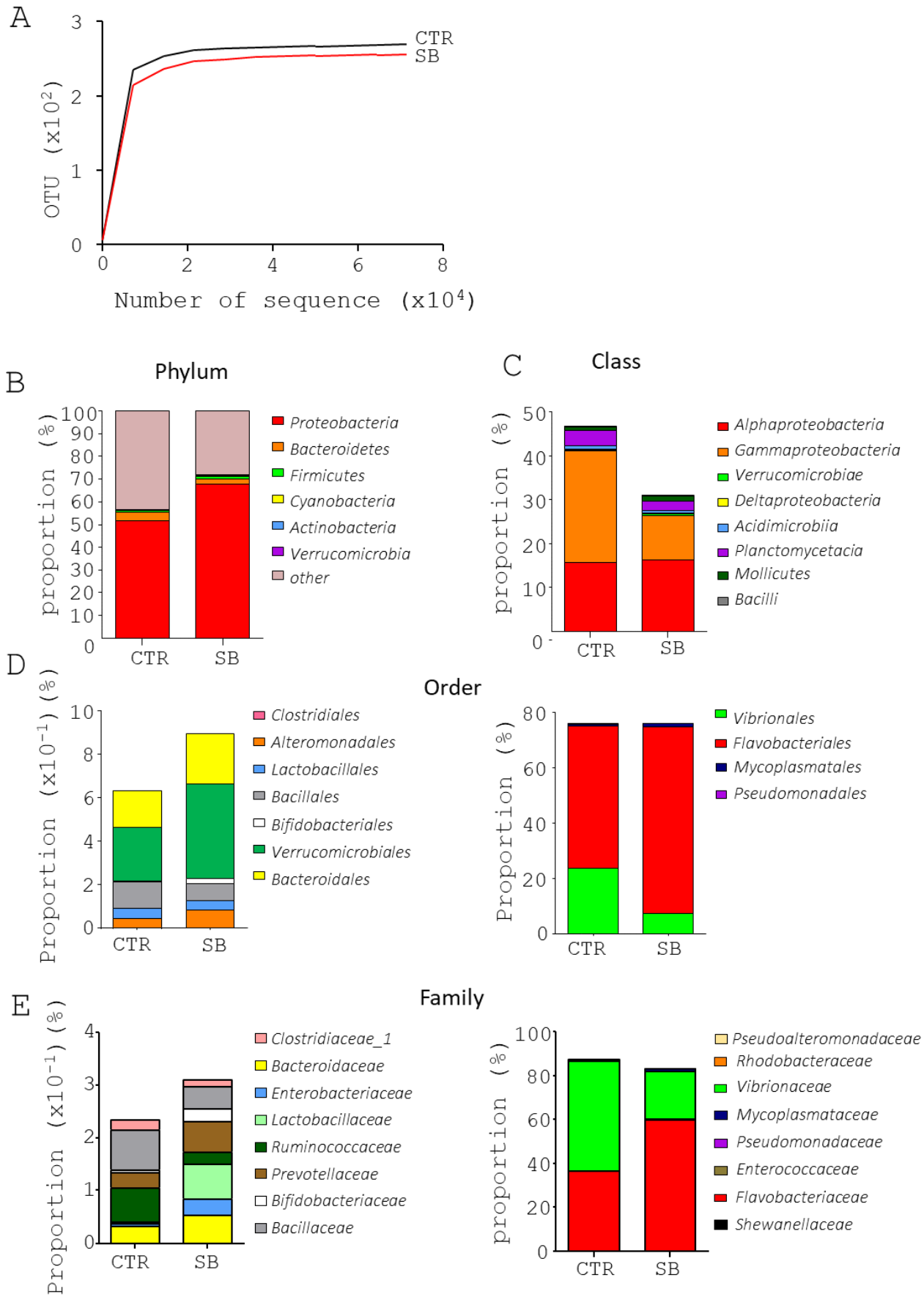
A
OTU (x10²)
Number of sequence (x10⁴)
CTR
SB
B
Phylum
proportion (%)
Proteobacteria
Bacteroidetes
Firmicutes
Cyanobacteria
Actinobacteria
Verrucomicrobia
other
C
Class
Alphaproteobacteria
Gammaproteobacteria
Verrucomicrobiae
Deltaproteobacteria
Acidimicrobiia
Planctomycetacia
Mollicutes
Bacilli
D
Order
Proportion (x10⁻¹) (%)
Clostridiales
Alteromonadales
Lactobacillales
Bacillales
Bifidobacteriales
Verrucomicrobiales
Bacteroidales
Vibrionales
Flavobacteriales
Mycoplasmatales
Pseudomonadales
E
Family
Clostridiaceae_1
Bacteroidaceae
Enterobacteriaceae
Lactobacillaceae
Ruminococcaceae
Prevotellaceae
Bifidobacteriaceae
Bacillaceae
Pseudoalteromonadaceae
Rhodobacteraceae
Vibrionaceae
Mycoplasmataceae
Pseudomonadaceae
Enterococcaceae
Flavobacteriaceae
Shewanellaceae

