Bacillus siamensis* I7B strain as heavy metal quencher and probiotics; isolated from gut of *Stolepherous commersonni

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Abstract

Probiotic products have been administered in food, medicine, dairy and fermentation industry to enhance the beneficial functions in the host. In the present study gut bacteria of *stolepherous commersonii* have been isolated and subjected to screening for heavy metal resistance. A potent heavy metal resistant bacterial strain of gut microbiota was subjected to assessment for its probiotic and antioxidant potential. Four bacterial strains namely I7A, I7B, I7C, and I8 were isolated and the strain I7B showed heavy metal resistant potential (upto 20 mg/ml of Cr, Cr, and Pb). The strain I7B showed high sensitivity to Streptomycin (10 µg), low sensitivity to Rifampin (5 µg) and resistant to pencillin (2 µg, 10 µg). Atomic absorption spectrometry studies showed that the I7B strain possess maximum heavy metal (Cu) removal ability of 81.64 % in textile dye effluent and the strain was identified as *Bacillus siamensis* by 16S rRNA gene sequencing studies. Hence I7B strain was named as *Bacillus siamensis* I7B and has been submitted in NCBI with accession number MN404539. *Bacillus siamensis* I7B strain possess high acid tolerance (90.9 %), high bile tolerance (96.9 %) and nonhemolytic. The I7B strain showed auto aggregation ($28 \pm 0.011\%$) and coaggregation potential ($27 \pm 0.16\%$) which could be a feasible characteristic of potent probiotic strain. Cell free Supernatant of *Bacillus siamensis* I7B strain possess good DPPH free radical scavenging ability ($59.43 \pm 0.002\%$) **and moderate hydroxyl radical scavenging ability** ($27.6 \pm 0.010\%$). Biopreservation effect of *Bacillus siamensis* I7B strain on tomato puree showed 72 % growth inhibition against *A. niger* for 15 days.

Introduction

Food contamination is the global concern worldwide. Heavy metal defile is caused by 3 main routes via ingestion, inhalation and dermal contact (Abtahi et al. 2017). Still, beyond 90% of heavy metal defile in human exposure is through food contamination. These heavy metals find accumulated in rice, vegetables, and fish. The consumption of contaminated fish or substances may enter the food chain pathway resulting in adverse effects in biota (Al-Saleh and Abduljabbar 2017). Among several crops, rice can predominantly accumulate heavy metals leading to critical human health risks (Djahed et al. 2018). Cd, As, Cr, Ni, pollution in food is owing to administration of contaminated water (Omar et al. 2015), metal-based pesticides, and chemical fertilizer (Djahed et al. 2018) in agriculture. Approximately 90% of total metal intake in humans is because of consumption of heavy metal befouled vegetables (Ametepey et al. 2018; Pajevic et al. 2018). Cd, Al, Cr, and As contamination was found in cereals and vegetables. Accumulation of heavy metals in human organs and tissues end up in the kidney, cardiovascular system, and nervous system toxicity (Owolabi and Hekeu 2010).

Studies confirmed administering probiotics can protect animals and humans against adverse health effects caused by food contamination by maintaining the gut microbiota (Abdel-Megeed 2020), which aid in reducing oxidative stress (Chattopadhyay et al. 2019), stimulation of gene expression in host, increasing the intestinal barrier (Duan et al. 2020), and at last enhancing the host ability to assimilate xenobiotics (Wang et al. 2018). Lactobacillus strains were proved to reduce chronic and acute heavy metals poisoning (Reddivari et al. 2017).

Probiotics are live microorganisms with beneficial activity to living organism when administered in adequate quantity (Hill et al. 2014). In trend, probiotics and their metabolic compounds have become a global research hotspot and various probiotic products have been administered in food, medicine, dairy and fermentation industry to enhance the beneficial functions in the host (Abatenh et al. 2018; Day et al. 2019). Probiotics have been isolated from fruit juices, grains, honeycomb, and soil, fish, beef, pork (Siripornadulsil et al., 2014), salted crab (Senthong et al. 2012), and seafood (Nanasombat et al. 2014). In dairy industry probiotics are the sources from cheese, yogurt (Mahmoudi et al 2014), and milk. Some groups of probiotics belonging to the genera of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Bacillus*, *Clostridium*, *Bacteroides ovatus* (Shen et al. 2018), *Bacteroides xylanisolvens* (Tan et al. 2019), *Bacteroides fragilis* (Tan et al. 2019), *Akkermansia muciniphila* (Zhai et al. 2019), and *Faecalibacterium prausnitzii* (Wong et al. 2019) (Martin et al. 2017). LAB is found to be the major group of probiotics and among the LAB group *acidophilus*, *paracasei*, *casei*, *plantarum*, *crispatus*, *reuteri*, *rhamnosus*, *gasseri* and *bulgaricus* were widely used. *Bifidobacterium* and *Lactobacillus* are most familiar probiotics among LAB. *Pediococcus pentosaceus* is predominant in thai fermented foods containing fish and pork (Siripornadulsil et al. 2014). Probiotics are found to possess the following protective functions like Anti-pathogenic, anti-diabetic, anti-obesity, anti-inflammatory, anti-cancer, anti-allergic, and also have potential in maintaining urogenital health care (Kerry et al. 2017). Administration of probiotics by food and pharmaceutical industry is just adaptable due to supplementation through drugs, foods, dietary supplements, infant formulas and animal feed (T'elessy 2019).

Lactic acid bacteria (LAB) are known to produce a range of antifungal agents such as alcohols, lactic acid, acetic acid, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, phenyl derivatives, hydroxy fatty acids, peptides and bacteriocins against pathogenic infections (Ortiz-Rivera et al. 2017). Among the LAB isolates, *Lactobacillus kunkeei* in giant honey bees showing antagonist effect against yeast spoilage in honey. Moreover, administration of probiotic in bio preservative perspective not only preserve the food but also enhance the additional health benefits to the host. Probiotics resident in gut and food matrix, have the capacity to produce vitamins with good antimicrobial activity. Vitamin C produced by probiotics elevate the lipid acidity in the bacterial cell membrane, causing cleavage in the cell membrane and bacterial cell wall (Pedros-Garrido et al. 2019), significant in food industry for food preservation to increase the shelf life of the food samples.

The rich nutrient of the fish digestive tract gives a nourishing environment for the growth of bacterial cultures. In the digestive tract, the resident bacteria exhibit the wide and enzymatic potential which plays a major role in fish digestion (Kavitha et al. 2018). The gut bacteria can produce various enzymes, like proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes, which are responsible for the digestion of proteins, carbohydrates, cellulose, lipids, and chitin in the host (Ray et al. 2012) and provide the nutritional benefits to the host (Dutta et al. 2015).

In the aquatic system, the functioning of gut microbiota is similar to the terrestrial mammals (Talwar et al. 2018). The main aim to isolate the gut bacteria of fish is to ease the selection of probiotics, prebiotics and their compounds to improve the gut balance, nutritional performance and host health (Wang et al.

2018). The production of various chemical like caustic soda and fertilizers by various industries in the coastal area discharge the wastewater without proper treatment into the sea through canals. The external conditions like poor water quality create stress in fish and modifies the composition of gut microbes (Hossain et al. 2013; Sihag et al. 2012). Heavy metal resistant bacteria from marine environment have showed good resistance to heavy metal and many antibiotics (Nithya et al. 2011). For more than a decade, gut microbiota had gained interest of many researchers worldwide, which gives the biological interactions of harbored microbes and the intestinal function (Jandhyala et al. 2015). Hence, many studies focus on isolating indigenous and exogenous microbiota of aquatic animals with probiotic functionality (Reddy 2015).

However, it is apparent that attempt has not been made so far to isolate bacteria from the gut of edible fish (*Stolepherous commersonnii*). To make use of the valuable quantity of gut microbes of *Stolepherous commersonnii*, this study has been designed to isolate and characterize the gut bacteria of *Stolepherous commersonnii* as probiotics. Four indigenous bacterial strains namely I7A, I7B, I7C and I8 have been isolated from the gut of edible marine fish (*Stolepherous commersonnii*), and their probiotic properties were characterized.

