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A native strain of *Bacillus subtilis* increases lipid accumulation and modulates expression of genes related to digestion and amino acid metabolism in *Litopenaeus vannamei*

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ABSTRACT

In the field of shrimp aquaculture, the utilization of probiotics represents a promising avenue, due to the well-documented benefits conferred by these microorganisms. In the current study, a *Bacillus subtilis* strain, referred to as strain E, was isolated from the gastrointestinal tract of the shrimp *Litopenaeus vannamei* and subsequently identified via molecular methods and phylogeny. The probiotic potential of strain E was characterized, and its application as a feed shrimp additive was evaluated in a 45-day experiment. Several parameters were assessed, including zootechnical performance, muscle tissue proximate composition, hepatopancreas lipid concentration, and the expression of genes associated with digestion, amino acid metabolism, and antioxidant defense mechanisms in various shrimp tissues. Although no significant impact on zootechnical performance was observed, supplementation with strain E led to an increase in lipid concentration within both muscle and hepatopancreas tissues. Furthermore, a marked decrease in the expression of genes linked to digestion and amino acid metabolism was noted. These findings suggest that the addition of the *B. subtilis* strain E to shrimp feed may enhance nutrient absorption and modulate the expression of genes related to digestion and amino acid metabolism.

1. Introduction

Shrimp farming is a significant contributor to the global aquaculture industry's economic value, with the Pacific white shrimp (*Litopenaeus vannamei*) representing the most commercially produced crustacean, as per FAO's, 2020 report. This species has garnered popularity among farmers due to its adaptability to diverse environmental conditions, rapid growth rate, and compatibility with commercial feeds. However, the pursuit of enhanced productivity often results in suboptimal management practices, particularly concerning high stocking densities, which can stress the ecosystem and potentially invite disease outbreaks. As a consequence, viral and bacterial diseases have become increasingly prevalent on shrimp farms. For instance, the White Spot Syndrome (WSS) virus can lead to massive losses within days (Wen et al., 2014), and bacterial infections, especially those caused by *Vibrio* species, can result in substantial mortality rates (Zokaeifar et al., 2012a, 2012b). Moreover, proper nutrition is an essential consideration in shrimp farming, given that feed constitutes the most significant production cost

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component in many countries (Shang et al., 1998; Karim et al., 2014; Nisar et al., 2021; Valenti et al., 2021). Fishmeal, a primary protein source in commercial feed, is becoming increasingly scarce and expensive. Plant-based protein alternatives, though more sustainable, have demonstrated negative impacts on fish nutrition due to the presence of anti-nutritional compounds (Azeredo et al., 2017; Estruch et al., 2018). An emerging solution to enhance feed digestibility and efficiency lies in the application of probiotic bacteria that produce and secrete digestive enzymes. These beneficial microorganisms can potentially mitigate some of the negative impacts associated with alternative protein sources and contribute to healthier, more sustainable shrimp farming practices.

Numerous bacterial species are recognized as probiotics, with certain *Bacillus* species gaining particular attention due to their notable pathogen antagonistic activity, extracellular enzyme production (including amylases, cellulases, lipases, and proteases), and heat resistance (Yu et al., 2009; Banerjee and Ray, 2017). One such species is *Bacillus subtilis*, a Gram-positive, non-pathogenic, spore-forming bacterium widely employed for oral bacterial therapy, prophylaxis of gastrointestinal disorders, and enhancing water quality and animal survival in aquaculture (Shen et al., 2010; Lee et al., 2017). Although *B. subtilis* is primarily recognized for its presence in the gastrointestinal tracts of animals, it can also be found in diverse environments, both terrestrial and aquatic, owing to the existence of strain-specific genes that augment its adaptability (Earl et al., 2008).

Given the potential benefits of *Bacillus* species, the present study aimed to isolate *B. subtilis* strains adapted to the gastrointestinal tract of the Pacific white shrimp, *L. vannamei*, perform a probiotic characterization of the isolated strains, and evaluate their effects when included in the feed. Specifically, the study assessed the impact of these strains on zootechnical performance, muscle proximate composition, lipid accumulation in the hepatopancreas, and the expression of genes related to digestion, amino acid metabolism, and antioxidant defenses in L. *vannamei*.

2. Material and methods

2.1. Isolation of bacteria from the gastrointestinal tract of shrimp

Shrimps with an average weight of approximately 7 g ($n = 30$) were housed in 150 L tanks, maintained under the following conditions: an average temperature of 29 °C, salinity of 30 g/L, alkalinity of 150 mg/L as $CaCO_3$, and total suspended solids averaging 500 mg/*L. prior* to intestine removal, the shrimps were subjected to a two-day fasting period to clear their gastrointestinal tracts. The shrimps were then euthanized via hypothermia, followed by sterilization in formaldehyde (50 mg/L) for five minutes to eliminate external bacteria. Afterward, they were rinsed with sterile water for one minute to remove the disinfectant, as per the method described by Boonthai et al. (2011). Intestinal samples were obtained by pooling the intestines from four individual shrimps, resulting in a total of four combined samples. These samples were subsequently macerated in 0.9% NaCl solution. The macerated samples were then diluted (1:10, *v*/v) in 0.9% NaCl for plating on Luria-Bertani (LB) solid medium, with duplicate plates prepared for each sample. Following a 16-h incubation at 35 °C, bacterial colonies were morphologically characterized, and the most frequently occurring morphotypes were isolated for subsequent identification through molecular taxonomy and probiotic characterization.

2.2. Molecular identification and phylogenetic analysis

For the molecular identification of bacterial colonies isolated from the gastrointestinal tract of shrimp, both the ribonuclease III-encoding gene (*rnc*) and *16S rRNA* were utilized. The *rnc* gene is deemed essential for *B. subtilis* (Herskowitz and Bechhofer, 2000). Although *rnc* is not typically employed in molecular taxonomy, a phylogenetic analysis of multiple bacterial ribonucleases conducted by Condon and Putzer (2002) concluded that *rnc* ranks among the most conserved genes within the Eubacteria Kingdom. Prior analysis using the BLAST tool from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) demonstrated that, despite its conservation, the *rnc* gene possesses variable regions capable of enabling taxonomic differentiation among *Bacillus* species (data not shown).

DNA was extracted from strains of differing morphotypes using a heat-based method. Bacterial cells were collected from individual colonies, suspended in 10 μL of ultrapure water, and incubated for five minutes at 95 °C. Following a brief centrifugation, 1 μL of the supernatant was directly used in polymerase chain reactions (PCR) to amplify both the *16S rRNA* and *rnc* genes, employing degenerate primers designed specifically for *Bacillus* species (Table 1). All PCR reactions utilized Platinum Taq DNA Polymerase (Invitrogen, Brazil), with the protocol executed according to the manufacturer's instructions. The thermocycler was set for an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 60 s (extension), ending with a final extension step at 72 °C for 5 min. PCR products were analyzed via 1% agarose gel electrophoresis. Amplified fragments were purified using the Quick PCR Purification Kit (Invitrogen, Brazil) and subsequently sequenced. Resulting sequences were input into GenBank's BLAST tool to obtain gene identity indices.