Fig. S3. Experimental design and analysis of 16S rDNA sequencing analysis of gut microbiota in shrimp. (A) Rarefaction curves of bacterial OTUs in the fecal bacteria of control shrimp (CTR) and shrimp fed with 1% SB (Fig. 1) using the 16S rDNA NGS analysis. (B-E) The 16S rDNA NGS analysis indicated the relative abundance of gut bacteria at the level of phylum (B), class (C), order (D) and family (E).

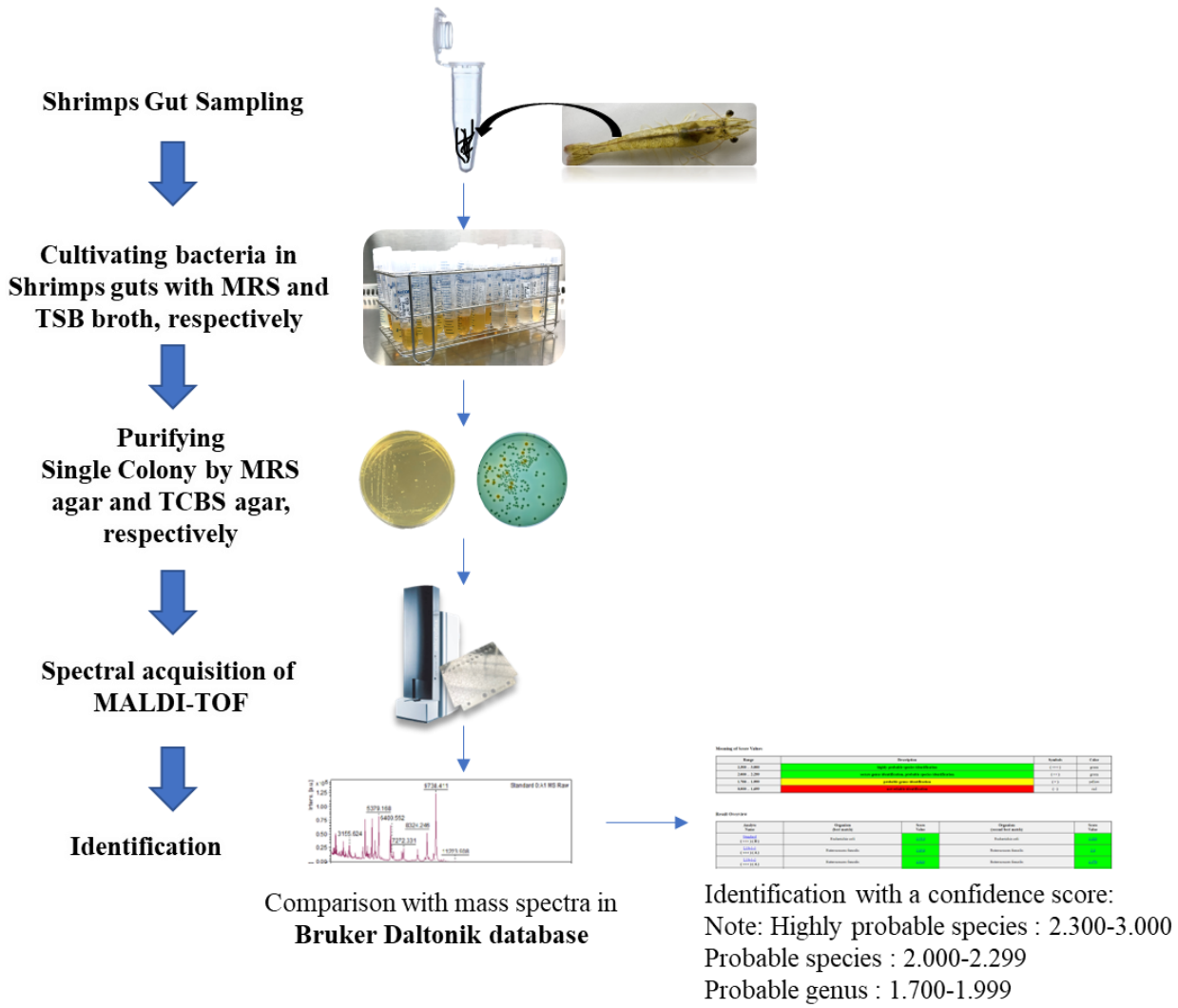

Fig. S4. Intestinal bacteria isolation, screening, and identification of probiotics and pathogens from shrimp gut. The procedure of intestinal bacteria collection, cultivation, screening single colony, MALDI-TOF MS analysis and identification using Biotype 3.1 against an in-house database. A mixture of three shrimps' guts were collected and screened for 16-48 single strains of probiotics and pathogens. Then, the single strains were identified by MALDI-TOF MS analysis.