Materials And Methods

Sample collection

The Commerson's anchovy (*Stolephorous commersonnii*) with an average weight of 50g was collected from the Thoothukudi marine region, Southeast coast of Tamil Nadu, India. The study area is located between 8°19' - 9°22'N latitude and 77°40' - 78°23'E longitude.

Isolation of gut bacteria

The fish is washed with sterile water and then carefully placed aseptically within laminar air flow chamber on ice slabs. The fish gut was dissected out and homogenized with 5 ml of normal saline under sterile condition. Fungal contaminants were removed by placing the homogenate in the boiling water bath at 80°C for 20 min. The homogenate was serially diluted with PBS to 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilution, and spread plate using L rod in the nutrient agar media. Plates were incubated at 37°C for 24 – 48 h. Individual colonies were picked out and purified using quadrant streaking method in the nutrient agar media. The pure isolates were revived using nutrient broth and stored at -80°C in 40% glycerol (Kavitha et al. 2018).

Screening for heavy metal resistance

For the selective screening of heavy metal resistant bacteria, the isolates were screened for heavy metal resistance using the agar diffusion method using Marzan et al. (2016). Briefly, the purified bacteria were

streaked in the plates containing 20 ml of minimal agar medium supplemented with different concentrations (10, 20, 30, 40, 50 and 60 mg/ml) of chromium, cadmium, copper and lead. The plates were incubated at 37°C for 48h. The potent isolate is chosen for further studies.

Bioremediation of heavy metals in textile dye effluent

Heavy metals present in solution were identified by flame atomic absorption spectrometer (AAS, model 220 AA B6; Varian). The bioremediation of Heavy metals was analysed in textile dye effluent collected from Arulpuram Common Effluent treatment plant, Tirupur. Four isolates I7A, I7B, I7c and I8 were inoculated in 20 ml of nutrient broth at 37°C for 24 h. 5 ml of culture was inoculated in 100 ml of sterilized textile dye effluents and incubated for 32 h and the sample was centrifuged at 9500 rpm for 10 min. The supernatant was analysed for heavy metal removal by atomic absorption spectrometry. The heavy metal concentrations were evaluated using appropriate blanks and standards for calibration (Jafarian and Ghaffari 2017). The result was interpreted using the formula as follows:

$$\% \text{removal} = \frac{\text{Heavy metal utilized (ppm)}}{\text{Heavy metals in textile dye effluent (ppm)}} \times 100$$

The heavy metal utilized = Heavy metals present in the effluent before treatment (ppm) – Heavy metal present in the effluent after treatment (ppm)

Morphological characterization of potent isolate

Morphological characteristics of potent isolate I7B was assed using gram staining and FESEM analysis. The potent isolate I7B was grown in nutrient broth at 37°C overnight in a rotary shaker. Gram-staining was performed using the method described by Ajayi et al. (2017). FESEM analysis is performed using Steffi et al. 2021. In brief, the potent isolate I7B grown mid-log phase is pelleted by centrifugation using 10,000 rpm for 10 min. The cells were fixed using 2.5% glutaraldehyde for 1 h, air dried and dehydrated using ethanol gradient. The cells were visualized in QUANTA – 250 FEG electron microscope.

Identification of potent isolate

The genomic DNA of the potent isolate I7B were isolated by HiPurA® Bacterial Genomic DNA purification kit. The purity of the DNA was checked using nanodrop and agarose gel electrophoresis. The isolated DNA were amplified by PCR using PCR master mix (Qiagen) and universal primers forward (518F) 5' CCAGCAGCCGCGGTAATACG- 3' and reverse primer (800R) 5' 34 TACCAGGTATCTAATCC-3', under the following conditions; initial denaturation 94°C for 5 min, followed by 25 cycles each consisting of 94°C of 2 min, 55°C for 1 min and 72°C for 2 min, following a final extension step at 72°C for 10 min. The PCR products were analyzed using agarose gel electrophoresis. The amplified DNA were sent for 16s rRNA sequencing. The obtained nucleotide sequences were compared with the available sequences in the

National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed with MEGA 7 software by the neighbor-joining method. The sequences were deposited in the NCBI GenBank (Adeoti et al. 2021).

Antibiotic susceptibility assay

The selected potent strain was tested for the antibiotic susceptibility against the selected antibiotics using the disc diffusion method. Six different antibiotic discs were used, namely Erythromycin: 15 µg/disc, tetracycline (30 µg), streptomycin (10 µg), kanamycin (30 µg), penicillin (10µg and 2 µg), and Rifampin(5µg). Antibiotic discs were placed on the Muller-Hinton agar plates and incubated at 37°C for 24 h. The antibiotic sensitivity of bacteria towards antibiotics was recorded by measuring the Zone of inhibition (mm) (Kavitha et al. 2018).

Screening of probiotic potential

The acid tolerance test

The tolerance of the isolate to acid were estimated by previous report by R.P Shastry et al. 2021. The potent strain grown overnight (approximately 1×10^8 CFU/ml) is inoculated into 5 ml of tryptone soya broth, and the pH was adjusted to 1.5 and 3.0 using hydrochloric acid. The culture was then incubated at 37°C and the viable bacterial counts were measured and depicted as bacterial growth count at 0, 1, 2, and 3 h time interval on nutrient agar plates.

$$\text{Cellviability\%} = \frac{\log\text{CFUofviablecellssurvived}}{\log\text{CFUofinitialcells}} \times 100$$

Assay of Bile salt tolerance

The bile tolerance test was carried out according to previous report by Chen Pei et al. 2014. Firstly, aliquot (approximately 1×10^8 CFU/ml) of potent strain grown overnight culture was inoculated into 10 ml nutrient broth which is supplemented with and without 0.3% and 0.7% bile salt and cultures were then incubated at 37°C. The growth medium with 0% bile salt served as control. The absorbance was measured at OD₆₀₀ for the time intervals 0h, 2 h, 4h and 24 h and was estimated against the corresponding uninoculated blank samples. Survivability of the isolates was represented by the following formula:

$$\text{CellViability(\%)} = \frac{\log\text{CFUofviablecellswithbile}}{\log\text{CFUofviablecellswithoutbile}} \times 100$$

Aggregation activity

Aggregation activity consisting of both auto-aggregation and co-aggregation was estimated in accordance with the method reported by Li Y et al. (2019). Pathogenic strain, *Staphylococcus aureus* and the potent strain was incubated separately at 37°C for 18 h and then the supernatant was harvested by centrifugation at 10,000 rpm for 10 mins. The cells were washed twice and resuspended in PBS of approximately 1×10^8 CFU/ml was used for the experiment. For determining auto-aggregation ability, 4 ml of the isolate suspension was individually added into sterile tubes and suspended thoroughly. The tubes were placed at room temperature with no agitation and a 150 µl aliquot of the upper suspension was taken after an incubation of 5 hours. The absorbance was determined at 600nm by using a microplate reader. Percentage Auto aggregation was calculated using the formula:

$$\text{Autoaggregationpercentage(\%)} = \frac{(A_0 - A_t)}{A_0} \times 100$$

Where A₀ corresponds to the absorbance at 0 h, and A_t corresponds to the absorbance of upper suspension after 5h.

For determining the co-aggregation of potent strains with the pathogens, equal amounts (2 ml) of isolates and pathogen cultures were mixed in sterile tubes and vortexed thoroughly. These tubes were placed at room temperature with no agitation and a 150 µL aliquots of upper suspension was taken at a time interval of 0 and 4 h and the absorbance was determined at 600nm absorbance by using a microplate reader. Coaggregation percentage was calculated as follows

$$\text{Coaggregationpercentage(\%)} = \frac{((A_{\text{pat}} + A_{\text{probio}}) - 2 \times A_{\text{mix}})}{(A_{\text{pat}} + A_{\text{probio}})} \times 100$$

Where A_{pat} is the absorbance of the pathogens and A_{probio} are the absorbance of the isolate at 0 h and A_{mix} is the absorbance of the mixed suspensions at 4 h.

Hemolytic activity assay

Hemolytic activity assay was conducted for the potent strain following the method reported by Samson J S (2020). Blood agar plates supplemented with 5% goat blood were streaked with the isolates and incubated for 48 h at 37°C. After incubation the hemolytic zones were observed. A presence of clear halo around the colonies indicates positive hemolysis (Beta- hemolysis) and a greenish zone around the colonies indicate negative hemolysis (Alpha-hemolysis) or the absence of clear zones around the colonies indicate no hemolysis (Gamma-hemolysis).

