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ISOLATION, CHARACTERIZATION, AND IDENTIFICATION OF PROBIOTIC LACTIC ACID BACTERIUM FROM *SABENG*, A PHILIPPINE FERMENTED DRINK

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ABSTRACT

The Philippines has many traditional fermented food products still awaiting research. One of these is *sabeng,* a probiotic drink prepared by fermenting washed unpeeled raw sweet potatoes in rice washing. Lactic acid bacteria (LAB) were isolated from *sabeng* and evaluated as potential probiotic. The isolates were screened for acid tolerance, bile tolerance, antioxidant activities [% 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and reducing power], cell hydrophobicity, antimicrobial activities, and enzyme activity. Following isolation and purification, six LAB isolates were obtained: SF37, SF38, SF39, SF40, SF41, and SF42. After screening, only SF42 was found to be acid- and bile-tolerant. It had a % DPPH scavenging activity of 39.39%, an absorbance of 0.48 in the reducing power equivalence assay and a higher than 90% hydrophobicity. It was subjected to antimicrobial assay and found to have antimicrobial activities against *Bacillus subtilis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Vibrio parahaemolyticus*. Using API ZYM, SF42 was also found not to produce ß-glucuronidase, a carcinogenic enzyme, thereby supporting its safe use as probiotic. Sequence identification using BLAST showed SF42 as *Lactobacillus plantarum* at 100% similarity.

Key words: *Lactobacillus plantarum,* probiotics, Philippine fermented foods

INTRODUCTION

The isolation and screening of microorganisms from natural sources have always been the most powerful means of obtaining useful and genetically stable strains for industrially important products (Adnan and Tan 2007). Nowadays, the focus in the isolation of microorganisms is to obtain potential probiotic bacteria, generally lactic acid bacteria (LAB). For microorganisms to be considered as potential probiotics, they should have the ability to strongly adhere to epithelial cells in the gastrointestinal tracts of hosts as well as to survive under unfavorable conditions including low pH and high concentration of bile salts (Lee et al. 2016). In addition, it should have antioxidant activities and antimicrobial properties against common pathogens like *Salmonella typhi, Staphylococcus aureus,* and *Escherichia coli.*

Sabeng is a traditional Philippine vinegary or non-alcoholic fermented probiotic drink produced only in the Cordillera region. It is part of the regular diet of Cordilleran people and is taken

with other food dishes or drunk as a mild alcoholic beverage (Picpican 2003). It is made by placing washed unpeeled sweet potatoes in an earthen jar, adding boiling rice washing (or water) and naturally ferment for a minimum of three days, after which the liquid portion is harvested. The harvested liquid is sour with a strong putrid odor and is used as a broth in tamarind pork stew. However, the continuous existence of *sabeng* is in peril with the slow dying out of age-old traditions among our indigenous tribes. In addition, *sabeng* manufacture has become sparse since it is prepared only during very special occasions like harvest festivities or funerals, and only by old locals. In fact, the introduction of commercial liquors into indigenous communities has slowly eroded the need to produce *sabeng*. Hence, it is important to study this traditional fermented food in particular and all Philippine fermented foods in general before these become extinct, so as to fully elucidate the richness of our food culture as well as to document the varied fermented foods and fermentation processes available in the country.

In the Philippines, traditional fermented foods abound in provinces mainly because of the need to prolong the shelf life of the harvest, fermentation being a very effective and guaranteed means of food preservation. Fermentation in food processing has the advantage of production of desirable enzymes, prevention of the development of undesirable flavors and odors, and of enzymatic degradation, flavor and aroma enhancement, raw material preservation, synthesis of healthy components, digestability improvement, and enhancement of appearance (color and physical state). It involves the conversion of food components into simpler substances by microbial action, such as yeasts and lactic acid bacteria (LAB), which are bacteria of interest because these are usually analyzed for their probiotic characteristics. Thus, here, we aimed to screen, characterize, and identify LAB isolated from *sabeng*. *Sabeng* was chosen to partially elucidate the microflora of this local product but also with the expectation that isolation of probiotics from this product will highlight the importance of fermented foods in the Philippines and promote research on them. This study was conducted from June, 2016 to December, 2017at the Institute of Food Science and Technology, University of the Philippines Los Banos, Laguna, Philippines and Korean Food Research Institute, Jeollabuk-do, Republic of Korea.