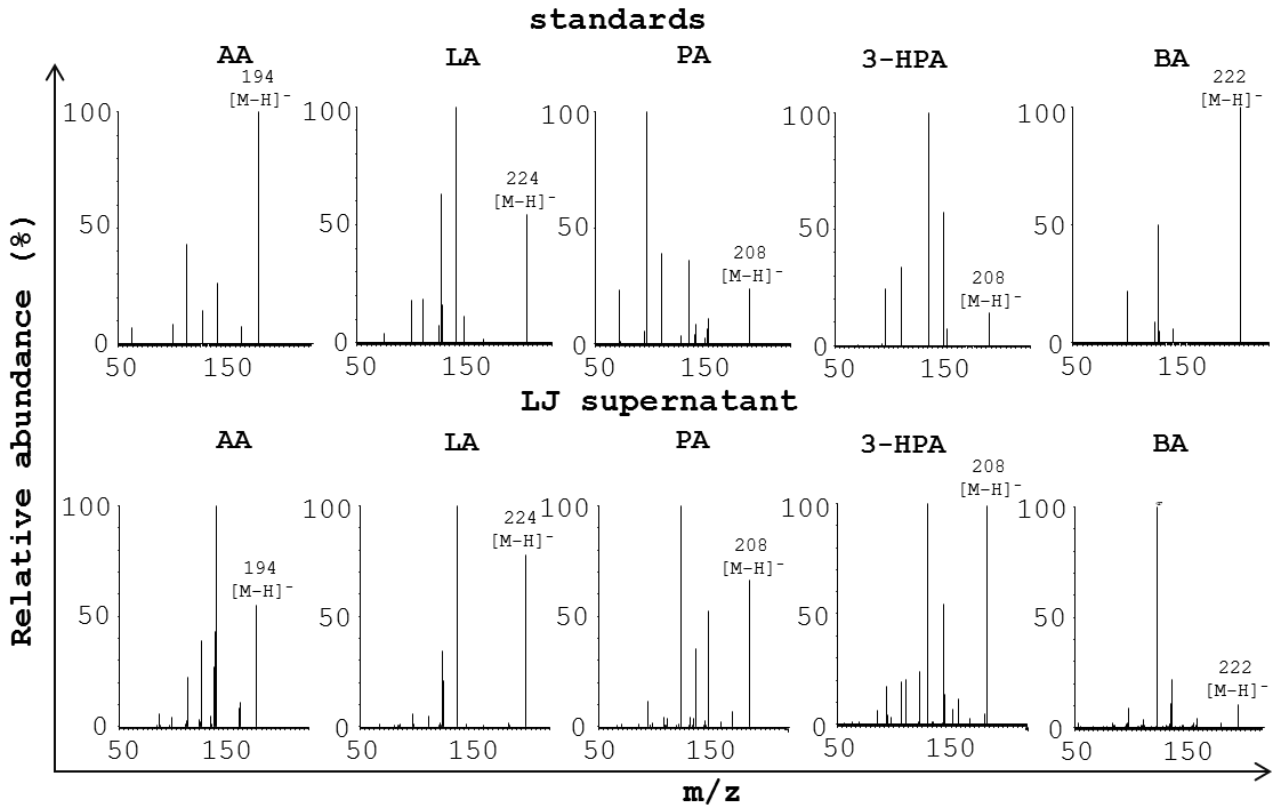

Fig. S5. Identification of 5 antimicrobial (5AM) produced by *L. johnsonii*. The 5 AM in the supernatant of *L. johnsonii* grown in MRS broth (bottom) as well as standard compounds (top), including acetic acid (AA), butyric acid (BA), lactic acid (LA), propionic acid (PA), and 3-hydroxypropionic acid (3-HPA), were subjected to LC-MS/MS. Representative MS/MS profiles of the 5 ion signals corresponding to the 5 AM are indicated. The quantification of antimicrobial metabolites (AM) in each group was obtained based on area under curve of LC histograms (Fig. 4E).

