Asia-Pacific Journal of Science and Technology: Volume: 30. Issue: 02. Article ID.: APST-30-02-11. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by Research Department,
Khon Kaen University, Thailand

Evaluation of probiotic functional properties of *Pediococcus pentosaceus* BBS1 and *Lactiplantibacillus plantarum* BBS13 strains isolated from Lao traditional fermented bamboo shoots (*Nor mai som*)

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Received 21 April 2024
Revised 21 June 2024
Accepted 5 July 2024

Abstract

Probiotics are increasingly popular in the food industry. Nevertheless, there remains a need for greater assessment of the probiotic potential of the lactic acid bacteria (LAB) by evaluating under-researched strains, from sources such as fermented bamboo shoots. The aim of this study was to evaluate the functional probiotic properties of *Pediococcus pentosaceus* BBS1 and *Lactiplantibacillus plantarum* BBS13 strains isolated from *Nor mai som*. *In-vitro* tests for probiotic properties revealed that both strains were tolerant to simulated gastric juice after 3h of incubation at pH 2.5 supplement with pepsin and also tolerant to small intestinal juice after 4h incubation at pH 8.0 with 0.3% oxgall bile salt. Bacterial adhesion to xylene (non-poplar solvent) and chloroform (acidic monopolar solvent) were obtained for BBS1 (36.88% and 40.17%) and BBS13 (39.54% and 49.33%), respectively. The highest 1-diphenyl-2-picrylhydrazyl radical-scavenging activity was found in BBS13 at 77.41 % and BBS1 at 72.74%. Both were positive for proteolytic and linamarase activities, promising biotechnological applications. The strains inhibited not only closely related species but they were also effective against several pathogenic microorganisms (*Bacillus cereus* DMST 5040, *Escherichia coli* ATCC 25922, *E. coli* O157: H17, *Listeria innocua* 026, *Staphylococcus aureus* DMST 8840, *Salmonella typhimurium* DMST 15674). A safety assessment showed that those strains were susceptible to many antibiotics administered to humans and animals, and showed no hemolytic activity. Results revealed that the two strains have potential probiotic properties and are considered safe to be applied as starter culture in fermented bamboo shoots and related food products.

Keywords: *Pediococcus pentosaceus* BBS1, *Lactiplantibacillus plantarum* BBS13, potential probiotic bacteria, *Nor mai som*

1. Introduction

Lactic acid bacteria (LAB), which are used to ferment food, are "generally regarded as safe" (GRAS), have functional properties for various food products and therapeutic applications such as modification of the intestinal microbiota, enhancing animal growth, reducing blood cholesterol levels, boosting nutrient utilization, induction of barrier function, and immunomodulation [1]. The FAO/WHO [2] defines probiotics as “live microorganisms which when administered in adequate amount confer a health benefit on the host”.

Traditional fermented foods are abundant with probiotic microorganisms as reported by numerous studies that have been conducted in the recent past. This marked interest on probiotic microorganisms can be attributed to

their functional and safety features. *L. plantarum* spp. and *P. pentosaceus* spp., which can be found in a variety of sources, including vegetables, fermented dairy products, and animal manures, exhibit probiotic properties, antioxidant activity, cholesterol-lowering, and immunological effects [3]. Probiotic strains *P. pentosaceus* R1 and *L. plantarum* spp., isolated from Harbin dry sausages, tolerated the human gastrointestinal tract (GI), and possess antioxidant activity [4]. LAB with probiotic potential properties have been isolated from silages of different species of forage plants, cocoa beans, and artisanal salami, and were mostly *L. plantarum* strains [5].

Although use of LAB probiotics in fermentation is gaining interest, there is still a need for more evaluation of the bacteria's unique characteristics from different sources, such as fermented bamboo shoots. With the dearth regarding the source and species of *P. pentosaceus*, it makes sense to evaluate *P. pentosaceus* from various sources [3]. Due to the high acidity of the fermented bamboo shoots, it is expected that the *Nor mai som* might harbor LAB with high tolerance to acidic conditions and may offer favorable environment for the enrichment of potential probiotic LAB. Furthermore, bamboo is a well-known plant that produces cyanogenic glycosides. Taxiphyllin (p-hydroxylated mandelonitrile) is one glycoside, the main anti-nutritional factor present in bamboo shoots. Ingestion of cyanogenic glycosides commonly leads to cyanide poisoning [6]. Screening and characterizing LAB that can produce beneficial substances such as bioactive compounds or enzymes that play an essential role in cyanogen reduction, making them valuable in the food industry [7].

In line with the FAO/WHO [3] guidelines for the evaluation of probiotics in food, a candidate probiotic must be resistant to gastric acidity and bile salt, show antagonism against pathogenic bacteria, and adhere to gut tissues. It should be susceptible to antibiotics, negative for biogenic amine production and virulence factors, and non-hemolytic. The aim of the current study was to evaluate, using *in-vitro* assay, the potential probiotic properties of *P. pentosaceus* BBS1 and *L. plantarum* BBS13 isolated from *Nor Mai Som*, a traditional fermented bamboo shoots of Laos.