MATERIALS AND METHODS

Sampling

Samples of *sabeng* were collected from a household in Barangay Samoki, Bontoc, Mt. Province. These were placed in plastic bottles, kept at refrigerated temperatures, and immediately processed for microbial isolation within 24 h of sampling.

Isolation and purification

Isolation was performed by inoculating aliquots of the samples into De Man, Rogosa and Sharpe (MRS) broths with 0.3% ox gall, and incubated at 30 and 37°C. After an 18 h incubation, 1 mL of each culture was pour-plated on MRS agar with sodium azide and bromcresol purple and incubated again at 30 and 37°C for 18 h. Colonies that grew anaerobically within the yellow region of the solid MRS medium were isolated and purified for screening.

Acid tolerance test

Growth at acidic pH (3.0) was determined using the method of Pringsulaka et al. (2015) and Taheri et al. (2009) with some modifications. The isolates were cultured in MRS broths adjusted to pH 3.0 by 1 M HCl addition. After incubation for 18 and 36 h, the absorbance of the cultures at 600 nm was determined using a UV-Vis spectrophotometer (Thermo Scientific Multiskan GO, Leicester, UK). Cultures with an absorbance greater than 0.050 were then appropriately diluted and pour-plated in MRS agar for the determination of cell count.

Bile tolerance test

Bile tolerance was determined according to Ji et al. (2015). Isolates were inoculated into MRS broth and incubated at 37 °C for 18 h. Then, 1 mL of each culture was added to 9 ml of MRS broth containing 0.3% (w/v) bile salt; the resulting culture was incubated at 37°C for 24 h. Positivity for bile

tolerance was determined by measuring the absorbance of the culture at 660 nm using a UV-Vis spectrophotometer (Thermo Scientific Multiskan GO, Leicester, UK). An absorbance equal to or greater than 0.050 was considered positive for growth and therefore for bile tolerance.

Antioxidant activity assay

DPPH scavenging activity. The antioxidant activity of the cell-free supernatant (CFS) of the isolates was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay according to Prakash et al. (2001) and Gowri and Ranjan (2011). Freshly grown cultures of the isolates were centrifuged at 1,500 xg at 10 °C for 10 min. 1 mL of the CFS of each isolate was carefully pipetted out into a test tube. The CFS was kept in the dark until analysis. 1 mM DPPH was then prepared by dissolving 4 mg of DPPH in 100 mL of methanol. The CFS of each isolate was diluted to 5 mL with deionized water in a test tube, into which 1 mL of 1 mM DPPH was added. The solution was left to stand for 30 min in the dark. A blank was prepared using uninoculated MRS broth instead of the CFS of each isolate. After standing, the solution was read of its absorbance at 517 nm using a UV-Vis spectrophotometer (Thermo Scientific Multiskan GO, Leicester, UK). Antioxidant activity expressed as % DPPH scavenging activity was then calculated using the formula as follows:

$$\%DPPH\ Scavenging\ Activity = \frac{Abs\ standard}{Abs\ sample - Abs\ standard}\ x\ 100$$

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Reducing power assay. Reducing power assay was done using the method of Gowri and Ranjan (2011). 100 µL of the CFS was diluted to 500 µL volume with distilled water. To each isolate, 1.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1.5 ml of 1% potassium ferricyanide were added in succession. The mixture was then incubated at 50°C for 20 min followed by the addition of 2.5 ml of 10% trichloroacetic acid. The solution was then centrifuged at 3500 rpm for 5 min at 4°C. 1 ml of the solution was diluted to 1.5 ml using distilled water and 300 µL of 0.1% ferric chloride was added. The absorbance of the resulting solution was read at 700 nm using a UV-Vis spectrophotometer (Thermo Scientific Multiskan GO, Leicester, UK).