For the phylogenetic analysis, only the ribosomal 16S gene sequence obtained for the *B. subtilis* strain E was used. Initially, the sequence was submitted to the NCBI's BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which was set to search in the "Reference RNA sequences" database (refseq_rna), excluding *B. subtilis* itself (taxid: 1423) from the search. Thus, it was possible to identify 16S sequences from other species related to *B. subtilis*, which might form groups with a close genetic relationship. Additionally, a search was conducted for sequences only within the group of *B. subtilis* strains to identify the closest strains. After an initial filtering, the set of sequences selected for the 16S phylogenetic reconstruction were: *B. subtilis* strain E (OQ793592.1), *B. subtilis* strain JCM1465 (NR113265.1), *B. subtilis* strain DSM10 (NR027552.1), *B. tequilensis* (NR104919.1), *B. vallismortis* (NR113994.1), *B. velezensis* (NR116240.1), *B. amyloliquefaciens* (NR116022.1), *B. nematocida* (NR115325.1), *B. siamensis* (NR117274.1), *B. haynesii* (NR157609.1), *B. licheniformis* (NR118996.1), *B. swezeyi* (NR157608.1), *B. sonorensis* (NR113993.1). To determine a sequence representing the outgroup, a search was conducted excluding all *Bacillus* species. The species chosen to represent the outgroup was *Rossellomorea arthrocnemi* (NR181775.1). All sequences were organized into a txt file in fasta format. From this point on, all subsequent analyses used the MEGA11 software (Tamura et al., 2021). The first step was to perform a progressive multiple alignment of the sequences, using the "muscle" option of MEGA11. Next, the phylogenetic tree was constructed using the Neighbor-joining method, with the following parameters: Test of phylogeny: Bootstrap method, Number of Bootstrap replications: 10,000, Model/Method: p-distance, Substitutions to include: transitions + transversions, Rate Among sites: Uniform rates, Gap/missing data treatment: Pairwise deletion.

Table 1
Degenerate primers used in PCR reactions to amplify the *rnc* and 16 s rRNA genes from *Bacillus* species.

Primer	Sequences (5′-3′)	Amplicon (nt)
RNC3-F1	tgwwtcaagcatttacrcattcatck	573
RNC3-R	attcwcgrttgtgvgcwggkcct	
RNC3-F2	tcatcktatgtgaatgagcatcgraa	548
RNC3-R	attcwcgrttgtgvgcwggkcct	
RNC3-F3	yccgrcwatgagygaaggagakt	414
RNC3-R	attcwcgrttgtgvgcwggkcct	
16S 27F	agagtttgatcmtggctcag	1514
16S 1492R	tacggytaccttgttacgactt	

2.3. Phenotypic characterization of isolated strains

To assess phenotypic characteristics, strains were initially cultured in Tryptone Soy Broth (TSB) for 16 h at 35 °C. Subsequently, the Gram staining technique was employed for the morphological and structural characterization of the bacterial cell wall. This method classifies bacteria as either Gram-positive or Gram-negative, depending on their response to specific dyes (Tortora et al., 2012).

For the evaluation of exopolysaccharide (EPS) production, the Congo red test was conducted. In this test, strains were grown on solid medium containing 0.8 g of Congo red dye per 1 L of Agar Brain Heart Infusion (BHI), supplemented with 36 g of sucrose. The plates were incubated at 35 °C for 24 h and then maintained at room temperature for an additional 48 h, following the methodology adapted from Freeman et al. (1989). EPS production is indicated by a change in the inoculum's color to black on the Congo Red Agar plate.

To assess the ability to adhere, the strain was inoculated in TSB medium and incubated at 35 °C for two days. Following this initial incubation, 200 μL of the culture was transferred in triplicate to sterile polystyrene microplates containing 96 “u”-shaped wells and incubated once more at 35 °C for two days, without shaking. Subsequently, the inocula were removed, and the wells were washed three times with sterile distilled water and then dried in an oven at 60 °C for one hour. After drying, 200 μL of a 1% crystal violet solution was added to each well and incubated for one minute. This was followed by three additional washes with distilled water and air-drying at room temperature, according to the methodology adapted from Christensen et al. (1985). Positive adhesion on polystyrene microplates is evidenced by the presence of aggregates and a purple hue in the wells after drying.

The bacterial isolate underwent screening for extracellular enzymes with potential relevance in digestive processes or as virulence factors. Tests were conducted according to established protocols in the literature, with the isolate being inoculated onto specific media: caseinase (Milk Agar), gelatinase (Tryptone Soy Agar - TSA, supplemented with 0.5% gelatin), amylase (Nutrient Agar supplemented with 0.1% soluble starch) (Rodrigues et al., 1993), and cellulase (Carboxymethylcellulose Agar - CMC) (Teather and Wood, 1982).

To assess hemolytic activity, the isolated strains were inoculated onto a Shrimp Hemolymph Agar medium, consisting of 1 mL of L. *vannamei* hemolymph containing 200 ppm of Rose Bengal, following the methodology proposed by Chang et al. (2000). The presence of a hemolytic zone can be observed as a clear halo around the inoculum. *Vibrio parahaemolyticus* IOC 18950, a known pathogen, was used as a positive control for hemolytic activity.

To evaluate the response to various antibiotics, the bacterial strain was cultured in TSA medium, incubated at 35 °C for 24 h, and then adjusted to 0.5 on the McFarland scale using a 1% saline solution. A spectrophotometer with a wavelength reading of 625 nm was used to make this adjustment. One unit on the McFarland scale corresponds to a homogeneous suspension of approximately 1.5×10^8 *E. coli* cells per mL. The strain was then spread onto Muller Hinton Agar (MHA) plates using a sterile swab, and commercial discs containing the antibiotics nalidixic acid (30 μg), chloramphenicol (30 μg), florfenicol (30 μg), and oxytetracycline (30 μg) were placed on the inoculum. The plates were incubated for 24 h at 35 °C, and the diameters of the inhibition zones around the discs were measured using a digital caliper. The results were interpreted based on resistance, intermediate susceptibility, or susceptibility to antibiotics, following the methodology proposed by Charteris et al. (1998).

The evaluation of the selected strains' tolerance to variations in temperature, pH, and salinity is a critical step in assessing their suitability for use as probiotics in shrimp farming. Probiotic strains must be able to withstand the environmental conditions of the aquaculture system where they will be applied. Temperature tolerance: The ability of the strains to tolerate a range of temperatures was evaluated by culturing them in LB medium and incubating them at either 4 °C or 40 °C for up to two days. Growth, indicated by the turbidity of the medium, suggests that the strains can survive and thrive under these temperature conditions. This is crucial since temperature can vary significantly in aquaculture systems, and a good probiotic should be able to adapt to these variations. pH tolerance: The tolerance of the strains to different pH levels was tested by growing them in LB medium adjusted to pH levels of 5.0 and 9.0 and incubating them at 35 °C for up to two days. Growth under these pH conditions, as evidenced by the turbidity of the medium, indicates that the strains can adapt to different pH levels. This is important as the pH in aquaculture systems can vary due to factors such as water quality, waste accumulation, and algal blooms. Salinity tolerance: The ability of the strains to tolerate different salinities was evaluated by culturing them in LB medium adjusted to salinities of 0, 30, and 80 g/L, followed by incubation at 35 °C for up to two days. Growth in the presence of different salinity levels, indicated by the turbidity of the medium, suggests that the strains can adapt to varying salinity levels. This is essential for shrimp farming, where salinity can fluctuate depending on factors such as location, season, and water exchange practices. The ability of the strains to tolerate these environmental variations is a crucial aspect of their potential application as probiotics in shrimp farming. Strains that exhibit good tolerance to temperature, pH, and salinity changes are more likely to be effective in promoting the health and productivity of shrimp in aquaculture systems.