2. Materials and methods

2.1 Bacterial Strains and growth media

Strain BBS1 and BBS13 used in this study were previously isolated from *Nor mai som* sample and were identified as *Pediococcus pentosaceus* BBS1 (GenBank accession number, OQ363204) and *Lactiplantibacillus plantarum* BBS13 (GenBank accession number, OQ363205). *L. plantarum* BS (BIOTECH 10287) [8] was used as positive control provided by the Biotechnology for Industry, Energy, and the Environment Program (BIEEP), National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB). They were cultured in de Man, Rogosa and Sharpe (MRS, HiMedia, India) medium under microaerophilic conditions at 37°C. All strains were maintained as stock cultures at –80°C in 30% glycerol until further use.

2.2 Characterization of antibacterial compounds produced by BBS1 and BBS13 strains

Strains BBS1 and BBS 13 were tested for production of antibacterial compounds [9], with minor modifications. A 24 h culture of the strains (10% v/v, 10^8-10^9 CFU/ml) was inoculated into MRS broth (100 ml) and incubated for 18-24 h at 37°C. The entire culture broth was harvested and centrifuged at 4,000 x g for 10 min at 4°C. The supernatants were collected and used in the experimental treatments as follows: (i) direct use of the cell-free supernatant (CFS); (ii) CFS neutralized to pH 6.5 using 1M NaOH to discount the antimicrobial effect of organic acids (NCFS); (iii) NCFS treated with catalase (1mg/ml; Sigma-Aldrich Corporation) to exclude the inhibitory effect of hydrogen peroxide and incubated at 37°C for 1h (NCFSC); (iv) NCFSC with added protease (NCFSCP) (1mg/ml; Sigma-Aldrich Corporation) and incubated at 37°C for 3h to confirm the presence of a bacteriocin-like inhibitory substance (BLIS). All the samples were filter-sterilized (0.22 µm) and heated for 5 min at 90°C to remove contaminations and kept on ice. Antibacterial activity was determined via a well-diffusion assay. Nutrient agar and MRS soft agars (0.8% agar, w/v) (48°C) were seeded with each individual indicator (10^6 CFU/ml) (Table 1), including *B. cereus* DMST 5040, *E. coli* ATCC 25922, *E. coli* O157: H17, *L. innocua* 026, *L. plantarum* BS, *L. sakei* JCM 1157, *P. acidilactici* 3G3, *S. aureus* DMST 8840, and *S.* Typhimurium DMST 15674 and then vortexed. All samples were poured into sterile Petri dishes, and after solidification of the agar, a cork borer was used to create wells of uniform diameter (5 mm). Aliquots (50 µl) from each treatment were placed into each well and incubated at 37°C for 24 h. MRS broth (50 µl) was used as control. The zone of inhibition is equated to the antimicrobial activity, and the result is reported as the diameter of the inhibition zone, measured using a Vernier calliper. Isolates that showed inhibition, were presumed to be producing a BLIS. All the indicators were obtained from the BIEEP laboratory, BIOTECH, UPLB.

Table 1 Antibacterial activity of the cell-free supernatant (CFS) and enzymatic activities of the strains BBS1 and BBS13.

Strains	Diameter of inhibition zone (mm)*	
	BBS1	BBS13
Food spoilage and foodborne pathogenic bacteria		
B. cereus DMST 5040	$23.26\pm0.00^{Aa**}$	23.81 ± 0.88^{Aa}
E. coli ATCC 25922	17.99 ± 0.61^{Bc}	19.87 ± 0.52^{Bb}
E. coli O157:H17	12.36 ± 0.01^{Dab}	11.04 ± 0.16^{Dc}
L. innocua 026	15.70 ± 0.43^{Cc}	16.87 ± 0.01^{Cb}
S. aureus DMST 8840	16.50 ± 0.23^{Cab}	15.75 ± 0.51^{Cbc}
S. Typhimurium DMST 15674	10.37 ± 0.07^{Db}	10.86 ± 0.09^{Dab}
Closely related species		
L. sakei JCM 1157	12.41 ± 0.00^{Da}	11.72 ± 0.14^{Db}
P. acidilactici 3G3	$0.00 \pm 0.00^{Fg**}$	6.51 ± 0.06^{Ee}
L. plantarum BS	0.00 ± 0.00^{Fg}	5 ± 0.02^{Ef}
Enzymatic activities		
Proteolytic activity (Protein hydrolysis index, PHI) *	4.69 ± 0.00^{b}	$5.32\pm0.52^{a**}$
Linamarase activity (U/ml)*		
Treatment with fresh bamboo shoots	$55.33\pm0.57^{a**}$	51.66 ± 0.57^{b}
Treatment with cassava peel powder	11.66 ± 1.04	18.33 ± 0.57

*Values are given as mean ± standard deviation (SD) from triplicate experiments.
**Significant differences in row are indicated by different capital letters, and significant differences in column are presented by different small letters by the LSD test ($P < 0.05$).

2.3 Enzymatic activity screening

2.3.1 Proteolytic activity

Strain BBS1 and BBS13 were assayed for proteolytic activity by the agar well diffusion method [10], with minor modifications. Casein agar (g/l), composed of beef extract 1.5; yeast extract 1.5; peptic digest of animal tissue 5; NaCl 5; casein 10; dextrose 1; agar 15; and bromocresol green (BCG) 0.015 was used as plating medium (pH 8.0). The plates were punctured using a cork borer to make wells of uniform diameter (5 mm). Then, 50 µl of overnight-cultures were inoculated into each well, followed by incubation at 37ºC for 24-48 h. MRS broth (50 µl) was used as control. A clear zone around the puncture indicated protease production. The ratio R/r was used to compute the protein hydrolysis index PHI, where R is the diameter of the entire clear zone and r is the diameter of the agar well inoculated with strains BBS1 and BBS13.