Free radical scavenging ability of selected strain

DPPH radical scavenging assay

The capability of the potent strains to scavenge DPPH was examined by Chen Pei et al. (2014). 1 ml of intact cells and CFS was mixed separately with 0.2 mM of freshly prepared DPPH solution and left for reacting for a time period of 30 min in dark. Deionised water was used as the blank. The decrease in absorbance at 517 nm reveals the scavenging of DPPH using the isolates. The ability to scavenge was calculated as follow:

$$\text{DPPHscavenging\%} = 1 - \frac{\text{sampleOD}}{\text{blankOD}} \times 100$$

Hydroxyl radical scavenging ability

The ability of the potent strains to scavenge hydroxyl radicals was estimated by Chen Pei et al. (2014). 1 ml intact cells and CFE was mixed separately in 1 ml of 2.5 mM 1, 10-Phenanthroline, 1 ml of PBS (pH 7.4), and 1 ml of 2.5 mM FeSO₄. Addition of 1 ml of 20 mM H₂O₂ starts the reaction and then the mixture is incubated at 37°C for 90 mins. The ability of the isolates to scavenge hydroxyl radical was then determined by measuring the increase in absorbance of the sample at 536nm. The scavenging ability was calculated as follow:

$$\text{Hydroxyl radical scavenging\%} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{(\text{Absorbance of sample} - \text{Absorbance of blank})} \times 100$$

Assessment of bio-preservation potential of selected strain

Fungal biomass growth inhibition

Fungal biomass growth inhibition by CFS of potent strain was determined following the method by Abouloifa *et al.* 2021 with some modifications. Into a flask containing 10 ml of sterile nutrient broth, 1 ml of the sterile CFS of the isolate was aseptically inoculated and then 1 ml of the fungal spore of *Aspergillus niger* were inoculated into the broth. Fungal culture without CFS was used as control. The fungal biomass was collected by using Whatman grade I filter after an incubation of 5 days at 25°C. The fungal biomass was then dried in an oven at 100°C for 18 h and the percentage biomass growth inhibition was calculated by the formula:

$$\text{Biomass growth inhibition (BI \%)} = \frac{\text{Tc} - \text{Tt}}{\text{Tc}} \times 100$$

Tc = The total fungal biomass obtained without CFS in grams (control)

Tt = The total fungal biomass obtained with CFS in grams.

Bio-preservation of tomato puree

The bio-preservation of potent strain was evaluated on tomato puree, using the method of Abouloifa *et al.* 2021. Briefly twenty grams of tomato puree was prepared from fresh tomato, having an initial pH of 3.8, were introduced in sterile Petri dishes. An overnight grown I7B strain was centrifuged and 10^5 CFU/ml was spread on the surface of tomato puree, and then drop inoculated, in the centre of Petri dishes, with 100 μ l of spore suspension (10^5 CFU/ml) of *Aspergillus niger*. The control sample was added with 1 ml of sterile distilled water and inoculates in the same condition of the other assay. The plates were incubated at 25°C for 15 days, and the diameter of fungal colonies was measured. The percentage of fungal growth inhibition (I%) was calculated by the formula:

$$\text{Fungal growth inhibiton (I \%)} = \frac{\text{Tc} - \text{Tt}}{\text{Tc}} \times 100$$

Tc = The total fungal colony diameter (mm) obtained in control

Tt = The total fungal colony diameter obtained with tested isolates

Statistical analysis

All the experiments were performed in triplicates. The results attained were expressed as mean $\pm$ SD.

Results

Isolation of gut bacteria

Totally four bacterial strains were isolated from the gut of the *Stolipherous commersonnii* and they were labelled as I7A, I7B, I7C, and I8. Colony morphology showed that all the four isolates were circular, medium-sized, white color, flat, opaque and smooth.

Screening for heavy metal resistant bacteria

Observation of bacterial colonies in heavy metals (Cr, Pb, and Cu) loaded media indicated the capacity of bacterial tolerance against different concentrations (10, 20, 30 and 40 mg/ml) of heavy metals. Out of 4 isolates only 1 isolate (I7B) can grow in 10 mg/ml and 20 mg/ml of copper, chromium, and lead. The presence of bacterial colonies in the media containing 10 mg/ml and 20 mg/ml of copper, chromium, and lead exhibits the heavy metal resistant ability of isolate I7B after 48 h of incubation (Fig. 4.1).

Assessment of biosorption ability of heavy metal resistant bacteria

The textile dye effluent was treated with I7B strain and incubated for 32 hours for heavy metal biosorption studies. Tests were carried out to evaluate the residual Cu, Cd, Zn, and Pb in the samples. The concentration of Cu was observed in the textile dye effluent. Heavy metal removal ability of the gut bacterial strains was assessed and represented in Fig. 4.2. The dye effluent contains 0.599 mg/L of Cu without bacterial treatment and in bacterial strains I7A, I7B, I7C and I8 treated sample concentration of Cu falls to 0.161 mg/ml, 0.110 mg/ml, 0.124 mg/ml and 0.524 mg/ml after 32 hours of incubation (Table. 4.1). The heavy metal (Cu) removal rate of all the four strains were 73.12%, 81.64%, 79.30%, and 12.35%. From the results it is inferred that I7B showed maximum heavy metal removal ability among the four strains. Consequently, I8 strain showed minimum heavy metal removal (Cu) capacity in the textile dye effluent after 32 hours of incubation. The potent heavy metal degrading isolate I7B was carried out for further analysis.

Morphological characterization

Microscopic analysis of gram staining result revealed that the Isolate I7B is gram positive, rod shaped. Further, the FESEM analysis confirms the rod-shaped morphology of isolate I7B (Fig. 4.3).

Molecular characterization studies

To intensify the identification of selected bacterial isolate after preliminary morphological analysis, molecular identification of 16S rRNA gene is proceeded. The concentration of DNA isolated from I7B was 18.15 ng/ μ l and the ratio of absorbance was found to be 1.9. Hence, the isolated DNA can be selected for amplification using PCR. The amplicon of 1312 bp length were obtained after PCR amplification of the 16S rRNA gene and subjected to BLAST analysis. For the taxonomical identification of the selected isolate I7B, the phylogenetic tree was constructed indicated a considerable genetic homogeneity. The sequence showed 100% similarity with *Bacillus siamensis* ML121-1. Hence, it was named as *Bacillus siamensis* I7B and has been deposited in the database of National Center for Biotechnology Information (NCBI) as *Bacillus siamensis* strain I7B with accession no MW404539. The evolutionary relationship was inferred with the Neighbor-Joining method using MEGA-X software is shown in Fig. 4.4 (Saitou and Nei 1987).

Antibiotic susceptibility assay

Antibiotic sensitivity assay results are shown in Table 4.2. I7B strain was resistant to Penicillin at both the concentration (2 μ g and 10 μ g). Whereas sensitive to Erythromycin (10 μ g, 15 μ g), Kanamycin (30 μ g),

Rifampin (5 µg), Streptomycin (10 µg) and Tetracycline (30 µg). I7B is more sensitive to Streptomycin (10 µg) and less sensitive to Rifampin (5 µg).

Table 4.1
Cu removal ability of I7B strain by Atomic Absorption Spectrometry

Isolates	Copper mg/ml	Metal removal (%)
		Copper
I7A	0.161±0.601	73.12
I7B	0.110±0.504	81.64
I7C	0.124±0.541	79.30
I8	0.525±0.432	12.35
Values are mean ± standard deviation		

Table 4.2
Antibiotic susceptibility test based on zone of inhibition (mm) to different antibiotics.