The antagonism test, as described, evaluates the potential for the selected bacterial strains to inhibit the growth of the pathogenic *Vibrio* strains *V. harveyi* and *V. parahaemolyticus*. This is an important assessment for a candidate probiotic strain in aquaculture, as it can indicate the ability of the probiotic strain to control or mitigate the presence of pathogens that could harm the shrimp population. In this test, *Vibrio* strains, which are known pathogens, are inoculated in the central streak of an agar plate. The bacterial strain being tested as a potential probiotic is then streaked perpendicular to the central streak, with a distance of about one centimeter between the streaks. The plate is incubated at 35 °C for 24 h to allow bacterial growth. After incubation, the interaction between the streaks is observed: Positive antagonism: If the growth of the central *Vibrio* streak is inhibited where it intersects with the perpendicular streak of the probiotic strain, it indicates that the probiotic has an antagonistic effect on the pathogen. Negative antagonism: If the growth of the central *Vibrio* streak meets the perpendicular streak without any inhibition, it suggests that the probiotic strain does not have an antagonistic effect against the pathogen. This test is crucial in evaluating the ability of a potential probiotic strain to prevent or suppress the growth of harmful pathogens in aquaculture systems. Probiotic strains with a strong antagonistic effect against pathogens like *Vibrio* spp. can help to reduce the prevalence of disease outbreaks, increase the survival rate of shrimp, and improve overall productivity in aquaculture systems.

2.4. Feed supplementation with the strain of B. subtilis isolated from the gastrointestinal tract of L. vannamei

The process for the supplementation of L. *vannamei* feed with *B. subtilis* strains involves cultivating the selected bacterial strain and the control strain (*B. subtilis* KM0), standardizing the cultures, washing the cells, and then diluting the pellets in saline solution so that the desired number of colony forming units (CFUs) are added to each gram of feed. Both the selected strain and the control strain are cultured in minimal salts medium with the addition of glucose and casein hydrolyzate. This step allows the bacterial strains to grow and multiply, which will provide sufficient biomass for the supplementation of the shrimp feed. After the overnight cultivation, the optical density (OD_{600}) of the bacterial cultures is measured and standardized to a specific value (in this case, 1.6). This step is important to ensure that a consistent number of bacterial cells are added to the shrimp feed, which is necessary for accurate comparisons of the effects of the selected strain and the control strain. The bacterial cultures are centrifuged to separate the cells from the

culture medium, and the pellets are washed with saline solution to remove any remaining medium or other contaminants. This step is crucial for ensuring that the bacterial cells added to the feed are free of unwanted substances. The washed pellets are diluted in saline solution so that each gram of feed receives a specific number of CFUs of probiotics (in this case, 10^{10} CFUs). This step ensures that the shrimp receive a consistent dosage of probiotics, which will help in assessing the effects of the supplementation on the shrimp's health and growth performance. The diluted pellets are added to the shrimp feed, ensuring that the feed is evenly coated with the bacterial solution. This step ensures that the shrimp receive the desired dosage of probiotics in their feed.

2.5. Experiment with L. vannamei

The experiment was conducted at the Marine Station of Aquaculture from the Federal University of Rio Grande (FURG, Brazil). Juvenile shrimp with an average weight of 0.63 ± 0.09 g were obtained from Aquatec (Rio Grande do Norte, Brazil). They were placed in tanks with a useful volume of 150 L and a stocking density of 266 shrimps/m^3. Before starting the experiment, the shrimp were acclimated to the experimental conditions without the addition of commercial probiotics. The physical and chemical parameters of the water were evaluated daily. The temperature was consistently maintained at 26 °C throughout the experimental period. Salinity and dissolved oxygen were kept at 30 g/L and 6 ± 0.5 mg/L, respectively. Aeration was continuously provided to each tank through a porous stone using an air blower. A daily water change of 35% was performed to maintain the nitrogen compounds below the shrimp's tolerance levels.

The experimental design of the study consisted of three different treatment groups, each conducted in quadruplicate. The treatment groups were defined as follows: Control group (Feed): The shrimps in this group were fed a commercial diet moistened with a 0.9% NaCl solution. *B. subtilis* KM0 strain group (Feed+KM0): The shrimps in this group received a commercial diet supplemented with the *B. subtilis* KM0 strain. Isolated shrimp strain group (Feed+E): The shrimps in this group received a commercial diet supplemented with the isolated shrimp strain, referred to as strain E. For 45 days, the shrimp in all treatment groups were fed twice daily with a commercial feed that contained 38% crude protein (manufactured by Guabi, Brazil). This time period was chosen because it is sufficient for the juvenile shrimp to reach commercial size under controlled conditions. The feeding rate started at 10% of the estimated biomass at the beginning of the experiment and was gradually reduced to 5% by the end of the experimental period.

The zootechnical performance of the shrimp was assessed using the following formulas:

- Weight gain (g) was calculated as the difference between the final weight (Pf) and the initial weight (Pi) of each shrimp. Weight gain (g) = Pf – Pi.
- Biomass gain was calculated as the difference between the final biomass (product of the average final weight and the number of individuals at the end of the experiment) and the initial biomass (product of the average initial weight and the initial number of individuals). Biomass gain = (Average Pf x number of individuals at the end) - (Average Pi x initial number of individuals).

- Feed conversion ratio (FCR) was calculated as the ratio of the total feed intake to the biomass gain. FCR = feed intake/biomass gain.
- Survival percentage was calculated as the ratio of the final shrimp population to the initial population, multiplied by 100. Formula: Survival (%) = (final shrimp population × 100)/initial population.

The results from these calculations were used to assess and compare the effects of the different treatments on the growth and survival of the shrimp.

2.6. Tissue collection

After the 45-day period, shrimps were selected for analysis. Five shrimps from each tank were euthanized and stored at −20 °C for proximate composition analysis of the muscle tissue. Additionally, for gene expression analysis, hepatopancreas and muscle tissues from four shrimps per replicate, totaling sixteen per treatment, were dissected and placed individually in 500 μL of Trizol Reagent according to the manufacturer's protocol. Whole shrimps were also reserved for histological analysis. The collected data and samples were then analyzed to assess the effects of the treatments on the shrimps.

2.7. Proximate composition analysis

In the proximate composition analysis, muscle tissue from five shrimps from each treatment was used. Moisture content was measured by drying the samples in an oven at 100 °C until they reached a constant weight. This technique removes all the water content in the tissue, and the remaining dry weight can then be used to determine the moisture content. Protein was quantified using the Kjeldahl method. This technique is widely accepted and involves a three-part process: digestion, distillation, and titration. Through the digestion phase, nitrogen in the tissue is converted to ammonia, which is then distilled and titrated to determine the nitrogen content. The nitrogen content is then used to estimate the protein content. Lipid content in the samples was measured using the Soxhlet method as described in the AOAC (2000) guidelines. The technique employs an organic solvent to extract lipids from the sample. Ash refers to the inorganic residue remaining after incineration of organic substances. For this analysis, the samples were incinerated in a muffle furnace at 600 °C for 6 h. The remaining ash represents the total inorganic content, providing information about the mineral content of the muscle tissue. Phosphorus in the samples was analyzed following the method proposed by Silva and Queiroz (2002).