2.3.2 Screening for linamarase activity

Strains BBS1 and BBS13 were carried out for linamarase enzyme, with slight modifications [11]. The actively growing LAB cultures (1% v/v, 10^8 -10^9 CFU/ml) were inoculated separately into 100 ml of sterile MRS broth containing fresh bamboo shoots (2%, w/w) and cassava peel powder (Sultan2 variety, 2%, w/w, positive control). All treatments were incubated at 30ºC for 24 h. The supernatants were collected and centrifuged (Centrifuge Combi-514R, Hanil Science Industrial, Korea) at 4,000 x g for 10 min at 4ºC. Ammonium sulfate (7.65 g) was added to 50 ml of supernatants and maintained in water bath set at 15ºC. Then, the solution was stirred until the ammonium sulfate dissolved completely. Another round of centrifugation followed, and the supernatants were discarded. The precipitates containing the extracted enzyme was resuspended in 1 ml of 0.01 M sodium phosphate buffer (SPB, Sigma-Aldrich, pH 6.8) and stored in the freezer (-20ºC) until use.

One-half ml (0.5 ml) of the diluted extracted enzyme (dilution 200-folds = 5 µL enzyme + 995 µL 0.01 M SPB, pH 6.8) was added to 1 ml of the prepared PNPG (4-Nitrophenyl-β-D- glucopyranoside, 21 mg of PNPG in 0.01 M SPB, pH 6.8) solution. After incubation, for 60 minutes at room temperature, 2 ml of 0.2 M Borate Buffer (pH 9.8) was added, and the volume was increased to 4 ml with distilled water. The samples were read at 400 nm using a UV spectrophotometer. SPB was used as negative control and *L. plantarum* BS (BIOTECH 10287) was used as positive control. One unit of linamarase activity is the amount of enzyme producing a 0.001 change in absorbance at 400 nm/min under the described assay conditions [11].

2.4 Tolerance to simulated gastrointestinal juice

The ability of the strains to tolerate gastric and intestinal conditions were determined *in vitro* [12]. The strains were cultured in MRS for 18-24 h at 37ºC and harvested by centrifugation at 4,000 xg for 5 min at 4ºC. The obtained cell pellets were washed twice with 0.1M phosphate buffer saline (PBS), pH 7.2. To assess the possible hydrolysis of the bacteria as they are suspended in the human oral cavity, the samples were placed in a sterile electrolyte solution (SES) containing 100 µg/ml lysozyme and then incubated at 37ºC for 10 min. Thereafter, the bacterial

suspension was centrifuged, and the precipitate was washed two times with PBS (pH 7.2) in order to find out their response to the simulated gastric juice. The samples were resuspended in PBS (pH 2.5) composed of 0.3% (w/v) pepsin and incubated at 37ºC for 3 h after which, they were serially-diluted and spread-plated on MRS agar containing 0.12 g/l of bromocresol purple.

The strains were also determined for their tolerance to the conditions existing in the small intestine. The cell suspension was centrifuged and washed twice with PBS (pH 7.2) and resuspended in PBS (pH 8.0) containing 0.1% (w/v) porcine pancreatin (Sigma, Germany) and 0.3% (w/v) oxgall bile salt (Merck, Germany), and incubated at 37ºC for 4 h. The samples were serially diluted and inoculated on MRS agar plates containing 0.12 g/l of bromocresol purple. Viable cells were counted at the initial time (0 h) and 2, 3 and 4 h thereafter, for gastric and intestinal tolerance. Values were expressed as logarithms of colony- forming units per milliliter (CFU/ml).

2.5 Bacterial adhesion to hydrocarbon (BATH)

The adhesion of the two strains by surface hydrophobicity was studied [13]. Bacterial cells grown for 18-24 h in MRS broth were collected by centrifugation at 4,000 x g at 4ºC for 5 min. The cell pellet was washed twice with 10 mM PBS, pH 7.2, and suspended in PBS (pH 7.2). The cell concentration of OD_{600nm} (A_i) was adjusted to OD_{600nm} 4.0 for optimizing the bacterial cells to 10^8 CFU/ml. Then, an equal volume of xylene or chloroform was added to the prepared bacterial cells and vortexed for 5 min. The tubes were placed to stand at room temperature (30±2ºC) for 60 min to allow for the separation of the two phases. The upper aqueous phase was carefully removed, and its absorption was read at 600 nm. The percentage of BATH of the selected LAB adhering to the solvent was calculated using the following formula:

$$\text{BATH (\%)} = [(A_i - A_F)/ A_i] \times 100$$

where A_i is absorbance before addition of the solvent.
A_F is absorbance after the addition of the solvents.

2.6 Susceptibility to antibiotics

Antibiotic susceptibility of the two strains were examined according to the guidelines of the Clinical and Laboratory Standards Institute [14] and by the guidelines of the European Food Safety Authority [15]. The test was conducted using the broth microdilution method [16], with minor modifications. Briefly, the colonies of strains were suspended in 10 ml of sterile saline solution until they reached a turbidity of 1.0 on the McFarland scale. They were then diluted 500-fold in LSM medium (Iso-sensitest broth and MRS at a ratio of 9:1). Fifty-microliter portions of the diluted bacterial suspension were introduced into each well, which contained various concentrations of antibiotics in a 50 µl amount of LMS broth each well. The antibiotics and their commonly used in human and veterinary were evaluated in varied concentrations, including ampicillin (0.125-16 µg/ml), chloramphenicol (0.125-64 µg/ml), clindamycin (0.125-16 µg/ml), erythromycin (0.125-16 µg/ml), gentamicin (0.125-64 µg/ml), kanamycin (0.125-256 µg/ml), penicillin (0.125-64 µg/ml), tetracycline (0.125-256 µg/ml), and oxacillin (0.125-64 µg/ml). All antibiotics were dissolved in sterile distilled water, except chloramphenicol, and tetracycline which were dissolved in absolute ethanol.