Cell surface hydrophobicity

Cell surface hydrophobicity was determined according to Ji et al. (2015). Isolates were cultured at 37°C for 18 h in MRS broth and harvested by centrifugation at 3,500 rpm for 15 min at 4°C. The cells were washed twice with phosphate-buffered saline (PBS) solution and resuspended in the same buffer. Chloroform was added to the suspension in a 1:1 ratio. The mixture was vortexed for 2 min and left to stand for 30 min to separate the layers. The absorbances of the suspensions of the isolates (reading 1) and separated water-soluble layer (reading 2) were measured at 580 nm using a UV-Vis spectrophotometer (Thermo Scientific Multiskan GO, Leicester, UK) in replicate to calculate hydrophobicity using

$$.\ \%\ Hydrophobicity = \frac{OD580nm\ reading1 - OD580nm\ reading2}{OD580nm\ reading1}\ x\ 100$$

Antimicrobial activity assay

The antimicrobial activity of each isolate was tested against *Bacillus subtilis*, *Es. coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *St. aureus*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*. Fresh 18 h broth cultures of these bacterial pathogens were centrifuged at 500 xg and the supernatant was discarded. The precipitated cells were then suspended in physiological saline solution and adjusted to 10^{-6} to 10^{-7} CFU/ml. The suspension was spread-plated onto plate media specific for the test microorganisms (Luria-Bertani agar for *B. subtilis*, *Es. coli*, and *V. parahaemolyticus*; ATCC medium 2174 with agar for *En. faecalis*; trypticase soy agar for *K. pneumoniae*; brain heart infusion agar for *L. monocytogenes* and *St. aureus*; and nutrient agar for *Sa. typhimurium*) and incubated at appropriate temperatures. Then fresh overnight MRS broth cultures of the isolates were harvested by centrifugation at 5,000 xg for 10 min. The test

CFS and control MRS broth that were neutralized with 1 M NaOH (pH 6.8 ± 0.2) were then filter-sterilized (0.22 µm pore size; Sartorius Stedim France S.A.S., France), and 60 µL of aliquot of each filtrate was made to absorb onto 6-mm-diameter paper discs. The discs were carefully laid on the plates preinoculated with the target pathogens. The plates were incubated at 37°C for 8-12 h and checked for the appearance of a clear inhibition zone. Each assay was performed in triplicate (Menconi *et al.* 2014).

Enzyme activity

An API ZYM kit (API Biomerieux, U.S.A) was used to determine enzyme activity. The isolate was grown at 37°C for 18 h in MRS broth. The isolate was centrifuged and adjusted to McFarland 5. After inoculation, the culture was incubated for 4 h at 37°C. The addition of a surface-active agent (ZYM A reagent) to the cupules facilitated the solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and values ranging from 0-5 (corresponding to the colors developed) were assigned. The approximate number of free nanomoles of hydrolyzed substrate was determined on the basis of color strength determined from the color chart provided in the package insert: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; and 5, 40 nmol or higher.

16S rRNA sequencing

Genomic DNA was isolated using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the protocol suggested by the manufacturer, and the DNA obtained was stored at −20°C until further analysis. Amplification of 16S rRNA gene was performed from the genomic DNA of the isolate using the bacterial universal primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' (Martin and Collen 1998). A 25 mL reaction mixture of PCR was run with 10-100 ng of template DNA, 2.5 mL of 10× reaction buffer, 1.0 mL of 25 mmol/L $MgCl_2$, 0.5 mL each of 10 mmol/L dNTP, 1 mL each of 10 mmol/L primer, 1 U of Taq DNA polymerase, and sterile double-distilled water in a 25 mL final volume. A DNA thermal cycler (PTC-200, MJ Research Inc, USA) was used for thermal amplification, which was programmed as follows: an initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 1.5 min at 72°C, followed by a final elongation for 10 min at 72°C.