2.8. Gene expression analysis

RNA extraction was performed using Trizol Reagent (Invitrogen, Brazil) at a proportion of 100 mg of tissue for every 1 mL of reagent. The extracted RNA was treated with DNase I (Invitrogen, Brazil), and the concentration was determined spectrophotometrically, while quality was assessed by 1% agarose gel electrophoresis. Out of the 16 samples collected for total RNA extraction, only the top eight RNAs in terms of quality were used for complementary DNA (cDNA) synthesis. For cDNA synthesis, the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil) was used. Gene expression levels were determined by quantitative PCR (qPCR), according to Livak and Schmittgen (2001), with $n = 8$. Seven genes were analyzed in the hepatopancreas, of which five are related to digestion (amylase, *amy*; lipase, *lip*; trypsin, *tryp*; cathepsin B, *cathB*; chymotrypsin, *chymo*) (Flores-Miranda et al., 2015; Duan et al., 2018), and two related to amino acid metabolism (glutamine synthetase, *gs*; glutamate dehydrogenase, *gdh*) (Lage et al., 2018). In muscle tissue, two genes related to the antioxidant defense system were analyzed (glutathione peroxidase, *gpx*; superoxide dismutase, *sod*) (Sharawy et al., 2020). All primers used in the qPCRs are described in Table 2. Prior to gene expression analysis, the efficiency of the primers was tested in serial dilutions. The selected cDNA dilution was 1:10, and the efficiency and expression reactions were performed with the PowerUP SYBR Green Master Mix kit (Applied Biosystems, Brazil), following a program of 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 15 min. The *40S-s24*, *ef1α*, and *60S-121* reference genes were used for normalization of gene expression data. The stability of the reference genes was determined using the geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002). All reactions were performed on the QuantStudio 3 Real-Time PCR system (Applied Biosystems, Brazil).

Table 2
Analyzed genes and primers used in qPCR reactions.

Gene	Sense (5′-3′)	Antisense (5′-3′)	Amplicon (nt)	GenBank
amy	ctctggtagtgctgttggct	tgtcttacgtgggactggaag	116	AJ133526
lip	actgtctcctctgctcgtc	atggtttctggaataggtgttt	131	XM027365317
tryp	cggagagctgccttaccag	tcggggttgttcatgtcctc	141	X86369
chymo	ggctctcttcatcgacg	cgtgagtgaagaagtcgg	182	XM037943862
cathB	ggatgtaacggaggcttc	ctgtatgctttgcctcca	248	XM027359505
gdh	aggttgtggaggaccagttg	ccgtggatcatctcgtaggt	166	EU496492
gs	ttccgtctcctgaaataccg	aggagccttgggaatgaagt	193	JN620540
gpx	agggacttccaccagatg	caacaactccccttcggta	117	AY973252
sod	tggagtgaaaggctctggct	acggaggttcttgtactgaaggt	175	DQ005531
40S-s24	caggccgatcaactgtcc	caatgagagcttgccttcc	204	XM027373709
60S-l21	gttgacttgaagggcaatg	cttcttggcttcgattctg	246	XM027359925
ef1a	ccaccctggccagattca	gcgaacttgcaggcaatg	75	DQ858921

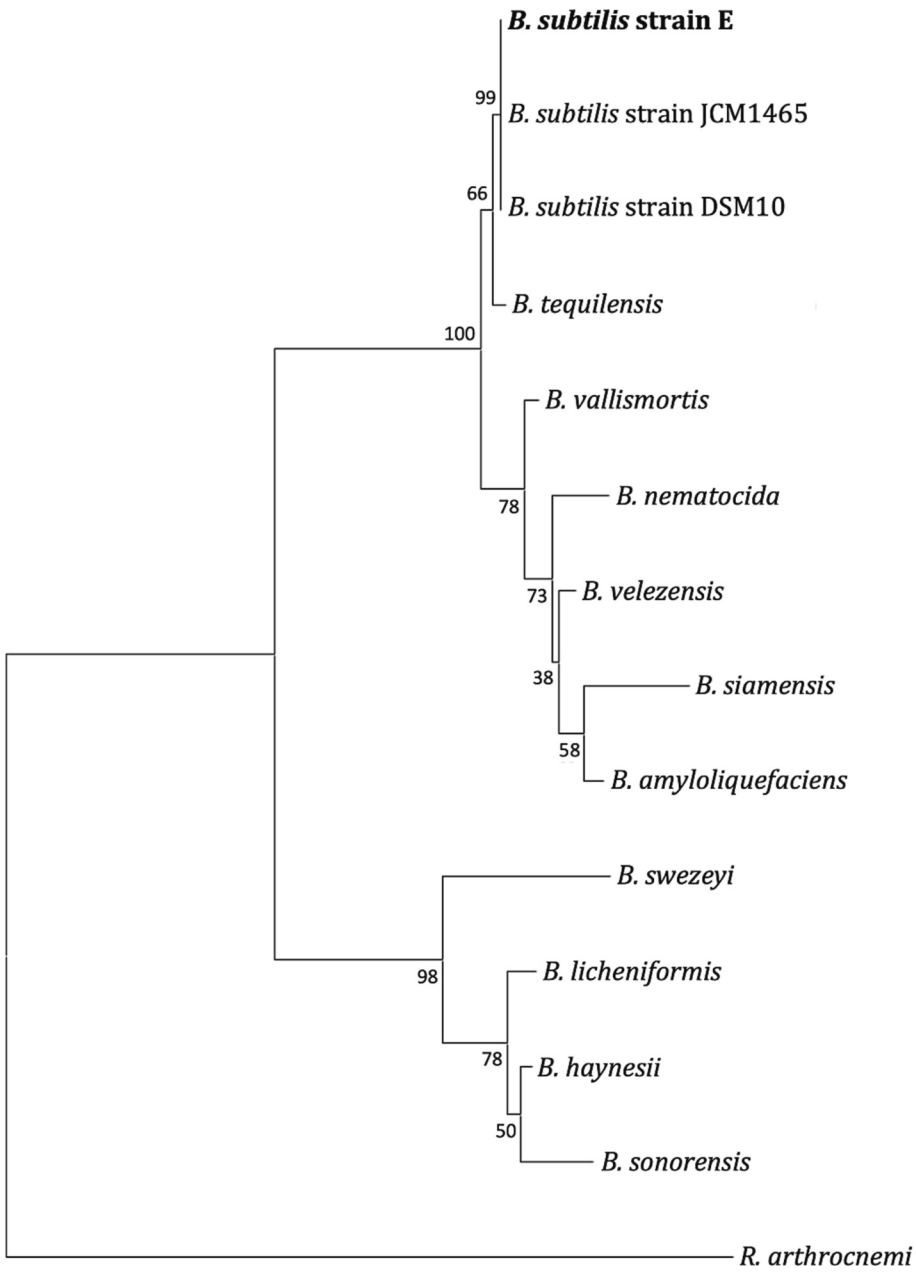

Fig. 1. *Bacillus* spp. phylogenetic tree inferred from nucleotide sequences of 16S ribossomal gene. The tree was reconstructed using the Neighbor-joining method, with the p-distance model and using 10,000 bootstraps, whose values (%) are shown at each node. *R. arthrocnemi* served as outgroup to root the tree.