Name of the antibiotics	Zone of inhibition (mm)	I7B
Erythromycin (10 µg)	17	S
Erythromycin (15 µg)	16	S
Kanamycin (30 µg)	19	S
Pencillin (2 µg)	0	R
Pencillin (10 µg)	0	R
Rifampin (5µg).	13	S
Streptomycin (10 µg)	25	S
Tetracycline (30 µg)	24	S

Screening for probiotic potential of potent strain

Acid tolerance test

To determine the survival ability of I7B strains in the host gastrointestinal tract, the viability percentage of cells in two different acidic pH 1.5 and 3.0 were tested and the obtained results are summarized in Fig. 4.5. I7B strain shows 100% survivability in both the pH 1.5 and 3 at 0 h of incubation. The highest survivability of 90.9% was found in pH 1.5 at 1 h incubation and gradually decreased to 81.8% and 82.7% at 2 and 3 h of incubation. At pH 3 the strain showed 76.6% and 68.2% of survivability in 1 and 2 h of

incubation period respectively. The minimum survivability of 65.5% was observed in pH 3.0 at 3 h incubation. The result showed the high acid tolerance capacity of the I7B strain to withstand the acid conditions of stomach.

Assay of Bile salt tolerance

A potent probiotic strain should possess bile tolerance capacity which is essential to survive, grow and exert its activity in the small intestine. Fig. 4.6 demonstrates the viability percentage of cells with different concentrations of bile salt treatments at 0-h, 2-h, 4-h, and 24-h of exposure. The I7B strain showed a survival rate of 100% in both the concentrations of the bile salts (0.3% and 0.7%). The survivability percentage of viable cells in 0.3% of bile was 96.9%, 91%, and 87% in 2, 4, and 24 h of incubation respectively. At 0.7% of bile concentration, the percentage of viable cells declined to 90%, 87%, and 80% after 2, 4, and 24 h of incubation. which indicates the tolerance level of the I7B Strain in a high concentration of bile environment, an essential feature of probiotics.

Aggregation activity

Auto-aggregation is a process which demonstrates the interrelation of probiotic strain forming colonization in the gastrointestinal tract through adhesion of probiotic strain to epithelial cells. Auto-aggregation property of I7B strain after 5 h of incubation was found to be 28 ± 0.011 %.

The results of auto-aggregation give the potential of self-aggregate competence of probiotic strain in adhesion to epithelial cells and mucus membrane.

The coaggregation potential of I7B strain with *staphylococcus aureus* was studied and *in vitro* studies serve as a primary screening of the finest probiotic strain. Results shows that I7B could coaggregate up to 27 ± 0.16 % with *Staphylococcus aureus*. Coaggregation of I7B strain with *staphylococcus aureus* enables them to form the barrier that facilitate in colonization of pathogen in the gastrointestinal tract.

Hemolytic activity assay

The hemolytic activity of the I7B strain was recorded by observing no clear zone or greenish zone around their colonies on the blood agar plate (Fig. 4.7). Hence, it illustrated the Gamma-hemolysis property of the I7B strain. It refers to the non-hemolytic property of the I7B strain. Hence it is safe and does not lyse red blood cells during probiotic administration.

Free radical scavenging ability of selected strain

DPPH radical scavenging ability

In order to investigate the antioxidant capacity of I7B strain, *in vitro* antioxidant assay has been performed. The scavenging DPPH capacity of intact cells and CFS were evaluated. The percentage of DPPH scavenging of intact cell and CFS was found to be $55.58 \pm 0.003\%$ and $59.43 \pm 0.002\%$ respectively (Table 4.3). Results shows good DPPH scavenging activity of I7B strain.

Table 4.3
Anti-oxidant activity of *Bacillus siamensis* I7B strain

Strain	Scavenging rate of DPPH%		Scavenging rate of hydroxyl radicals%	
	Intact cells	Cell Free Supernatant	Intact cells	Cell Free Supernatant
I7B	55.58 ± 0.003	59.43 ± 0.002	25.15 ± 0.009	27.6 ± 0.010
The anti-oxidant activity of <i>Bacillus siamensis</i> I7B strain was done in triplicates. The percentage of survival was expressed as an average from three experiments $\pm$ standard deviation.				

Hydroxyl radical scavenging ability

In this study, both the intact cells and the CFS of the selected I7B strain showed the ability to eliminate the hydroxyl radical. The hydroxyl radical scavenging ability of intact cells and CFS showed $27.6 \pm 0.010\%$ and $25.15 \pm 0.009\%$ respectively (Table 4.3). The higher hydroxyl radical scavenging activity of the I7B strain might be due to production of metabolic compounds.

Assessment of bio-preservation potential of selected strain

Bio-preservation potential of *Bacillus siamensis* I7B strain was assessed with fungal biomass assay. Food bio-preservation potential of the *Bacillus siamensis* I7B strain was evaluated on tomato puree.

Fungal biomass growth inhibition assay

The fungal biomass growth inhibition was evaluated with the strain I7B. The obtained values for the cell-free supernatant of the I7B strain against fungus, *Aspergillus flavus* are reported in Table 4.4. The I7B strain inhibits the growth of *A. niger* up to 42%.

Table 4.4 Anti-fungal activity of *Bacillus siamensis* I7B strain against *A. niger*

Isolates	Biomass inhibition (%)		
	Biomass (g)		Inhibition %
	Test(g)	Control(g)	
I7B	0.3395 ± 0.03	0.593 ± 0.10	42

Fungal biomass growth inhibition assay was performed in triplicates. The percentage of survival was expressed as an average from three experiments $\pm$ standard deviation.

Bio-preservation effect of *Bacillus siamensis* I7B on tomato puree

Bio-preservation effect of I7B on tomato puree was tested. Test were performed in a petridish containing tomato puree and *A. niger*. The selected strain I7B was spread on the surface of the tomato puree. After 15 days of incubation at 25 °C, the growth of *A. niger* was observed and showed in Fig. 4.8 and Table 4.5. Inhibition percentage results that the I7B strain has the potential to control the growth of *A. niger* in tomato puree up to 72%. From this result, it is evident that our I7B strain possesses good bio-preservative potential.

Table 4.5
Inhibition (%) of *A. niger* in tomato puree by I7B strain

Isolates	Fungal growth inhibition %	
	Inhibited diameter (cm) (Control – 9cm)	Inhibition %
I7B	2.5	72

Discussion

Fish gut act as a reservoir of exclusive microorganisms which aid in maintaining the health of the host. To study the beneficial activity of the gut microbes, the bacteria must be isolated and further characterized. Nowadays, probiotics were gained focus for their heavy metal detoxification in living system (Giri et al. 2019). Heavy metals contamination through food entering the stomach may affect the metabolism of the gut microbiome and mutates their function (Tian et al. 2012). It is evident from Gao et al. (2017) and Xia et al. (2018) studies in mice model that Pb exposure causes changes in amino acid, bile acid, nitrogen, and vitamin E metabolism which develop oxidative stress and disrupt the metabolic function of gut microbiota. Zhai et al. (2017) reported the influence of heavy metals like Cd, Pb, Cu, and Al towards the gut microbiota in the mice model and cause reduction of *Akkermansia* (probiotic bacteria) after heavy metal exposure. Furthermore, the gut microbiome act as a physical barrier to control oxidative stress and maintain the pH by altering the absorption and metabolism of heavy metals (Coryell et al. 2018). Hence, it is essential to select heavy metal tolerant bacterial strain while choosing a potent probiotic strain. On this aspect we have isolated four heavy metal resistant bacterial isolates and assessed for heavy metal degradation.

In our study, we have isolated four bacterial isolates I7A, I7B, I7c and I8, and out of four isolates only I7B showed resistance to 10 mg/ml and 20 mg/ml of copper, chromium, and lead. Atomic absorption spectrometry results clearly demonstrated the removal of Cu on treatment with I7B strain in the textile dye effluent. Hence, we have chosen isolate I7B for morphological, phenotypic and probiotic characterization. Morphological observation of gram staining and FESEM analysis proved the gram-positive nature of I7B isolate. Phenotypic characterization using 16s rRNA sequencing revealed that the strain in *Bacillus siamensis* and has been submitted in NCBI as *Bacillus siamensis* I7B strain with accession no MW404539.

Antibiotic sensitivity is an important parameter to safety assessment as the antibiotic resistant nature of the probiotic is not desirable (European Food Safety Authority, 2012). *Bacillus siamensis* I7B strain was resistant to Penicillin, Whereas sensitive to Erythromycin, Kanamycin, Rifampin, Streptomycin and Tetracycline. The antibiotic-resistant trait also stipulates that the isolates would survive in the condition by resisting the dreadful situation that occurred because of occasional high antibiotic concentrations.