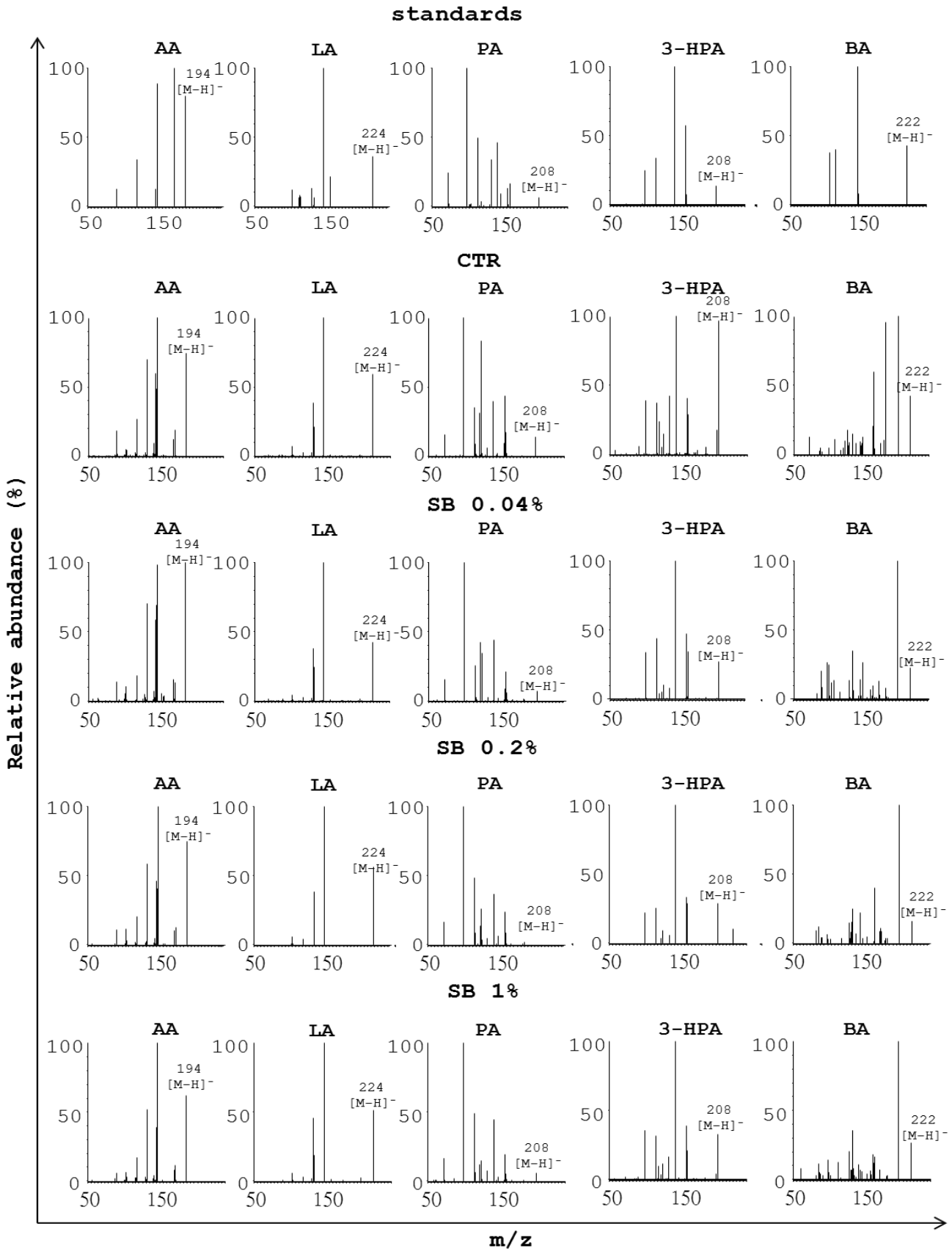
standards
AA
LA
PA
3-HPA
BA
194 [M-H]-
224 [M-H]-
208 [M-H]-
222 [M-H]-
CTR
SB 0.04%
SB 0.2%
SB 1%
Relative abundance (%)
m/z

Fig. S6. Identification of five antimicrobial metabolites (5AM) in shrimp guts. Eighty-six-day-old shrimp were fed with standard diet (CTR) and a diet containing SB at three indicated dosages for 4 weeks. Their gut digesta were subjected to LC-MS/MS analysis, followed by quantification of 5 AM (μg/g of guts), including acetic acid (AA), butyric acid (BA), lactic acid (LA), propionic acid (PA), and 3-hydroxypropionic acid (3-HPA). Representative MS1 profiles of the 5 ion signals corresponding to the 5 AM standards are indicated (1st row). MS/MS profiles of the 5 AM present in CTR-feeding shrimp gut (2nd row) and three indicated SB-feeding shrimp gut (3rd to 5th rows) are shown. The quantification of antimicrobial metabolites (AM) in each group was obtained based on area under curve of LC histograms (Fig. 5E).