2.9. Histological analysis

Whole shrimps were injected with Davidson's solution (11.5% acetic acid, 22% formalin, 33% ethanol) and maintained in this solution for 48 h. Subsequently, the hepatopancreas were dissected and transferred to a container containing 70% ethanol. Following successive dehydration steps in increasing concentrations of ethanol, the hepatopancreas were cleared in xylene and embedded in Paraplast at 60 °C. The embedded hepatopancreas were sectioned into 3 μm thick slices. Histological sections were stained with Hematoxylin and Eosin (Bell and Smith, 1993) to enumerate lipid storage cells. Lipid vacuoles were observed and counted under a compound microscope at 40× magnification.

2.10. Statistical analysis

To assess potential differences among treatments, a one-way analysis of variance (ANOVA) was employed. Normality and heteroscedasticity were evaluated using the Shapiro-Wilk and Levene tests, respectively. When significant differences among treatments were detected, post-hoc comparisons of means were conducted using Tukey's test. All numerical data are presented as mean ± standard error. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Isolation and identification of bacterial strains from the gastrointestinal tract of L. vannamei

Four strains, characterized as Gram-positive, were isolated from the gastrointestinal tract of shrimp. The PCR products of the *rnc* and *16S rRNA* genes for these four strains were sequenced. The amplicons obtained from strain E showed 100% identity with the *B. subtilis* sequences for both genes available in GenBank (data not shown). Strain E was selected for subsequent experiments because it belongs to a species recognized as Generally Recognized as Safe (GRAS) by the US Food & Drug Administration (FDA; https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras). The *rnc* (460 bp) and *16S rRNA* (1418 bp) sequences obtained from strain E were submitted to the National Center for Biotechnology Information (NCBI) and received the GenBank accession numbers OQ817988 and OQ793592, respectively.

3.2. Phylogenetic tree

The consensus phylogenetic tree is shown in Fig. 1. This tree was reconstructed with 13 sequences of the 16S ribosomal gene from 11 different *Bacillus* species, with *R. arthrocnemi* as the outgroup. *B. subtilis* was represented by three strains, strain E (the focus of this study), and strains JCM1465 and DSM10. The phylogenetic reconstruction reveals the formation of three main branches, supported by significant bootstrap values (> 50%). The first branch consists of the three *B. subtilis* strains and *B. tequilensis*. Strain E forms a sub-clade with the other two *B. subtilis* strains with a bootstrap value of 99%, definitively indicating that it belongs to the *B. subtilis* species. This first branch forms a larger, highly significant branch (bootstrap 100%) with a group consisting of *B. vallismortis*, *B. velezensis*, *B. amyloliquefaciens*, *B. nematocida*, and *B. siamensis*. The third and most distant branch (bootstrap 98%) is formed by *B. haynesii*, *B. licheniformis*, *B. swezeyi*, and *B. sonorensis*. The high bootstrap values observed in the phylogenetic reconstruction demonstrate that the choice of *R. arthrocnemi* as the outgroup for tree rooting was appropriate.

3.3. Phenotypic characterization of the strain E of B. subtilis

Strain E demonstrated positive adherence to a polystyrene microplate but did not exhibit the ability to produce exopolysaccharides (EPS). Regarding enzymatic activity, strain E did not produce gelatinase or cellulase. However, activity was detected for caseinase and amylase. Moreover, strain E did not exhibit β-hemolysis activity. Stress resistance tests revealed that strain E could grow at 40 °C but not at 4 °C. In terms of pH, strain E could grow at pH 9 but not at pH 5. With respect to salinity, strain E was capable of growing under all tested salinities (0, 30, and 80 g/L). The antibiogram demonstrated that strain E is sensitive to the antibiotics tested (nalidixic acid, chloramphenicol, florfenicol, and oxytetracycline), with inhibition diameters ≥18 mm. In the pathogen antagonism test, strain E exhibited positive antagonism against both *V. harveyi* and *V. parahaemolyticus*, as shown in Fig. 2.

3.4. Zootechnical performance and proximate composition of muscle tissue

The zootechnical performance results are presented in Table 3. There were no significant differences among the experimental groups for all variables analyzed ($p > 0.05$). As shown in Table 4, no significant differences were detected among the experimental groups with respect to body moisture, crude protein, ash, and phosphorus ($p > 0.05$). However, shrimp fed the diet supplemented with strain E had a significantly higher lipid content compared to those fed the diet supplemented with the KM0 strain ($p < 0.05$).

3.5. Relative expression of genes related to digestion

The relative expression of *cathB*, *chymo*, *tryp*, *lip*, and *amy* genes is presented in Fig. 3. Only the expression of the *amy* gene remained unchanged with the addition of *B. subtilis* strains to the diet. The expression levels of the *chymo*, *tryp*, and *lip* genes were significantly reduced ($p < 0.05$) in both the Feed+KM0 and Feed+E treatments. In contrast, the *cathB* gene showed reduced expression only in the Feed+E treatment ($p < 0.05$) when compared to the Feed+KM0 treatment.

3.6. Relative expression of genes related to amino acid metabolism

The expression levels of *gs* and *gdh* genes are depicted in Fig. 4. The expression of the *gdh* gene was reduced in both treatments with *B. subtilis* strains ($p < 0.05$), whereas the *gs* gene exhibited reduced expression only in the Feed+E treatment ($p < 0.05$).

3.7. Relative expression of genes related to antioxidant defenses

The expression of *gpx* and *sod* are shown in Fig. 5. The treatments with the strains of *B. subtilis* did not produce alterations in the two analyzed genes ($p > 0.05$).

3.8. Histology of the hepatopancreas

The result of lipid vacuole counts in shrimp hepatopancreas is shown in Fig. 6. Shrimp fed with diet supplemented with both *B. subtilis* KM0 and *B. subtilis* strain E had a statistically higher number of lipid vacuoles (31.55 ± 0.74 and 32.68 ± 1.89, respectively) compared to the control treatment (Feed; 29.28 ± 1.13).