Purified PCR products were sequenced through requested service from Macrogen Inc. (Seoul, Korea) with an automated DNA sequencer using 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' as sequencing primers. Sequence similarity was determined using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Sampling

During the fermentation of traditional fermented foods, various organisms are involved, with LAB being the most common microorganisms that play a biopreservation role, contributing to the flavor and controlling the growth of pathogenic and spoilage organisms. Samples of *sabeng* were collected from one household in Bontoc, Mountain Province. This was fortuitous sampling since *sabeng* production, had already stopped in the area and adjacent vicinities. It only happened that a member of the *datu* tribe passed away, necessitating the manufacture of a traditional alcoholic beverage for the burial ceremony. The *sabeng* (Fig 1.) sampled had been prepared and stored for 2 days in a traditional earthen jar kept in a dark corner in the house before the sampling was done. It had a very strong putrid odor that lingered in the mouth when drank.

Fig. 1. Sabeng

Preliminary screening

Following the isolation and purification procedures, six probable LAB strains were isolated from the samples on the basis of their acid production (indicated by a yellow zone) and anaerobic growth on MRS supplemented with bromcresol purple. These were given the codes SF37, SF38, SF39, SF40, SF41, and SF42. Excluding SF41, which was found to be gram-negative, all were confirmed to be LAB on the basis of their characteristics: gram-positive, catalase-negative, anaerobic, acid-producing, and non-spore-forming. The five isolates were further screened for acid and bile tolerance, two of the most crucial properties of probiotic bacteria that determine the ability of these bacteria to survive in the small intestine and consequently their capacity to play their functional role as probiotics. This will ensure that the screened isolates will survive in the gut. Potential probiotics should have high acid tolerance since these should be able to survive passage through gastric juices in the stomach that are generally extremely acidic (pH 2.5-4.0). These should also be able to tolerate bile salt at a concentration of 0.3% ox gall, a level normally encountered in the human intestine (Sjovall 1959). The ability of microorganisms to survive depends on their bile resistance (Gilliland et al. 1984, Gilliland 1987). Bile entering the duodenal section of the small intestine has been found to reduce bacterial survival, which may be due to the breakdown of the bacterial cell membrane consisting mainly of lipids and fatty acids, which are very susceptible to destruction by bile salts. Hence, the success of a probiotic depends on the selected strain possessing bile-resistant qualities. Bile resistance is an important characteristic that enables *Lactobacillus* to survive and grow in the intestinal tract (Jin et al. 1998).

In terms of acid tolerance, S42 clearly showed positive growth in MRS broth at pH 3 based on the cell optical density readings, registering absorbances of 0.241 and 0.437at 600 nm, as well as based on the plate counts of the MRS broth cultures of 3.04 x 10^{-3} and 5.63 x 10^{-4} on MRS agar after 18 and 24 h of incubation, respectively. In terms of bile tolerance, it showed positive growth in MRS broth with 0.3% ox gall after 24 h of incubation at 37°C, as indicated by an absorbance of 0.156 at 660 nm. All the other isolates showed absorbances below 0.05 at 660 nm in both tests. From the results of the screening, only SF42 showed both acid and bile tolerances (Table 1).

Table 1. Potential probiotic properties of screened isolates from *sabeng*

Isolates	Acid Tolerance (pH 3)	Bile Tolerance	% DPPH Scavenging Activity	RA (Abs)	% Hydrophobicity
SF37	-	-	38.35	0.46	100
SF38	-	-	46.74	0.43	96.12
SF39	-	-	41.90	0.52	99.45
SF40	-	-	38.57	0.36	93.12
SF42	+	+	39.39	0.48	100