Gastric juice and bile acids have great antimicrobial activity by breaking the cell membrane of microbiota and cause DNA damage (Ruiz et al., 2013). Hence, bacteria resident in the intestine could tolerate bile and acidic conditions to enhance the ability to colonize and perform metabolic activities in the gut (Amin et al. 2017). The stomach has low pH of 2.5 – 3.0, which is essential for digestion of food and serves as a barrier to guard the host from pathogen infection. *Bacillus siamensis* I7B strain has high acid tolerance capacity at pH 1.5 and 3, which exhibits the potential of *Bacillus siamensis* I7B strain to withstand the acid conditions of stomach. Similarly, Govindaraj et al. (2021) studied the survival of *L. fermentum* isolated from the intestine of freshwater fish *C. mrigala* in acidic (pH 2.0) medium. *Bacillus amyloliquefaciens* from *Labeo rohita* showed a wide range of tolerance from pH 2 – 9 (Khan et al. 2021). Percentage of bile in the intestine of human is 0.3% (W/V) (Guo et al. 2012). Therefore, a potent probiotic strain should possess bile tolerance capacity which is essential to survive, grow and exert its activity in the small intestine. Bile tolerance assay of *Bacillus siamensis* I7B strain indicates good tolerance to bile environment, an essential feature of probiotics. Amenogbe et al. (2021) showed tolerance of 76.16% in *Pantoea agglomerans*, 54.06%, and 65.38% of *Bacillus sp.* and *Bacillus cereus* isolated from intestines of cobia fish in 0.5% bile salt after 3 h of exposure. Niu et al. (2021) showed above 96% of tolerance to 0.3% (W/V) of bile at 0, 1, 2, and 3 h of incubation by *B. amyloliquefaciens* SK4079 and *B. subtilis* marine finfish (*Paralichthys olivaceus*). Literatures shows that most of the bacteria isolated from fish gut possess acid and bile tolerance ability.

Aggregation activity assay is a preliminary screening test used for selecting potent probiotic strain, which demonstrates the colony forming ability of the microorganism's causing precipitation in the suspended medium. The aggregation ability is interrelated to the adhesion of microbes to host cells, i.e., A strain with good aggregation property can adhere to different types of host cells effectively (Malik et al. 2013). Auto-aggregation (which takes place between microorganisms of the same strain) is a process in which the interrelation of probiotic strain forming colonization in the gastrointestinal tract through adhesion of probiotic strain to epithelial cells. Whereas Co-aggregation test reveals the interrelationship of probiotic

adhesion to pathogenic strain. The co-aggregation property of probiotic strain prevents the pathogenic colonization in the host intestine (Balakrishna 2013). Auto-aggregation of *Bacillus siamensis* I7B strain was $28 \pm 0.011\%$ after 5h of incubation and co-aggregation with *S. aureus* showed up to $27 \pm 0.16\%$. The auto-aggregation result portrays the self-aggregate competence of *Bacillus siamensis* I7B strain in adhesion to mucus membrane and epithelial cells. Sahoo et al., 2015 studied auto-aggregation of 27.55–62.92% in LAB isolated from gut of *Labeo rohita* and *catla catla*. The auto-aggregation capacity of *L. fermentum* showed 66.6% and 62.4% in *L. lactis*. The *L. fermentum* and *L. lactis* show higher co-aggregation against *A. hydrophila* (Govindaraj 2021). Khan et al. (2021) reported 65.1% of auto-aggregation property of *Bacillus amyloliquefaciens* and 43.94% of co-aggregation against *A. hydrophila*. From previous results of probiotic strain, it is apparent that *Bacillus siamensis* I7B strain possess moderate autoaggregation and coaggregation property with *S. aureus*. Co-aggregation of *Bacillus siamensis* I7B strain with *staphylococcus aureus* enables them to form the barrier that facilitate in colonization of pathogen in the GI tract.

Hemolysis is a virulent factor, which causes infection in the resident living organisms via skin and mucous membranes (Ramesh et al. 2015; Nandi et al. 2017). Assessment of hemolytic activity of the strain is an important test to assess the safety of probiotic strain (Argyri et al. 2013). Hemolytic activity is important to assess the virulence factor of strain to lyse blood cells in the host. Hence, absence of hemolytic activity strain was characterized as an important property to assess a safe probiotic strain (Argyri et al. 2013). *Bacillus siamensis* I7B strain showed non-hemolytic property.

Oxidative stress is a major problem during heavy metal toxicity which causes elevated lipid peroxidation and reduction of antioxidant enzyme activity. This assay was performed in both intact cells and cell-free supernatant of the selected bacterial strains I7B. Intact cells act more effectively protecting intestinal epithelial cells against oxidative stress due to heavy metal ingestion. Cell-free supernatant was noticed to be absorbed by the small intestine and enters the bloodstream, involved in defense mechanism to prevent damage to other organs like kidney and liver due to oxidative stress (Ojekunle et al. 2017). The scavenging of DPPH radical is a measure of calculating the reduction of stable DPPH to antioxidant with electron-donating ability (Kim et al. 2018). The DPPH radical scavenging method has been used as an important tool to evaluate the anti-oxidant property of probiotic strains. *B. licheniformis* reported $53.74 \pm 0.89\%$ DPPH activity isolated from the intestine of a freshwater teleost fish rohu (*Labeo rohita*) (Khan et al. 2021). Similarly, *Bacillus amyloliquefacins* from the intestine of *Labeo rohita* showed 63.2% of DPPH activity (Khan et al. 2021). *Bacillus siamensis* I7B strain showed good DPPH scavenging activity of $55.58 \pm 0.003\%$ in intact cells and $59.43 \pm 0.002\%$ in the CFS. From the results it is inferred that selected I7B strain have considerable DPPH scavenging activity when compared to other strains. Hence, I7B can scavenge the oxidative stress caused due to consumption of heavy metal contaminated through food habits.

Hydroxyl radical seems to be the most reactive oxygen radical causing damage to living cells. Hence, it is essential to scavenge the hydroxyl radical to inhibit oxidative damage (Zhang et al. 2011). Antioxidant molecules produced by the probiotics can remove and neutralize free radicals, protecting them from

cellular damage (Yadav et al. 2009). These antioxidant metabolites produced by probiotics work with free radicals by donating a hydrogen atom to free radicals (Adnan et al. 2009). The hydroxyl radical is the most reactive oxygen radical and cause cell damage. And therefore, it is very important to reduce the amount of reactive oxygen by scavenging it. Upon *Bacillus siamensis* I7B analysis, the scavenging rate of CFS was higher than that of the intact cells, i.e., $25.15 \pm 0.009\%$ of hydroxyl radical scavenging activity upon intact cells treatment and $27.6 \pm 0.010\%$ in CFS. The hydroxyl radical scavenging activity of *Lactobacillus* LM07 and LM19 was 7.83% and 86.64% (Delgado et al. 2021). *Weisella confuse* exhibited 24.65% of hydroxyl radical scavenging in CFE and 16.95% in IC (Sharma et al. 2018). Previous reports confer the higher hydroxyl radical scavenging activity of the *Bacillus siamensis* I7B strain might be due to production of metabolic compounds by the bacteria.

To study the bio-preservative potential of *Bacillus siamensis* I7B strain they are subjected for fungal biomass assay. The I7B strain inhibits the growth of *A. niger* up to 42%. Previous report also supports the results of the present study. Abouloifa, et al. (2021) isolated fourteen *Lactobacillus* strains from the traditional fermented green olives, the CFS of the bacteria have antifungal activity towards various yeasts and molds. The CFS of all the fourteen strains showed around 75 – 85% of biomass inhibition against *Aspergillus niger*. The biomass inhibition of *Lactobacillus* strain was higher in yeast than in molds (Nayyeri et al. 2017). Lactic acid bacteria strain showed 100% biomass inhibition against *A. flavus* after 7 days of incubation at aerobic conditions (Marie et al. 2018). Moreover, *Baillus siamensis* I7B strain can I7B strain has the potential to control the growth of *A. niger* in tomato puree up to 72%. The biomass inhibition of *Lactobacillus* against *A. niger* ranges between 42.05 to 70.59 mm (Abouloifa et al. 2021). *Plantarum* treated tomato puree delayed the fungi and bacterial growth up to 25 – 30 days. Whereas *acidophils* treated tomato puree delayed the fungi and bacterial growth up to 35 days extension of shelf life (George et al. 2020). Oranusi et al. (2013) reported the antifungal activity of *L. lactis* against *Aspergillus*, *Penicillin*, *Mucor* and *Rhizopus*. Similarly, *L. acidophilus* and *Bifidobacterium bifidum* showed prevention against *A. niger* in brained white cheese surface (Moghanjoui et al. 2020). From this result, it is evident that our I7B strain possesses good bio preservative potential.