4. Discussion

In the first stage of this study, a bacterial strain with probiotic potential was isolated from the gastrointestinal tract of shrimp L. *vannamei*. This strain, named strain E, was molecularly and phylogenetically identified as a strain of *B. subtilis*, a species recognized as GRAS by the US FDA. Considering that probiotic bacteria tend to adapt to specific environments due to the presence of genes related to adaptability, it can be concluded that probiotics applied in shrimp farming isolated from shrimp itself can have a greater impact on the benefits already known to be produced by such bacteria. In fact, the isolation of probiotics from shrimp has been shown to be a valid practice for shrimp farming,

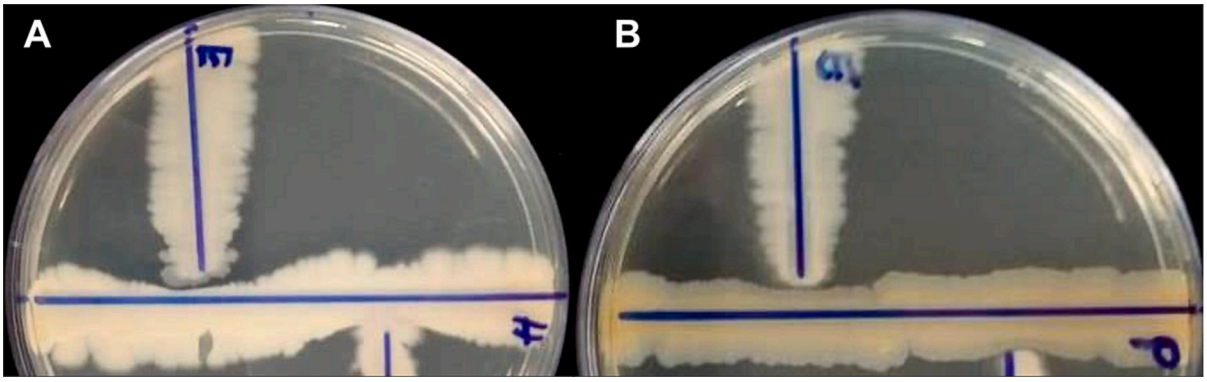

Fig. 2. Antagonism test of *Bacillus subtilis* strain E (vertical streak) to *Vibrio harveyi* (horizontal streak in A) and *Vibrio parahaemolyticus* (horizontal streak in B).

Table 3
Zootechnical performance of *Litopenaeus vannamei* fed with commercial feed or commercial feed supplemented with different *Bacillus subtilis* strains.

	Feed	Feed+KM0	Feed+E
Initial weight (g)	0.61 ± 0.06	0.62 ± 0.05	0.66 ± 0.14
Final weight (g)	5.44 ± 0.49	5.09 ± 0.45	4.90 ± 0.53
Weight gain (g)	4.84 ± 0.49	4.47 ± 0.42	4.27 ± 0.66
Feed conversion ratio	1.39 ± 0.13	1.50 ± 0.15	1.60 ± 0.24
Survival	100%	100%	100%

Table 4
Proximate composition of *Litopenaeus vannamei* muscle fed with commercial feed or commercial feed supplemented with different *Bacillus subtilis* strains. Moisture, ash, proteins and lipids are expressed as g/100 g of wet matter. Phosphorous is expressed as mg/100 g of wet matter.

	Feed	Feed+KM0	Feed+E
Moisture	74.56 ± 0.63	73.97 ± 0.22	74.77 ± 0.82
Ash	1.63 ± 0.27	1.53 ± 0.23	1.54 ± 0.39
Protein	18.97 ± 1.74	19.26 ± 0.44	18.86 ± 0.87
Lipid	0.75 ± 0.65^{ab}	0.47 ± 0.43^{a}	1.22 ± 1.56^{b}
Phosphorus	79.24 ± 9.03	98 ± 1.41	102.86 ± 5.79

Different letters represent statistically significant differences ($p < 0.05$).

enhancing the control or inhibition of pathogenic bacteria, zootechnical performance, digestive enzyme activity and host immune responses against pathogens or physical stress (Zuo et al., 2019; Kim et al., 2020; Wang et al., 2020; Li et al., 2021).

Strain E was phenotypically characterized in terms of its probiotic potential. First, it is necessary to assess the presence of certain virulence factors in candidate probiotic strains. One of these is gelatinase activity. This enzyme is an extracellular endopeptidase that promotes the hydrolysis of bioactive compounds such as gelatin, collagen and hemoglobin, being considered a harmful enzymatic activity in strains intended for use in aquaculture (Muñoz-Atienza et al., 2013). Strain E did not show gelatinase activity, which is a positive characteristic of this strain. Another important virulence factor is the β-hemolysis activity, which makes iron available to the microorganism and can cause anemia and edema in the case of a vertebrate host (Vesterlund et al., 2007). Strain E also did not show β-hemolysis activity, which is another positive factor to be considered. In addition to the virulence factors that need to be absent, the probiotic strain must also have additional characteristics, such as the expression of extracellular enzymes and adhesion to surfaces. The presence of enzymes such as caseinase and amylase, for example, is a positive factor for probiotic candidates, as it makes nutrients bioavailable, aiding in the digestive processes of the hosts (Banerjee and Ray, 2017). Strain E showed both caseinase and amylase activity, making it a candidate for a probiotic that can help in the digestive processes of shrimp.

Although strain E does not produce exopolysaccharides (EPS), it was able to adhere to the polystyrene plate. Adhesiveness is an important feature in probiotic candidates, as it is well established that bacteria capable of colonizing the surface of the intestinal mucosa are more crucial in maintaining nutrition, physiology and animal immunity than are free-living bacteria (Banerjee and Ray, 2017). Bacterial adhesion can occur non-specifically, when based on physicochemical factors, or specifically, involving adhesin molecules on the surface of adherent bacteria and receptor molecules on epithelial cells (Salminen et al., 1996). Furthermore, the adherence to the intestinal tract of hosts by probiotic

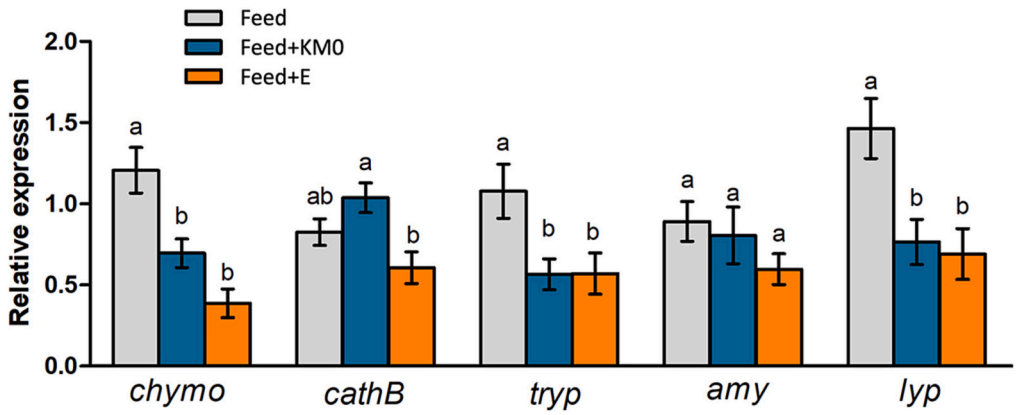

Fig. 3. Relative expression of genes related to digestion in *Litopenaeus vannamei* hepatopancreas fed diets supplemented with different strains of *Bacillus subtilis*. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *B. subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. One-way ANOVA was used for each gene separately. Different letters represent statistically significant differences ($p < 0.05$).