2.7 Hemolytic Activity

Hemolytic activity was evaluated for strains BBS1 and BBS13 [13]. MRS agar infused with sheep blood (5%, v/v) was prepared and actively-growing strains were streaked on the medium. Incubation followed at 37ºC for 24-48 h. Hemolysis was classified as follows: appearance of a clear halo surrounding the colonies (β-hemolysis), partial hydrolysis manifested as green-hued zones around the colonies (α-hemolysis), or absence of clear zones around the colonies (γ-hemolysis). Strain exhibiting γ–hemolysis was considered as non-hemolytic.

2.8 Antioxidant Activity

The antioxidant activity of the two strains under were analyzed, with minor modifications [17]. The strains were grown in MRS broth for 18-24 h (10^8-10^9 CFU/ml) and the supernatants were harvested using centrifugation at 4,000 x g at 4ºC for 10 min. The 2,2-diphenyl-1-picrylhydrazyl (DPPH, Alfa Aesar, USA) radical scavenging activity was performed for antioxidant activity. Briefly, an equal volume of 0.1ml of prepared samples and prepared ethanolic DPPH solution (0.4 mmol/L) were mixed. Then, the mixtures were allowed to react for 30 min in the dark. The samples were measured for absorbance at 517 nm using UV-visible spectrophotometer, and the ethanol-mixed DPPH solution was used as a control (blank). Scavenging activity was calculated using the formula:

$$\text{DPPH scavenging activity (\%)} = [(\text{A blank} - \text{A sample})/ \text{A blank}] \times 100$$

where A is absorbance of the samples at 517 nm.

2.8 Statistical analysis

The Statistical Package for the Social Science software (IBM SPSS, version 20; SPSS, Inc., Chicago, IL) was used for statistical analysis. One-way analysis of variance (ANOVA) was considered statistically significant at $P < 0.05$. All data were expressed as mean ± standard deviation. Multiple comparisons between different groups were performed by Tukey and Least Significant Difference (LSD) tests.

3. Results and discussion

3.1 Antibacterial substances produced by BBS1 and BBS13 strains

Inhibitory activity of the both strains against indicators by agar well diffusion test is shown in Table 1. CFS from the set-up using the strains revealed a significant difference ($p < 0.05$) on levels of inhibition towards the pathogenic bacteria, and closely related species tested. *L. plantarum* BS was used as a positive control because it inhibited *L. sakei* JCM 1157 for antibacterial compounds. This reference strain could not be applied to test for all indicators since antimicrobial activity is dependent on species-and strain-specific. The largest inhibition zone was observed with *B. cereus* DMST 5040 (approximately 23 mm), followed by *E. coli* ATCC 25922 (17 to 19 mm), *L. innocua* 026 and *S. aureus* DMST 8840 (15-16 mm), and *E. coli* O157:H17 and *S. typhimurium* DMST 15674 (10-12 mm). The strains were also found to inhibit closely related species, including *L. sakei* JCM 1157, *P. acidilactici* 3G3, and *L. plantarum* BS, except strain BBS1, which did not deter the proliferation of *P. acidilactici* 3G3, and *L. plantarum* BS. The above results indicate that pathogenic bacteria in fermented foods can be controlled without affecting the beneficial probiotic community, suggesting an interesting technological application in fermented foods [9].

When the CFS was neutralized to pH 6.5 (NCFS) to discount the influence of organic acids and when the NCFS was treated with catalase (NCFSC) to eliminate the inhibitory effect of H_2O_2, no antimicrobial effects were observed against any of the indicators. A similar observation, the antimicrobial activity of CFS from presumptive LAB, isolated from fresh vegetable sources, was performed by microtitration, and revealed activity against indicators such as *B. cereus*, *L. monocytogenes*, *L. lactis*, *L. casei*, molds, and yeast strains, but they were inactivating when tested by adding the LAB-CFS with pH > 6.0 [18]. In general, bacteriocin-producing LAB strain expression is typically due to growth-dependent metabolites, regulated by environmental conditions, the growth phase of the productive strain, as well as specific responses toward indicators [19]. Therefore, it can be concluded that the inhibitory action of BBS1 and BBS13 in the present study is highly associated with the combination of these organic compounds such as lactic acid, acetic acid, diacetyl, citric acid, butyl acid, or more.