Conclusion

In this present study four bacterial strains namely I7A, I7B, I7C, and I8 were isolated from the gut of *stolepherous commersonii*. Among the four isolates, isolate I7B showed more heavy metal resistant potential and atomic absorption spectrometry studies showed the maximum heavy metal (Cu) removal ability of 81.64% of I7B strain in textile dye effluent. The I7B strain is found to be gram-positive rod-shaped bacterium and it was identified as *Bacillus siamensis* by 16S rRNA gene sequencing studies. The potent heavy metal resistant strain was named as *Bacillus siamensis* I7B and has been submitted in NCBI GenBank as *Bacillus siamensis* strain I7B with accession number MN404539. Antibiotic susceptibility test of *bacillus siamensis* I7B showed high sensitivity to Streptomycin (10 µg) and less sensitive to Rifampin (5 µg) and resistant to pencillin (2 µg, 10 µg). The study confirmed that *Bacillus siamensis* I7B strain possess significant probiotic potential with high acid and bile tolerance, good aggregation ability, effective antibiotic susceptibility and non-hemolytic activity. *Bacillus siamensis* I7B

strain possess good DPPH free radical scavenging ability and moderate hydroxyl radical scavenging ability. *Bacillus siamensis* I7B strain inhibits the growth of *A. niger* up to 42%. Biopreservation effect of *Bacillus siamensis* I7B strain on tomato puree showed 72% growth inhibition against *A. niger* for 15 days. However, further *in vivo* studies are essential to assess the probiotic strain in animal models.

Declarations

Conflict of interest

All authors declare that they have no conflict of interest.

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Author's contribution

AUTHOR NAME	Credit role
DANIEL RAJA FEMIL SELTA	Framing the work plan and specific objectives, Writing- original manuscript and data preparation, methodology, formal analysis.
SABNA BS	Data curation
RAMASAMY MAHENDRAN	Supervision, Visualization of data
BENCY THANKAPPAN	Conceptualization, scientific suggestions and manuscript corrections
GAYATHRI MUTHUSAMY	Validation
VINOTH BALASUBRAMANIAN	Supported in methodology
ANGAYARKANNI JAYARAMAN	Provided work space, instrumentation facilities and manuscript corrections.
KAVITHA RANGASAMY	Work approval, relevant suggestions and overall support.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Reference

1. Abatenh E, Gizaw B, Tsegay Z, Tefera G, & Aynalem E (2018) Health benefits of probiotics. *Journal of Bacteriology Infectious Disease* 2(1):8–27.
2. Abdel-Megeed R.M (2020) Probiotics: a promising generation of heavy metal detoxification. *Biol. Trace Elem. Res.* <https://doi.org/10.1007/s12011-020-02350-1>
3. Abouloifa H, Gaamouche S, Rokni Y, Hasnaoui I, Bellaouchi R, Ghabbour N, Asehrou A et al (2021) Antifungal activity of probiotic *Lactobacillus* strains isolated from natural fermented green olives and their application as food bio-preservative. *Biological Control* 152:104450.
4. Abtahi M, Fakhri Y, Conti GO, Keramati H, Zandsalimi Y, Bahmani Z, Ghasemi SM et al (2017) Heavy metals (As, Cr, Pb, Cd and Ni) concentrations in rice (*Oryza sativa*) from Iran and associated risk assessment. A systematic review. *Toxin Reviews* 36:331–341.
5. Adeoti OM, & Usman AT (2021) The Molecular Characterization of Rhizobacteria Isolates from Saki, Nigeria. *European Journal of Biology and Biotechnology* 2(2):26-38.
6. Adnan M, Patel M, Hadi S (2017) Functional and health promoting inherent attributes of *Enterococcus hirae* F2 as a novel probiotic isolated from the digestive tract of the freshwater fish *Catla catla*. *Peer J* 5:e3085. <https://doi.org/10.7717/peerj.3085>
7. Ahn JH, Jo YH, Kim SB, Turk A, Oh KE, Hwang BY, Lee MK et al (2018) Identification of antioxidant constituents of the aerial part of *Plantago asiatica* using LC–MS/MS coupled DPPH assay. *Phytochemistry Letters* 26:20-24.
8. Ajayi AA, Bibi O, Oniha MI, Atolagbe OM, Anosike SO, Onibokun EA (2017) Studies on *Staphylococcus aureus* isolated from pimples. *Pakistan Journal of Biological Sciences* 20(7):350-354.
9. Al-Saleh I, Abduljabbar M (2017) Heavy metals (lead, cadmium, methylmercury, arsenic) in commonly imported rice grains (*Oryza sativa*) sold in Saudi Arabia and their potential health risk. *International Journal of Hygiene and Environmental Health* 220:1168–1178.
10. Amenyogbe E, Huang JS, Chen G, Wang WZ (2021) Probiotic Potential of Indigenous (*Bacillus* sp. RCS1, *Pantoea agglomerans* RCS2, and *Bacillus cereus* strain RCS3) Isolated From Cobia Fish (*Rachycentron canadum*) and Their Antagonistic Effects on the Growth of Pathogenic *Vibrio alginolyticus*, *Vibrio harveyi*, *Streptococcus iniae*, and *Streptococcus agalactiae*. *Frontiers in Marine Science*.
11. Ametepey ST, Cobbina SJ, Akpabey FJ, Duwiejuah AB, Abuntori ZN (2018) Health risk assessment and heavy metal contamination levels in vegetables from tamale Metropolis, Ghana. *Int J Food Contam* 5:5.
12. Amin M, Adams M, Bolch CJ, Burke CM (2017) In vitro screening of lactic acid bacteria isolated from gastrointestinal tract of Atlantic Salmon (*Salmo salar*) as probiont candidates. *Aquacult Int* 25:485–498. <https://doi.org/10.1007/s10499-016-0045-6>
13. Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou EZ (2013) Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol* 33:282–291.

14. Balakrishna A (2013) In vitro evaluation of adhesion and aggregation abilities of four potential probiotic strains isolated from guppy (*Poecilia reticulata*). *Brazilian Archives of Biology and Technology* 56:793-800.
15. Chattopadhyay S, Khatun S, Maity M, Jana S, Perveen, H, Dash M, Maity PP et al (2019) Association of vitamin B 12, lactate dehydrogenase, and regulation of NF- κ B in the mitigation of sodium arsenite-induced ROS generation in uterine tissue by commercially available probiotics. *Probiotics and antimicrobial proteins* 11(1):30-42.
16. Chen P, Zhang Q, Dang H, Liu X, Tian F, Zhao J, Chen W (2014) Screening for potential new probiotic based on probiotic properties and α -glucosidase inhibitory activity. *Food Control* 35(1):65-72.
17. Coryell M, Mcalpine M, Pinkham NV, Mcdermott TR, Walk ST, 2018 The gut microbiome is required for full protection against acute arsenic toxicity in mouse models. *Nat. Commun* 9.
18. Day RL, Harper AJ, Woods RM, Davies OG, Heaney LM (2019) Probiotics: Current landscape and future horizons. *Future Science OA* 5(4):FSO391.
19. Djahed B, Taghavi M, Farzadkia M, Norzaee S, Miri M (2018) Stochastic exposure and health risk assessment of rice contamination to the heavy metals in the market of Iranshahr, Iran. *Food and Chemical Toxicology* 115:405–412.
20. Duan H, Yu L, Tian F, Zhai Q, Fan L, Chen W (2020) Gut microbiota: a target for heavy metal toxicity and a probiotic protective strategy. *Sci. Total Environ* 742:140429
<https://doi.org/10.1016/j.scitotenv.2020.140429>.
21. Dutta D, Ghosh K (2015) Screening of extracellular enzyme-producing and pathogen inhibitory gut bacteria as putative probiotics in mrigal, *Cirrhinus mrigala* (Hamilton, 1822). *International Journal of Fisheries and Aquatic Studies* 2(4):310-318.
22. European Food Safety Authority (2012). Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed. *EFSA Journal* 10:1–84.
23. Gao B, Chi L, Mahbub R, Bian X, Tu P, Ru H, et al (2017) Multi-omics reveals that lead exposure disturbs gut microbiome development, key metabolites, and metabolic pathways. *Chem. Res. Toxicol* 30:996–1005
24. George-Okafor U, Ozoani U, Tasie F, Mba-Omeje K (2020) The efficacy of cell-free supernatants from *Lactobacillus plantarum* Cs and *Lactobacillus acidophilus* ATCC 314 for the preservation of home-processed tomato-paste. *Scientific African* 8:e00395.
25. Giri SS, Jun JW, Yun S, Kim HJ, Kim SG, Kang JW, Kim SW, Han S. J, Park SC, Sukumaran V (2019) Characterisation of lactic acid bacteria isolated from the gut of *Cyprinus carpio* that may be effective against lead toxicity. *Probiotics and Antimicrobial Proteins* 11(1):65–73.
<https://doi.org/10.1007/s12602-017-9367-6>
26. Govindaraj K, Samayanpaulraj V, Narayanadoss V, Uthandakalaipandian R (2021) Isolation of Lactic Acid Bacteria from Intestine of Freshwater Fishes and Elucidation of Probiotic Potential for Aquaculture Application. *Probiotics and Antimicrobial Proteins* 1-13.