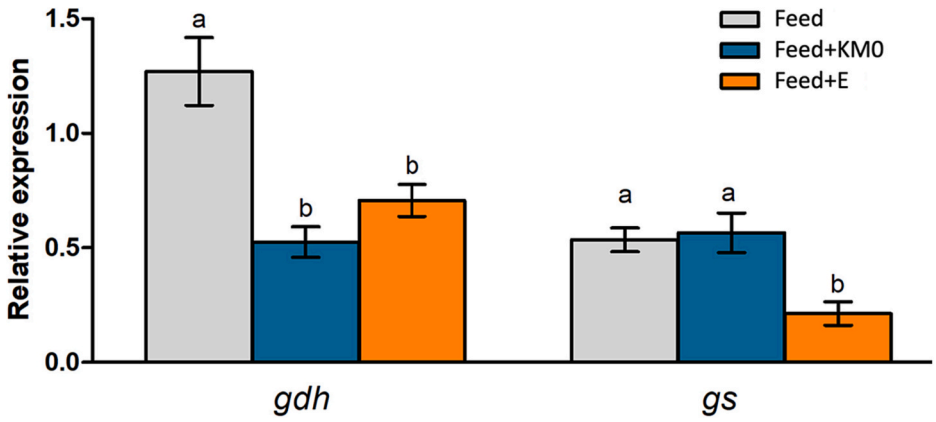

Fig. 4. Relative expression of genes related to amino acid metabolism in *Litopenaeus vannamei* hepatopancreas fed diets supplemented with different strains of *Bacillus subtilis*. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *B. subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. One-way ANOVA was used for each gene separately. Different letters represent statistically significant differences ($p < 0.05$).

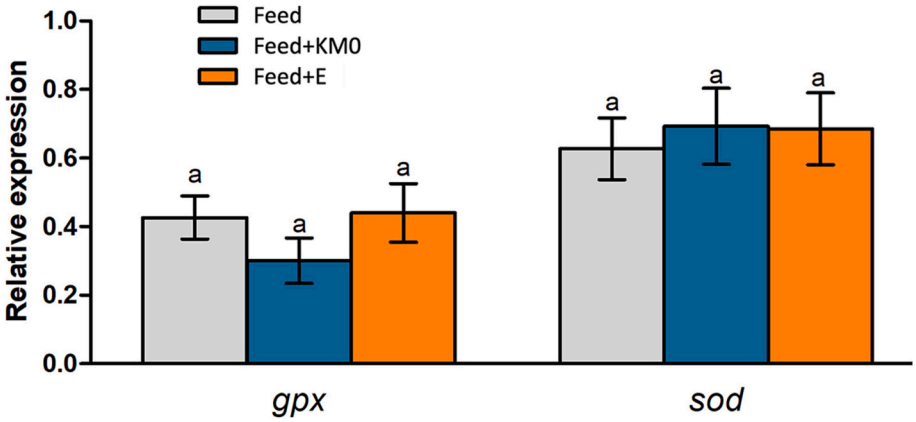

Fig. 5. Relative expression of genes related to antioxidant defenses in *Litopenaeus vannamei* muscle fed diets supplemented with different strains of *Bacillus subtilis*. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *B. subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. One-way ANOVA was used for each gene separately. Equal letters represent the absence of statistical differences ($p > 0.05$).

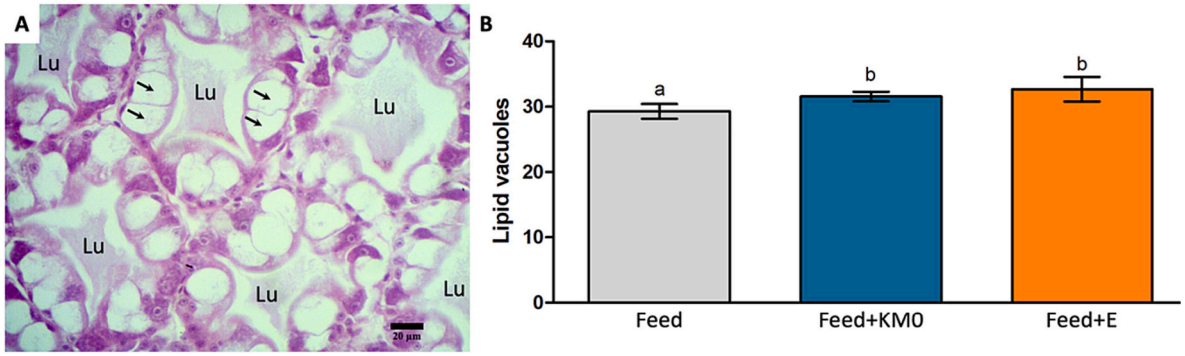

Fig. 6. Histological analysis of *Litopenaeus vannamei* hepatopancreas. A) Illustrative photo of the histological sections of shrimp hepatopancreas (40× magnification). Lu: lumen of the hepatopancreatic tubules; arrows indicate some examples of lipid vacuoles; B) Graphic representation of lipid vacuole counts. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *Bacillus subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. Data are expressed as mean ± standard error from four independent replicates. Different letters represent statistically significant differences (One way ANOVA; $p < 0.05$).

bacteria creates a competitive exclusion, preventing the establishment of pathogenic bacteria and reducing their harmful effects (Vieira and Pereira, 2016).

The stability of bacterial strains is an important factor for survival in a culture environment and for colonizing the host's gastrointestinal tract. *Bacillus* species are known to withstand high temperatures and drying processes, which makes them widely used as probiotics in shrimp feed (Yu et al., 2009). Stress tolerance tests showed that strain E can grow at high temperature (40 °C), as well as at basic pH and in a wide range of salinity (from 0 to 80 $g.L^{-1}$). Additionally, this strain was sensitive to different antibiotics, which demonstrates the absence of resistance genes. This feature is important because it reduces the possibility of the occurrence of horizontal transfer of genes related to antimicrobial resistance to other microorganisms present in the

environment. Also, strain E showed positive antagonism against *V. harveyi* and *V. parahaemolyticus*. *Vibrio* species are widely distributed in marine and estuarine environments and can act as agents of foodborne diseases, impacting public health [48]. In aquaculture, *V. harveyi* and *V. parahaemolyticus* have been considered as opportunistic pathogens, which can affect a range of marine species, causing high mortality in crops and may even affect the health of consumers (Cheng et al., 2010; Zokaeifar et al., 2012a, 2012b; Vieira and Pereira, 2016). Hostins et al. (2017) showed that the addition to water of a mixture of commercial probiotics containing *Bacillus* species decreased the abundance of *Vibrio* sp. in the gastrointestinal tract of L. *vannamei* raised in biofloc (BFT) or clear water. Thus, strain E can be considered an alternative tool for combating and preventing vibriosis that commonly affect shrimp farms.

In the second stage of this study, an experiment was carried out with L. *vannamei*, where three experimental groups were evaluated for 45 days. The first group of shrimps was fed only commercial feed. The second group was fed a commercial feed supplemented with the KM0 strain, originated from *B. subtilis* 168 and considered as a positive control. The third group was fed with feed supplemented with strain E. After 45 days, no difference was observed among the experimental groups in terms of zootechnical performance. Toledo et al. (2019) demonstrated, through meta-analysis, that the impact of probiotics on shrimp growth performance is not always positive, as the beneficial effects of probiotics are influenced by several related factors such as rearing conditions, method of administration, dosage, probiotic strain, and shrimp species. Thus, it can be inferred that strain E does not affect shrimp growth as strain KM0, already recognized for its probiotic potential.