3.2 Proteolytic activity

BBS1 and BBS13 exhibited protease activity, as shown by the clear zone that formed around the bacterial colony cultured on casein agar medium after 24 h of incubation (Table 1). The diameter of the proteolytic zone was calculated as PHI and statistically computed for significant differences ($P < 0.05$). BBS13 had PHI value of 5.32±0.52, while BBS1 gave a lower value of 4.69±0.00. Generally, proteolytic enzymes can be classified into three distinct groups based on their active pH levels: acidic (pH 5.0), near neutral (pH 6.5), and alkaline (pH 8.0) proteases [20]. Bromocresol green was added in casein agar medium to determine proteolytic activity on casein agar plates. The pH of the culture medium was maintained at a value of 8 in our study investigation. Therefore, the plate appeared as blue in color (data not shown). The BCG reagent significantly enhances the color intensity of the plates by binding to unhydrolyzed protein without impeding the proliferation and secretion of proteases [10]. The presence of a hydrolysis zone formed by our strains BBS1 and BBS13 indicates that the proteolytic enzymes produced by these bacteria are active extracellular, especially alkaline protease based on their active pH. There have been reports that LAB were able to generate and secrete extracellular proteolytic enzymes, are capable of hydrolyzing casein molecules into colorless peptide fragments, forming a clear zone around the culture on skim milk [20]. Increasing nutritional bioavailability is a promising biotechnological application of LAB's proteolytic activity. This is achieved when poorly absorbed products are converted, or agro-industrial waste products are used for human or animal consumption. Bone powder, for instance, is a high source of collagen protein and calcium, both of which are difficult for humans to absorb due to the triple-helix structure of collagen. In addition, fermentation by LAB with proteolytic activity may also confer antioxidant capacity. Nevertheless, enzymatic capacities produced

by LAB are dependent on species-and strain-specific differences in LAB enzymatic systems, such as the regulation of gene expression. The conditions of a food matrix, including the availability of protein substrate, pH, temperature, and bacterial growth phase also largely affect enzyme activities [21]. The current findings suggest that the strains coming from *Nor mai som* can efficiently produce extracellular proteolytic enzymes. However, the purpose of our study was just to preliminary screen of the proteolytic enzyme, which can be used as a guide to study on effect of pH extracellular proteolytic activity and their potential for extracellular amino acid production in particular products.

3.3 Linamarase screening

Taxiphyllin, a p-hydroxylated mandelonitrile tiglochinin, is a cyanogenic glucoside that is found in bamboo shoots [20]. Table 1 indicated significant differences ($p<0.05$) among all treatments. The linamarase activity of the strains from fresh bamboo shoots treatment was relatively high, amounting to 55.33±0.57 U/ml in BBS1 and 51.66±0.57 U/ml for BBS13. When the high cyanide containing Sultan 2 variety was used as reference, linamarase production by the strains appeared to be generally low when compared to the fresh bamboo shoots treatment. The values obtained were lower when compared to the findings of different species *Lactobacillus* and *Bacillus* spp., generate extracellular linamarase in the fermentation medium containing cassava peels, with enzyme activity ranging from 18 to 94 enzyme units [22]. Several investigators explained that the variation demonstrated by *Lactobacillus* spp., for linamarase production may be due to their isolation source and the effect of cultural conditions (pH, temperature, substrate concentration, incubation time, and inoculum volume), protein expression, and some genetic diversity [23]. There are two potential processes of cyanide degradation, the introduction of microbially-hydrolyzed enzymes to materials and the degradation of enzymes in the cell wall that allows contact between compartmentally separate cyanide and endogenous linamarase of materials [22-24]. There have been reports of linamarase activity in several bacterial strains, but limited studies on linamarase activity of *Lactobacillus* spp., in bamboo shoots fermentation [22]. The hydrolysis of cyanide contained in bamboo shoots may be attributable to the production of extracellular linamarase during bamboo shoots fermentation. Thus, this study explored the potential of the isolates to produce linamarase for application in the food industry, where they can be employed as starter cultures in fermenting bamboo shoots to develop such products with a lower cyanide content.

3.4 Tolerance to simulated gastrointestinal juice

In the current study, the tolerance of strains BBS1 and BBS13 to the GI tract was evaluated. Cell viability after exposure were compared with the control (pH 6.5) ($P < 0.05$). In the presence of simulated gastric fluid at a pH of 2.5 supplemented with pepsin (Figures 1(A)and 1(B)), the viable cell counts of BBS1 and BBS13 dropped to approximately 7 log CFU/ml after 3 h of incubation, which translates to around 80% and 76% of cells remaining, compared to the control (100%) respectively. Subsequently, both strains were examined in simulated small intestine juice at pH 8.0 composed of oxgall bile salt and pancreatin (Figures 1(C) and 1(D)). For BBS1, the number of viable cells slightly decreased to about 8 log CFU/ml after 2 h of incubation. Then after 3 and 4 h, the number of viable cells plummeted to just over 7 log CFU/ml, but the final cell concentration was still 76% relative to the control.

With regard to BBS13, there was no initial detectable variation in the cell count from the control. The survival numbers diminished to 8.05, 7.24, and 7.06 log CFU/ml, after incubation for 2, 3, and 4 h, respectively, which still showed a survival rate of 75% at the end. There have been reports that *P. pentosaceus* and *L. plantarum* strains isolated from ethnic pickled bamboo shoots were able to survive, about 43-94%, under gastric conditions after exposure to pH 2.0 at 2 h of incubation. Bile salt and pancreatin had no effect on the survival under intestinal conditions after 8 h of incubation. In the digestive system, gastric acid serves as a key host defense mechanism to stop infection from ingested pathogenic microbes [25]. A probiotic bacteria must enter the colon with a high concentration of live cells, roughly 10^5 to 10^6 CFU/ml, to have a positive impact on the health of the consumer [25]. Additionally, the gastrointestinal tract has a pH of 2.5 to 3.5 when there are foods present, as opposed to 1.5 to 2.0 when there are none. Under fed conditions, the bacteria's stomach digestion takes longer [26]. This research showed that BBS1 and BBS13 have the essential characteristics of probiotics since they are highly resistant to simulated gastric and intestinal fluids, making them potentially valuable in food products.