27. Guo CF, Zhang LW, Li JY, Zhang YC, Xue CH, Yi HX, Han, X et al (2012) Screening of bile salt hydrolase-active lactic acid bacteria for potential cholesterol-lowering probiotic use. In *Advanced Materials Research* 345:139-146.
28. Guo Z, Liu XM, Zhang QX, Shen Z, Tian FW, Zhang H, Sun ZH, Zhang HP, Chen W (2011) Influence of consumption of probiotics on the plasma lipid profile: A meta-analysis of randomised controlled trials. *Nutr. Metab. Cardiovasc Dis* 21:844–850. [CrossRef] [PubMed]
29. Hernández-Delgado NC, Torres-Maravilla E, Mayorga-Reyes L, Martín R, Langella P, Pérez-Pastén-Borja, R, Bermúdez-Humarán LG et al (2021) Antioxidant and Anti-Inflammatory Properties of Probiotic Candidate Strains Isolated during Fermentation of Agave (*Agave angustifolia* Haw). *Microorganisms* 9(5):1063.
30. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014) The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology* 11(8):506–514. <https://doi.org/10.1038/nrgastro.2014.66>
31. Jafarian V, Ghaffari F (2017) A unique metallothionein-engineered in *Escherichia coli* for biosorption of lead, zinc, and cadmium; absorption or adsorption? *Microbiology* 86:73–81
32. Jain S, Yadav H, Sinha PR (2009) Antioxidant and cholesterol assimilation activities of selected lactobacilli and lactococci cultures. *J Dairy Res* 76(04):385–391. <https://doi.org/10.1017/S0022029909990094>
33. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN (2015) Role of the normal gut microbiota. *World journal of gastroenterology* 21(29):8787.
34. Kavitha M, Raja M, Perumal P (2018) Evaluation of probiotic potential of *Bacillus* spp. isolated from the digestive tract of freshwater fish *Labeo calbasu* (Hamilton, 1822). *Aquac Reports* 11:59-69. <https://doi.org/10.1016/j.aqrep.2018.07.001>
35. Kerry RG, Patra JK, Gouda S, Park Y, Shin HS, Das G (2018) Benefaction of probiotics for human health: A review. *Journal of food and drug analysis* 26(3):927-939.
36. Khan MIR, Choudhury TG, Kamilya D, Monsang SJ, Parhi J (2021) Characterization of *Bacillus* spp. isolated from intestine of *Labeo rohita*—Towards identifying novel probiotics for aquaculture. *Aquaculture Research* 52(2):822-830.
37. Khan MIR, Kamilya D, Choudhury TG, Tripathy PS, Rathore G (2021) Deciphering the Probiotic Potential of *Bacillus amyloliquefaciens* COFCAU_P1 Isolated from the Intestine of *Labeo rohita* Through In Vitro and Genetic Assessment. *Probiotics and Antimicrobial Proteins* 1-13.
38. Li Y, Liu T, Zhao M, Zhong H, Luo W, Feng F (2019) In vitro and in vivo investigations of probiotic properties of lactic acid bacteria isolated from Chinese traditional sourdough. *Applied microbiology and biotechnology* 103(4):1893-1903.
39. Mahmoudi R, Zare P, Hassanzadeh P, Nosratpour S (2014) Effect Of *Teucrium Polium* Essential Oil On The Physicochemical And Sensory Properties Of Probiotic Yoghurt *Journal of Food Processing and Preservation* 38:880–888.

40. Malik S, Petrova MI, Claes IJJ et al (2013) The highly autoaggregative and adhesive phenotype of the vaginal *Lactobacillus plantarum* strain CMPG5300 is sortase dependent. *Appl Environ Microbiol* 79:4576–4585. <https://doi.org/10.1128/AEM.00926-13>
41. Marie KP, Ngoufack François Z, Edith Marius FK, Ciobotaru O, Matei F, Cornea CP, Israel-Roming F (2018) Antifungal activity of lactic acid bacteria isolated from peanuts, gari, and orange fruit juice against food aflatoxigenic molds. *Food Biotechnology* 32(4):237-256.
42. Martin R, Langella P (2019) Emerging health concepts in the probiotics field: Streamlining the definitions. *Frontiers in Microbiology* 10:1047. <https://doi.org/10.3389/fmicb.2019>
43. Marzan LW, Hossain M, Mina SA et al (2017) Isolation and biochemical characterization of heavy-metal resistant bacteria from tannery effluent in Chittagong city, Bangladesh: Bioremediation viewpoint. *Egypt J Aquat Res* 43(1):65-74. <https://doi.org/10.1016/j.ejar.2016.11.002>
44. Moghanjoughi ZM, Bari MR, Khaledabad MA, Almasi H, Amiri S (2020) Bio-preservation of white brined cheese (Feta) by using probiotic bacteria immobilized in bacterial cellulose: Optimization by response surface method and characterization. *LWT* 117: 108603.
45. Nanasombat S, Phunpruch S, Jaichalad T (2012) Screening and identification of lactic acid bacteria from raw seafood and Thai fermented seafood products for their potential use as starter cultures. *Songklanakarin J Sci Technol* 34:255–62.
46. Nandi A, Dan SK, Banerjee G, Ghosh P, Ghosh K, Ringø E et al (2017) Probiotic potential of autochthonous bacteria isolated from the gastrointestinal tract of four freshwater teleosts. *Probiot. Antim. Prot.* 9:12–21. doi: 10.1007/ s12602-016-9228-8.
47. Nayyeri N, Edalatian Dovom MR, Habibi Najafi MB, Bahreini M (2017) A Preliminary study on antifungal activity of lactic acid bacteria isolated from different production stages of Lighvan cheese on *Penicillium expansum* and *Rhodotorula mucilaginosa*. *Food Meas.* 11:1734–1744. <https://doi.org/10.1007/ s11694-017-9554-x>
48. Nithya C, Gnanalakshmi, B, Pandian SK (2011). Assessment and characterization of heavy metal resistance in Palk Bay sediment bacteria. *Marine environmental research* 71(4):283-294.
49. Niu KM, Kothari D, Lee WD, Lim JM, Khosravi S, Lee SM, Lee BJ, Kim KW, Han HS, Kim SK (2019) Autochthonous *Bacillus licheniformis*: Probiotic potential and survival ability in low-fishmeal extruded pellet Aquafeed. *Microbiology Open* 8(6):e00767. <https://doi.org/10.1002/mbo3.767>
50. Ojekunle O, Banwo K, Sanni AI (2017) In vitro and in vivo evaluation of *Weissella cibaria* and *Lactobacillus plantarum* for their protective effect against cadmium and lead toxicities. *Lett Appl Microbiol* 64(5):379–385. <https://doi.org/10.1111/lam.12731>
51. Omar NA, Praveena SM, Aris AZ, Hashim Z (2015) Health Risk Assessment using in vitro digestion model in assessing bioavailability of heavy metal in rice: A preliminary study. *Food Chemistry* 188:46–50.
52. Oranusi SU, Braide W, Oguoma OI (2013) Antifungal properties of lactic acid bacteria (LAB) isolated from *Ricinus communis*, *Pentaclethra macrophylla* and Yoghurts. *Global Advanced Research Journal of Food Science and Technology* 2(1):001-006.