[a]RA – Reducing activity

Antioxidant activity

Antioxidants with free radical scavenging activities may have great significance in the prevention and therapeutics of diseases involving oxidants or free radicals (Soares et al. 1997). Antioxidant activity is one of the multiple biological effects of polyphenolic compounds, such as flavonoids and phenolic acids, commonly found in plants (Brown and Rice-Evans, 1998; Gil et al. 1999; Kähkönen et al. 1999; Vinson et al. 1995). Thus, antioxidant activity in probiotics could help improve the resistance of the host against diseases. The antioxidant activity of the five isolates was determined by two methods: DPPH scavenging activity assay, which is based on the measurement of the scavenging activity for DPPH that reacts with a suitable reducing agent (Kamleshiya et al. 2012); and reducing power assay, in which the yellow color of the test solution changes to green/blue depending on the reducing power of each component (Wu et al. 2014). The reduction of the ferric cyanide complex to its ferrous form is due to the presence of reducers in the test solution. Note that the reducing capacity of a compound indicates its power to serve as an antioxidant (Laxmi et al. 2007; Nikhat and Subramanyam EVS, 2009).

DPPH scavenging activity is linearly correlated with antioxidant activity (Table 1). The three isolates with the highest antioxidant activities were SF38, SF39, and SF42. On the other hand, results of the reducing power assay showed SF39, SF42, and SF37 having the highest absorbance. The absorbance in reducing power assay is linearly correlated with reducing activity, and thus with antioxidant activity. Thus, the results of the two assays point to two different sets of isolates with high antioxidant activities, with SF39 and SF42 being high in both scavenging and reducing activities. However, SF39 is neither pH- nor bile-tolerant, but SF42 is, which prompted us to focus on SF42.

Cell hydrophobicity

Adhesion can provide information on the possibility of probiotics to colonize the intestines and may modulate the host immune system (Ji et al. 2015). For the isolates to adhere to epithelial cells, at least 85% hydrophobicity is required. Results showed high hydrophobicity for all five isolates (Table 1), indicating their high potential to serve as probotics since they can easily adhere to the gut, where they can exert their antioxidant activity through direct or indirect impact on the colonizing microbiota of the intestines by various mechanisms (Sanders 2011).

Antimicrobial analysis

Recently, there is increasing concern over the rampant and indiscriminate use of antibiotics for disease treatment and growth promotion in livestock as well as with the development of and rise in the number of antibiotic-resistant pathogens. This has led to increased interest in the application of probiotics and their antimicrobial metabolites as alternative antimicrobial strategies for the trestment and prevention of livestock diseases and infections. Thus, the importance of antimicrobial analysis is highlighted. Antimicrobial activity against pathogens is a desirable property of a potential probiotic strain (Shokryazdan 2014). The antipathogenic effect of LAB has been widely reported (Casey et al. 2007), and this effect is due largely to the production by LAB of substances with antibacterial properties, such as hydrogen peroxide, organic acids and bacteriocins (Tejero-Sarinena et al. 2012).

Only SF42, the only acid- and bile-tolerant isolate was subjected to antimicrobial analysis. SF42 has antimicrobial activities against *B. subtilis*, *En. faecalis*, *P. aeruginosa*, *St. aureus*, *Sa. typhimurium* and *V. parahaemolyticus* (Table 2). However, it did not show inhibition against *Es. coli*, which suggest the absence of fecal coliforms in fermented sweet potato from which the isolates were isolated, since antimicrobial activities develop in a given microorganism in response to the presence of other possible competitor or inhibitory microorganisms that may adversely affect its growth. Moreover, the antimicrobial activity of SF42 against, for example, *Sa. typhimurium*, is very important in relation to the probiotic potential of SF42 since *Salmonella* is one of the most common bacterial causes of diarrhea and the most common cause of foodborne-related hospitalizations and deaths linked to

salmonellosis (Wolfram 2017). SF42 can therefore be added to functional food products with the possible prevention or reduction of the risk factors of diseases due to these pathogens.