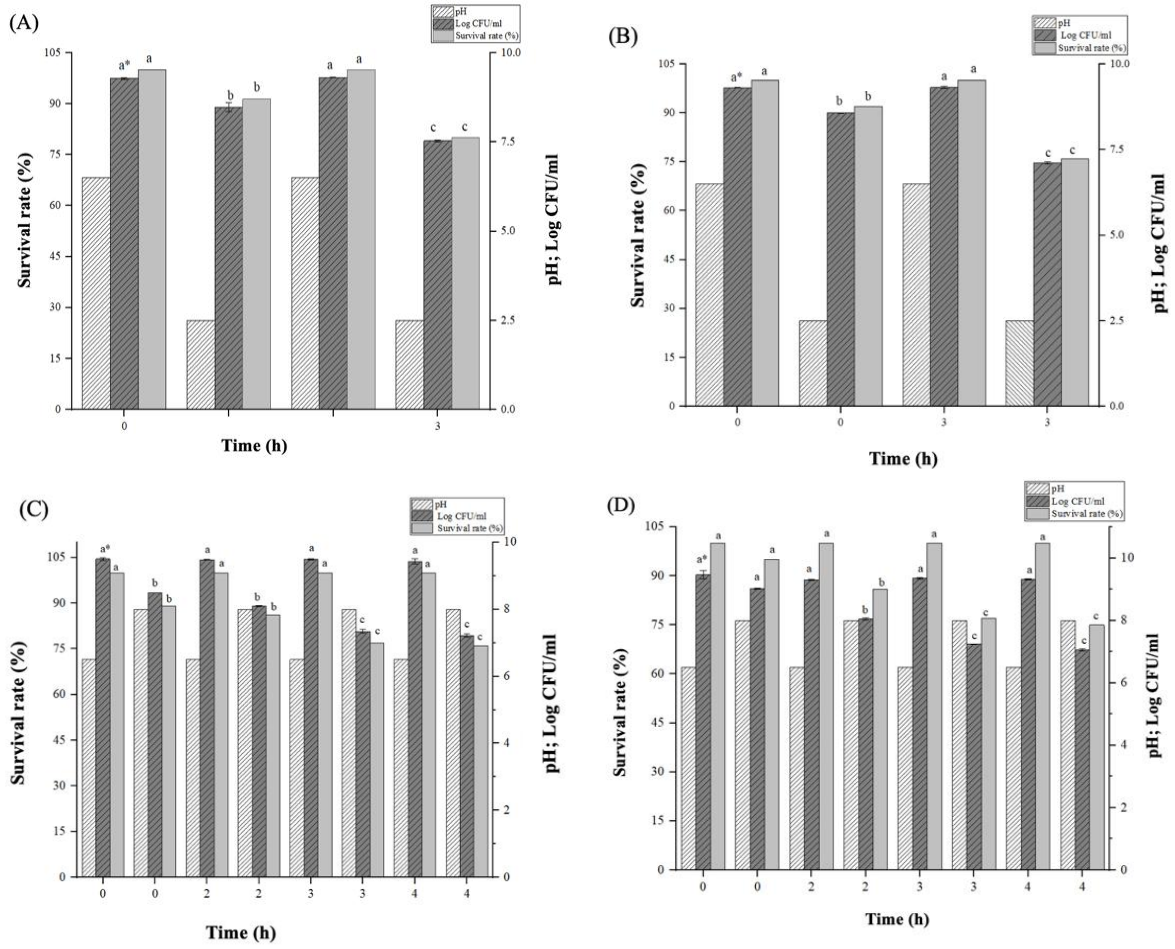

Figure 1 Viable cell counts of the strains BBS1 (A) and BBS13 (B) in simulated gastric juice after 3h of incubation at pH 2.5 supplemented with pepsin. Survival cell counts of BBS1 (C) and BBS13 (D) in small intestinal juice after 4 h of incubation at pH 8.0 supplemented with oxgall bile salt. pH 6.5 was used as a control for both conditions. Values are given as mean ± standard deviation (SD) from triplicate experiments. Percentage survival rate was calculated as [cell number (log CFU/ml) survived in gastric or small intestinal juice /cell number (log CFU/ml) of initial inoculated cell] x 100. *Significant differences based on cell counts are presented in different small letters using the LSD test (P <0.05).

3.5 Bacterial adhesion to hydrocarbon (BATH)

Adhesion to epithelial cell line is a major factor in the selection of potential probiotics. Bacterial adhesion is an intricate process involving non-specific (hydrophobic) and specific ligand-receptor interactions [17]. The hydrophobicity of the strains BBS1 and BBS13 were assessed in the current investigation using two solvents: xylene, utilized as an apolar solvent (bacterial adhesion), and chloroform used as a monopolar acid (electron acceptor for the bacteria) [13]. The percentage of cell adhesion among treatments are shown in Figure 2, statistically compared for significant difference ($P < 0.05$). Results indicated that the hydrophobic values derived for BBS13 was highly adherent to chloroform and xylene at 49.33% and 39.54%, respectively, in comparison with *L. plantarum* BS (positive control). Whereas, BBS1 showed identical behavior in terms of bacterial adherence and cell wall at 40.17% (chloroform) and 36.88% (xylene) compared to the control. Our study revealed that strains have a cell surface with higher acidity (chloroform) in comparison to the hydrophobicity values observed using xylene. *L. plantarum* strains showed adhesion properties to solvents ranging from 47% to 58% [27]. The hydrophobicity and auto-aggregation tests rely on the theory that the first attachment to the epithelial surface is a crucial prerequisite for the colonization of probiotic microorganisms. Hydrophobicity can be assessed by the presence of hydrophobic components in the outer membrane of the organism. It is suggested that hydrophobic interactions are important for the adherence of bacteria to epithelial cells. Despite the use of solvents in the test, it is feasible to qualitatively evaluate the polarity of the bacterial surface, which indicates its ability to adhere to non-polar surfaces such cell surfaces [13, 17]. The