53. Ortiz-Rivera Y, Sanchez-Vega R, Gutierrez-Mendez N, Leon-Felix J, Acosta-Muniz C, Sepulveda DR (2017) Production of reuterin in a fermented milk product by *Lactobacillus reuteri*: Inhibition of pathogens, spoilage microorganisms, and lactic acid bacteria. *Journal of dairy science* 100(6):4258-4268.
54. Owolabi JB, Hekeu MM (2014) Heavy metal resistance and antibiotic susceptibility pattern of bacteria isolated from selected polluted soils in Lagos and Ota, Nigeria. *Int J Basic Appl Sci* 4(6):6–12.
55. Pajevic S, Arsenov D, Nikolic N, Borisev M, Orcic D, Zupunski M, Mimica-Dukic N (2018) Heavy metal accumulation in vegetable species and health risk assessment in Serbia. *Environ Monit Assess* 190:459.
56. Pedros-Garrido S, Clemente I, Calanche JB, Condon-Abanto S, Beltran JA, Lyng JG, Whyte P et al (2020) Antimicrobial activity of natural compounds against *Listeria* spp. and their effects on sensory attributes in salmon (*Salmo salar*) and cod (*Gadus morhua*). *Food Control* 107:106768.
57. Ramesh D, Vinothkanna A, Rai AK, Vignesh VS (2015) Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in *Labeo rohita* against *Aeromonas hydrophila*. *Fish Shellf. Immunol* 45:268–276. doi: 10.1016/j.fsi.2015.04.018
58. Ray AK, Ghosh K, Ringo E (2012) Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition* 18(5):465-492.
59. Reddivari L, Veeramachaneni DNR, Walters WA, Lozupone C, Palmer J, Hewage MKK, Bhatnagar R, Amir A, Kennett MJ, Knight R, Vanamala JKP (2017) Perinatal bisphenol A exposure induces chronic inflammation in rabbit offspring via modulation of gut bacteria and their metabolites. *mSystems* 2:1–16. <https://doi.org/10.1128/msystems.00093-17>.
60. Ruiz L, Margolles A, Sánchez B (2013) Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front Microbiol* 4:396. <https://doi.org/10.3389/fmicb.2013.00396>
61. Sahoo TK, Jena PK, Nagar N, Patel AK, Seshadri S (2015) In Vitro evaluation of probiotic properties of lactic acid bacteria from the gut of *Labeo rohita* and *Catla catla*. *Probiotics Antimicrob Proteins*. doi:10.1007/s12602-015-9184-8
62. Samson JS, Choresca CH, Quiazon KMA (2020) Selection and screening of bacteria from African nightcrawler, *Eudrilus eugeniae* (Kinberg, 1867) as potential probiotics in aquaculture. *World Journal of Microbiology and Biotechnology* 36(1):1-10.
63. Senthong R, Chanthachum S, Sumpavapol P (2012) Screening and identification of probiotic lactic acid bacteria isolated from Poo-Khem, A traditional salted crab. Paper presented at the International Conference on Nutrition and Food Sciences.
64. Sharma S, Kandasamy S, Kavitha D, Shetty PH (2018) Probiotic characterization and antioxidant properties of *Weissella confusa* KR780676, isolated from an Indian fermented food. *LWT* 97:53-60.
65. Shastry RP, Arunrenganathan RR, Rai VR (2020) Characterization of probiotic *Enterococcus lactis* RS5 and purification of antibiofilm enterocin. *Biocatalysis and Agricultural Biotechnology* 101897.

66. Shen ZH, Zhu CX, Quan YS, Yang ZY, Wu S, Luo WW, Wang XY et al (2018). Relationship between intestinal microbiota and ulcerative colitis: Mechanisms and clinical application of probiotics and fecal microbiota transplantation. *World journal of gastroenterology* 24(1):5.
67. Sihag RC, Sharma P (2012) Probiotics: the new ecofriendly alternative measures of disease control for sustainable aquaculture. *J Fish Aquat Sci* 7:72
68. Siripornadulsil W, Tasaku S, Buahorm J, Siripornadulsil S (2014) Probiotic properties of lactic acid bacteria isolated from fermented food. *Intl J Biol food Vet Agri Eng* 8:364–6.
69. Steffi AP, Balaji R, Prakash, N. Rajesh, TP, Ethiraj S, Samuel MS, Chandrasekar N et al (2021) Incorporation of SiO₂ functionalized gC₃N₄ sheets with TiO₂ nanoparticles to enhance the anticorrosion performance of metal specimens in aggressive Cl⁻ environment. *Chemosphere* 133332.
70. T'elessy I (2019) Nutraceuticals. In R. B. Singh, R. R. Watson, & T. Takahashi (Eds.), *The role of functional food security in global health* 409–421. London: Academic Press.
71. Talwar C, Nagar S, Lal R, Negi RK 2018. Fish gut microbiome: current approaches and future perspectives. *Indian journal of microbiology* 58(4):397-414.
72. Tan H, Zhai Q, Chen W (2019) Investigations of *Bacteroides* spp. towards next-generation probiotics. *Food Research International* 116:637–644. <https://doi.org/10.1016/j.foodres.2018.08.088>
73. Tian F, Zhai Q, Zhao J, Liu X, Wang G, Zhang H, Zhang H, Chen W (2012) *Lactobacillus plantarum* CCFM8661 alleviates lead toxicity in mice. *Biol Trace Elem Res* 150:264–271.
74. Vijaya Kumar B, Vijayendra SVN, Reddy OVS (2015) Trends in dairy and non-dairy probiotic products – a review. *J Food Sci Technol* 52(10):6112–6124. <https://doi.org/10.1007/s13197-015-1795-2>
75. Wang J, Ji H, Wang S, Liu H, Zhang W, Zhang D, Wang Y, (2018) Probiotic *Lactobacillus plantarum* promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Front. Microbiol* 9:1953. <https://doi.org/10.3389/fmicb.2018.01953>.
76. Wang Y, Wu Y, Wang Y, Xu H, Mei X, Yu D, Wang Y, Li W (2017) Antioxidant properties of probiotic bacteria. *Nutrients*, 9(5):521. <https://doi.org/10.3390/nu9050521>
77. Wong CB, Odamaki T, Xiao JZ (2019) Beneficial effects of *Bifidobacterium longum* subsp. *longum* BB536 on human health: Modulation of gut microbiome as the principal action. *Journal of Functional Foods* 54:506–519. <https://doi.org/10.1016/j.jff.2019.02.002>
78. Xia J, Lu L, Jin C, Wang S, Zhou J, Ni Y, Jin Y et al (2018) Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 209:1-8.
79. Zhai Q, Li T, Yu L, Xiao Y, Feng S, Wu J, Chen W et al (2017) Effects of subchronic oral toxic metal exposure on the intestinal microbiota of mice. *Science Bulletin* 62(12):831-840.
80. Zhai Q, Liu, Y, Wang C, Qu D, Zhao J, Zhang H, et al (2019b) *Lactobacillus plantarum* CCFM8661 modulates bile acid enterohepatic circulation and increases lead excretion in mice. *Food Funct* 10:1455–1464.

Figures

Figure 1

Screening the heavy metal resistant bacteria

Figure 2

Cu removal (%) by gut bacterial isolates

Figure 4

Neighbor-Joining tree showing the phylogenetic position of I7B

Figure 5

Acid tolerance test of *Bacillus siamensis* I7B strain

The acid tolerance test was performed in triplicates. Values are mean $\pm$ standard deviation.

Figure 6

Bile tolerance assay of *Bacillus siamensis* I7B strain

The bile tolerance assay was performed in triplicates. Values are mean $\pm$ standard deviation.

Figure 7

Assessment of hemolytic activity

Absence of hemolytic activity in test sample

Figure 8

Bio-preservation of *Bacillus siamensis* I7B strain against *A. niger* on tomato puree