Table 2. Antimicrobial activities of isolate SF42 against the tested bacteria

Tested bacterium	Diameter of inhibitory zone (mm)
B. subtilis	12.7 ± 0.3
Es. coli	-
En. faecalis	8.7 ± 0.3
K. pneumoniae	-
L. monocytogenes	-
P. aeruginosa	9.8 ± 0.3
St. aureus	8.8 ± 0.3
Sa. typhimurium	9.2 ± 0.3
V. parahaemolytus	12.5 ± 0.5

Enzyme activity

SF42 was found to produce several important enzymes, namely, β-galactosidase, leucine arylamidase, acid phosphatase, and β-glucosidase in high amounts (Table 3). In addition, SF42 showed strong valine arylamidase, β-glucosidase, and *N*-acetyl- β- glucosaminidase activities. SF42 was not positive for β-glucuronidase, which has been associated with carcinogen formation since this enzyme, together with nitroreductase, is involved in the transformation of pro-carcinogens to carcinogen (Goldin 1990). Thus, the three isolates, with their confirmed safety, indeed show promise as probiotics.

Table 3. Enzyme activities of the isolate SF42.

	Enzyme	Activity range
1	Alkaline phosphatase	1
2	Esterase (C4)	0
3	Esterase lipase (C8)	1
4	Lipase (C14)	0
5	Leucine arylamidase	4
6	Valine arylamidase	4
7	Cystine arylamidase	1
8	Trypsin	0
9	α-Chymotrypsin	0
10	Acid phosphatase	3
11	Napthol-AS-BI-phosphohydrolase	1
12	α-Galactosidase	1
13	ß-Galactosidase	4
14	ß-Glucuronidase	0
15	α-Glucosidase	4
16	ß-Glucosidase	3
17	*N*-acetyl-ß-glucosaminidase	3
18	α-Mannosidase	0
19	α-Fucosidase	0

* A value ranging from 0 to 5 is assigned to the standard color: zero represents a negative; 5 represents a reaction of maximum intensity. Values 1 through 4 represent intermediate reactions depending on the level of intensity. The approximate activity may be estimated from color strength: 0 corresponds to a negative reaction; 1 corresponds to the liberation of 5 nmol of hydrolyzed substrate, 2 to 10 nmol, 3 to 20 nmol, 4 to 30 nmol, and 5 to 40 nmol or higher.

Sequence similarity

SF42 exhibited 100% sequence similarity with *Lactobacillus plantarum* JCM1149 strain based on BLAST results. This identity is completely in line with the characteristics of SF42 determined in the preliminary screening: gram-positive, catalase-negative, anaerobic, acid-producing, and non-spore-forming. *L. plantarum* is a transient resident probiotic bacterium in the human gut that effectively eliminates pathogenic bacteria in the human body. It is highly tolerant of stomach acid, can easily make the full trip from supplementation to mouth to intestines to colon to feces, and has an optimum growth temperature very close to the human body temperature. Thus, it is clear from this study that the probiotic properties of the Philippine traditional fermented food s*abeng* is due mainly to the action of lactic acid bacteria, specifically *L. plantarum.* Further study of the exact mechanism of action of this species is under way.

CONCLUSION

Lactic acid bacteria were isolated from *sabeng*, a Philippine fermented food, with the goal of isolating a potential probiotic for use as feed supplement. An acid- and bile-tolerant strain, coded SF42, was isolated which has good antioxidant activities, high cell hydrophobicity, and antimicrobial activities against *B. subtilis*, *En. faecalis*, *P. aeruginosa*, *St. aureus*, *Sa. typhimurium* and *V. parahaemolyticus*. Moreover, it does not produce ß-glucuronidase, a carcinogenic enzyme, thereby supporting its safe use as probiotics. This strain was identified to be *L. plantarum* JCM 1149. Thus, the Philippine traditional fermented food *sabeng* harbors potential probiotics. It is therefore crucial that research on the systematics of the fermenting microflora of our indigenous fermented foods be encouraged hereafter.

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