physically and chemically heterogeneous bacterial surface, including hydrophobic amino acids, fatty acid polysaccharides, and other constituents on the cell surface, may be primarily responsible for the varied hydrophobicity shown across the examined strains [28]. Since hydrophobicity is strain-specific, the current findings found that the strains BBS1 and BBS13 maintain adhesion probiotic properties in the gut, offering health benefits.

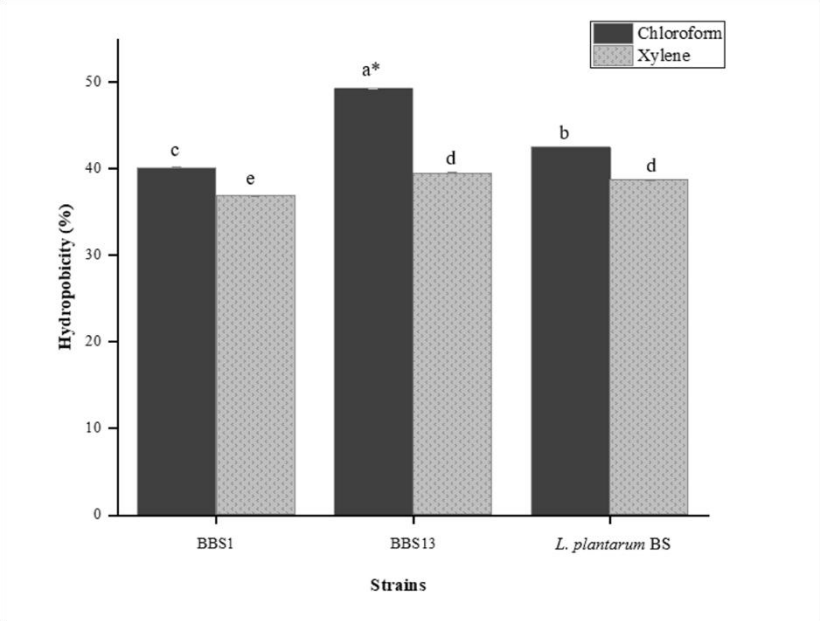

Figure 2 Adhesion ability of the strains BBS1 and BBS13 toward chloroform and xylene. Values are given as mean ± standard deviation (SD) from triplicate experiments. *L. plantarum* BS was used as a positive control. *Significant differences based on cell counts are presented in different small letters using the LSD test ($P < 0.05$).

3.6 Susceptibility to antibiotics

When applied as a selection criterion for promising probiotics, it must be ensured that the used strains are safe and incapable of transferring antibiotic-resistant genes to intestinal microflora. In this regard, antibiotic resistance was assessed according to the MIC breakpoint cut-off values [15] for each species. Strains BBS1 and BBS13 were susceptible to antibiotics that inhibit cell wall synthesis, such as ampicillin, penicillin, and oxacillin. Moreover, susceptibility to protein synthesis inhibitors, including chloramphenicol, clindamycin, erythromycin, gentamicin sulfate, tetracycline, and kanamycin were found in both strains, but resistance to kanamycin was observed in BBS1. *P. pentosaceus* strains isolated from ethnic pickled bamboo shoots exhibited kanamycin resistance and were susceptible to protein synthesis inhibitors found in *Lactobacillus* species [29]. Many LAB, particularly lactobacilli, are naturally or intrinsically resistant to specific antibiotics, as resistance to the aminoglycoside antibiotics (kanamycin, streptomycin, kanamycin, neomycin, and gentamicin), is intrinsic in LAB [30]. The different mechanisms involved in aminoglycoside intrinsic resistance result in the absence of cytochrome-mediated electron transport, hence inhibiting antibiotic uptake, as well as alterations in cellular permeability [31]. However, probiotics with intrinsic antibiotic resistance, such as resistance to glycopeptides and aminoglycosides that are commonly encountered in humans, may be advantageous for maintaining the gastrointestinal tract's equilibrium and minimizing antibiotic-induced diarrheas [31]. On the basis of its antibiotic resistance, it is reasonable to show that intake of the strains BBS1 and BBS13 does not pose any risk to humans.

3.7 Hemolytic activity

Probiotics should be devoid of hemolytic activity [2]. In the present study, strains BBS1 and BBS13 revealed γ- hemolysis (no hemolysis of blood cells) compared to *S. aureus* (positive control) (Figure 3). The result of the current research is in line with the study of [32] who found that *Bifidobacterium* strains and *Lactobacillu*s spp., did not exhibit hemolytic activity. Strains examined in this study did not present a risk to human health on account of their absence of hemolytic activity.