The analysis of the proximate composition of the muscular tissue of the shrimps in group E showed a significant increase of 2.6 times in the amount of lipids, when compared to the KM0 group. Likewise, the count of lipid vacuoles in the hepatopancreas showed that both strains of *B. subtilis* used in the present study increased the concentration of lipids in that tissue. Although, in the present study, a test of lipolytic activity of strain E was not performed, it is known that *B. subtilis* secretes two types of lipases (*lipA* and *lipB*), whose genes are differentially expressed depending on the growth conditions (Eggert et al., 2003). The observation that shrimp fed with E strain is favoring the concentration of lipids in both muscle and hepatopancreas may be an interesting feature from a commercial point of view, since shrimps have been considered an important source of functional lipids, especially phospholipids (Sun et al., 2020). Interestingly, Tsai et al. (2019) reported that *Bacillus subtilis* E20 increased apparent digestibility coefficients of L. *vannamei*, showing that supplementation with the probiotic enhanced the absorption of nutrients with consequent increase in growth performance. However, the authors did not observe an increase in the concentration of lipids in the shrimp tissues. This observation shows that each probiotic strain can generate specific effects on its hosts.

To evaluate the response of the shrimp to the addition of *B. subtilis* strains in the diet, the expression of genes related to digestion, amino acid metabolism, and antioxidant defenses were quantified. Relative expression of genes related to antioxidant defenses (*gpx* and *sod*) were not altered by treatments with probiotics. However, the digestion-related genes showed an almost homogeneous expression pattern of decreased transcriptional activity. Except for *amy*, which was not altered by the treatments, all the others had their expression reduced with the addition of probiotics in the shrimp diet. The *chymo*, *tryp* and *lip* genes were significantly downregulated in both probiotic treatments. As previously mentioned, *Bacillus* species are capable of producing and secreting lipases. Likewise, these bacteria can also secrete high amounts of proteolytic enzymes. According to Contesini et al. (2018), bacteria of the genus *Bacillus* are among those that most produce and secrete proteases with outstanding properties such as high stability in adverse environmental conditions such as extremes of temperature and pH, being resistant to the presence of organic solvents, detergents, and oxidizing agents. In a scenario where probiotic bacteria produce and secrete significant amounts of proteases and lipases, it is expected that the host intestine will decrease the production and secretion of such enzymes, starting with a decrease in the transcription rate of endogenous related genes. An interesting difference between the strain KM0 and the strain E was observed in the expression of the *cathB* gene, which encodes Cathepsin B. This cysteine protease was first identified in L. *vannamei* by Stephens et al. (2012). It is an enzyme that participates not only in the intracellular hydrolysis of proteins, but also in the extracellular hydrolysis of proteins soon after food ingestion, increasing the capacity of amino acid absorption by the intestine. The fact that the *cathB* gene was significantly reduced in shrimp treated with strain E in relation to strain KM0 indicates an interesting difference between the two probiotic strains. Although there is a lot of information about the production of proteases in *Bacillus* species, the opposite occurs when it comes to a more specific group such as cysteine proteases. Recently, Yamazawa et al. (2022) identified the *yabG* gene product of *B. subtilis* as a cysteine peptidase, which is related to spore formation. Thus, *B. subtilis* is also capable of producing and secreting cysteine proteases, and strain E seems to differ from strain KM0 in this specific characteristic.

Regarding genes related to amino acid metabolism, the *gdh* gene had its transcription rate downregulated in shrimp hepatopancreas in both treatments with probiotics. The enzyme glutamate dehydrogenase is present in the mitochondrial matrix of eukaryotic cells and catalyzes the oxidative deamination of glutamate to form α-ketoglutarate which can be used as fuel for the Krebs cycle to drive the electron transport chain and production of ATP by oxidative phosphorylation (Dawson and Storey, 2012). Furthermore, this enzyme is linked to several cellular processes, including ammonia metabolism, acid-base balance, redox homeostasis, lactate production, and lipid biosynthesis via oxidative generation of citrate (Plaitakis et al., 2017). Considering that glutamate dehydrogenase induces the production of Krebs cycle intermediates, and that one of these intermediates (citrate) is used for the synthesis of lipids, it is reasonable to hypothesize that the decrease in the transcription of the *gdh* gene is a response to an already high concentration of lipids in the hepatopancreas of shrimp that were treated with the probiotic strains. Another gene related to amino acid metabolism analyzed in the present study was *gs*. This gene encodes the enzyme glutamine synthetase, which catalyzes the formation of glutamine from glutamate and NH_4^+. Qiu et al. (2018) considered this enzyme as an important marker of ammonia stress in L. *vannamei*. These authors demonstrated that expression of *gs* is increased in shrimp exposed to high concentrations of ammonium and that hepatopancreas plays a key role in the response to stress caused by excess nitrogen. In the present study a significant decrease in *gs* expression was observed only in the hepatopancreas of shrimp treated with strain E, and this is another interesting difference between the two probiotic strains studied here. It is possible that the strain E has a greater capacity than strain KM0 to reduce the nitrogen compounds present in the rearing water and, thus, reduce the stress in shrimp. In fact, it is well established that the administration of *B. subtilis* strains confers benefits in maintaining the quality of rearing water in aquaculture (for review see Hlordzi et al., 2020).

New strains of *B. subtilis* have been reported for their distinct characteristics and potential applications in various fields. Ishnaiwer et al. (2022) reported two new strains of *B. subtilis* (CH311 and S3B) isolated from dog and snail feces, respectively, with strong in vitro activity against Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli*. Du et al. (2019) isolated strain WS-1 from pig feces and demonstrated its ability to inhibit pathogenic *E. coli* in vitro, as well as reduce diarrhea and mortality in newborn pigs when administered orally. New strains of *B. subtilis* have also been reported for the control of fruit diseases. Yánez-Mendizábal et al. (2011) showed that strain CPA-8 has in vitro activity against various fungi that attack fruits such as oranges, apples, and stone fruits after harvest. In the present study, a strain of *B. subtilis* (strain E) was isolated from the gastrointestinal tract of L. *vannamei*, which has several phenotypic characteristics that classify it as

a potential probiotic, with particular emphasis on its strong antagonistic activity in vitro against two *Vibrio* species. The supplementation of the feed with strain E did not change the zootechnical performance of the shrimp but increased the lipid concentration in muscle and hepatopancreas. Additionally, exposure to the strain E strongly decreased the expression of genes related to digestion and amino acid metabolism, suggesting that this potential probiotic can facilitate digestive processes and nutrient absorption. Thus, strain E can be considered an interesting tool in shrimp farming in terms of better use of nutrients present in the feed.

Ethics statement

According to Brazilian Federal Law No. 11,794/08, there is no need for permission from the Ethics Committees in Animal Experimentation to carry out experiments involving invertebrate animals. In any case, the experiments carried out here followed the main guidelines of the Declaration of Helsinki.

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CRediT authorship contribution statement

Luiza Medeiros: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Laura Dall'Agno:** Investigation. **Jade Riet:** Investigation. **Bruna Nornberg:** Investigation, Formal analysis, Writing – review & editing. **Raíza Azevedo:** Investigation, Formal analysis, Writing – review & editing. **Arthur Cardoso:** Investigation. **Jéssica Lucinda Saldanha da Silva:** Investigation. **Oscarina Viana de Sousa:** Investigation. **Victor Torres Rosas:** Investigation. **Marcelo Borges Tesser:** Investigation. **Virgínia F. Pedrosa:** Investigation. **Luis A. Romano:** Investigation. **Wilson Wasielesky:** Conceptualization, Resources, Writing – review & editing. **Luis F. Marins:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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