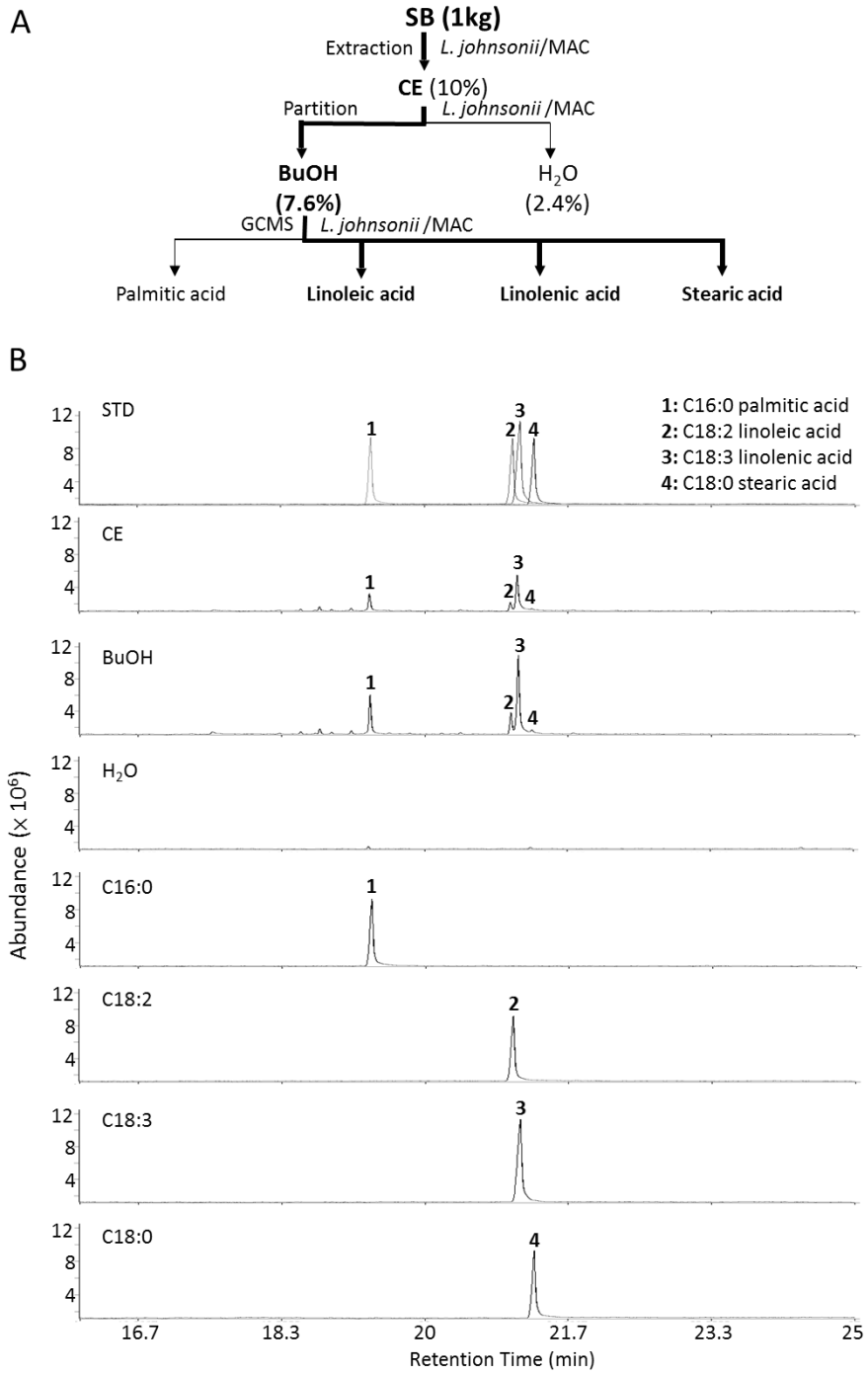

Fig. S7. Identification and quantification of active compounds of SB. (A) We partitioned the crude extract (CE) of SB into butanol (BuOH) and water (H_2O) fractions. Using the bioactivity-directed fraction and isolation strategy, we purified and identified four active compounds, including palmitic acid (**1**), linoleic acid (**2**), linolenic acid (**3**), and stearic acid (**4**), which could promote *L. johnsonii* growth, from the SB extract. (B) GC-GC/MS analysis was used to characterize the four fatty acids in standards (STD) and the CE, BuOH fraction, H_2O fraction, and pure compounds of SB.

References:

Apud, F.D. (1989). Recent developments in prawn pond culture. (Aquaculture extension pamphlet No. 1). Tigbauan, Iloilo, Philippines: Southeast Asian Fisheries Development Center, Aquaculture Department.

Margabandu, V., and Ramamurthy, D. (2015). Recent farming practices for culturing sustainable pacific White Shrimp, Peneaus vannamei. *International Journal of Science and Research* 4(2)**,** 9-12. ISSN (Online): 2319-7064.