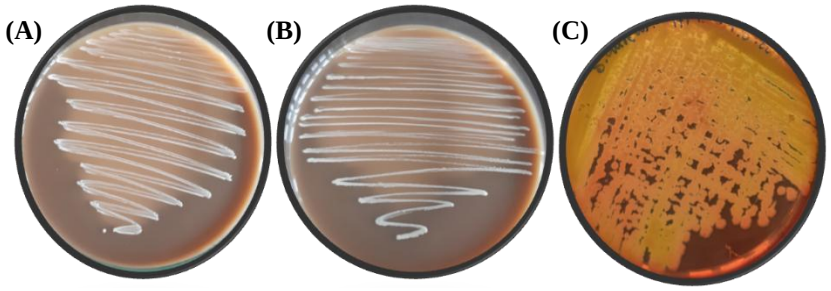

Figure 3 Hemolytic activity (γ–hemolysis) of the BBS1 (A) and BBS13 (B) strains. *S. aureus* DMST 8840 (C) was used as a positive control, indicating partial hydrolysis as manifested by green-hued zones around the colonies (α-hemolysis).

3.8 Antioxidant activity

In vivo generation of reactive oxygen species (ROS) occurs continuously and leads to lipid, protein, and DNA damage. The action of the LAB strains that have colonized the intestinal tract and metabolites of LAB enhance to eliminate ROS, thereby keeping the intestinal oxidation–reduction balance [33]. As shown in Figure 4, the CFS of strains BBS1 and BBS13 indicated antioxidant activity in varying degrees, but significantly lower compared to the positive control (ascorbic acid) which had 97.22% antioxidant activity. The scavenging activity of BBS13 of 77.41% was significantly higher than that of BBS1 (72.74%) but significantly ($p < 0.05$) higher than that of the reference strain *L. plantarum* BS (BIOTECH 10287) (68.35%). Despite the fact that there have been numerous studies on the bacterial antioxidant activity, the results of this work could not be directly compared to the published results due to the different methodologies used. However, these current findings were comparable to some published works indicating that antioxidant capability was strain-specific. DPPH free-radical-scavenging activity, a CFS of *L. plantarum* strain, isolated from kimchi exhibited antioxidant activity ranging from 21-40.97% [31]. It has been reported that *Lactobacillus* and *Pediococcus* strains in traditionally fermented alcoholic beverage from millet in Korea showed that the DPPH radical-scavenging activities ranged from 30-39%. Antioxidant efficacy is commonly measured using DPPH and does not exist naturally, methanol solutions stabilize it [33]. Some LAB, may have antioxidant action due to the synthesis of exopolysaccharides, lipoteichoic acid, and cell-surface proteins [34]. The formation of antioxidant components in bamboo shoots, such as phenolic compounds, flavonoids, and superoxide dismutases (SODs), might enhance strains to present antioxidant activity [34]. Therefore, strains BBS1 and BBS13 which demonstrated significant antioxidant properties, indicating their potential for application in fermented food products that promote health are recommended for development as starter cultures.

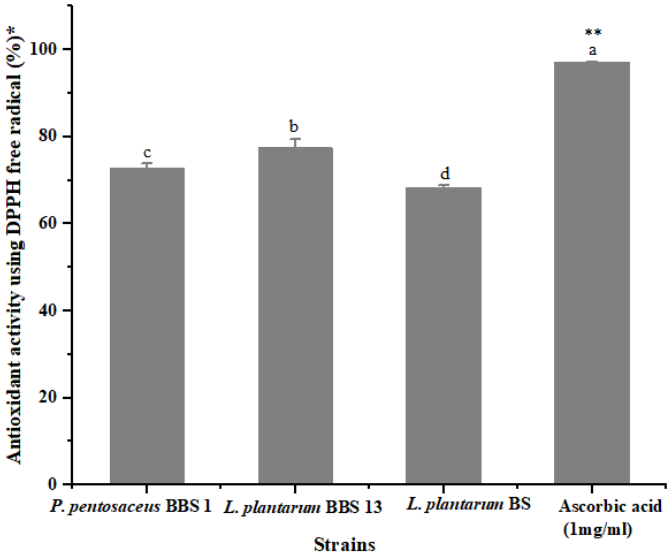

Figure 4 Antioxidant activity of the strains BBS1 and BBS13 performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. *L. plantarum* BS was used as a reference strain. The samples contained of the CFS of a 10^8-10^9 CFU/ml suspension. As, ascorbic acid was a standard (positive control). *Values are given as mean ± standard deviation (SD) from triplicate experiments with lowercase letters (a-d**) indicating significant differences ($P < 0.05$) by the LSD test.

4. Conclusion

Numerous sources of fermented foods contain LAB probiotics. This study found that *Nor mai som* can be another preferred source for LAB probiotic bacteria. *Pediococcus pentosaceus* BBS1 and *Lactiplantibacillus plantarum* BBS13 proved to have probiotic characteristics based on their ability to survive simulated GIT conditions, cell surface hydrophobicity, enzymatic, antibacterial, and antioxidant activities, and absence of safety concerns. This study may shed light on the two strains that have probiotic potential and could be used in functional foods and plant-based fermentation.

5. Acknowledgements

This research was supported by the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) and was conducted at the of BIOTECH, UPLB, Laguna, Philippines. The authors would like to thank Mr. Craig B. Jamieson, Director and founder of the Straw Innovations (native speaker) for English proofreading.

6. Conflict of interest

All the authors declare that there is no conflict of interest.

7. References

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