Isolation and Antimicrobial Activity of Fructophilic Lactic Acid Bacteria from Flowers in the University of the Philippines Diliman, Quezon City

Gil M. Penuliar*
Renz Joseph R. Artezuela
Institute of Biology
University of the Philippines Diliman

ABSTRACT

Fructophilic lactic acid bacteria (FLAB) is a recently discovered group of lactic acid bacteria that prefers fructose as carbohydrate source. The isolation of FLAB from fructose-rich niches like flowers, in particular, and the gut of insect pollinators suggests that it may be used as probiotics. The objective of this study was to determine if FLAB can be isolated from flowers at the Institute of Biology, University of the Philippines Diliman, and to screen them for antimicrobial activity against bacteria that are commonly associated with intestinal diseases. A total of 20 different isolates were obtained from 14 species of flowers. All isolates were identified as LAB, but only 17 out of 18 isolates were osmotolerant in 30% fructose, and only 8 out of 15 isolates had higher absorbance in Fructose Yeast Peptone Broth, which are characteristics of presumptive FLAB. Seven isolates exhibited inhibitory activity in at least three test bacteria in the primary screening and only four isolates had inhibitory activity in at least two test bacteria, particularly against *Enterococcus faecalis* and *Campylobacter jejuni,* in the secondary screening. DNA sequencing and phylogenetic analysis identified isolates MFPS 4.1 and MFRU 7.2 as *Weissella* spp. The *in vitro* antimicrobial activities of these isolates can be studied further for possible applications in food and medicine, and their low sequence similarities suggest that the isolates might be novel *Weissella* species.

Keywords: FLAB, antimicrobial activity, *Weissella* spp.

* *Corresponding Author*

INTRODUCTION

Fructophilic lactic acid bacteria (FLAB) is a unique group of lactic acid bacteria that preferentially utilizes fructose as their main carbohydrate source under anaerobic conditions. In addition, key characteristics of FLAB include a need for electron acceptors, such as pyruvate and oxygen, for effective glucose utilization, and poor carbohydrate fermentation capabilities (Endo et al. 2009). Studies have shown that FLAB can be isolated from fructose-rich environments such as flowers, fruits, fermented products made from fruits, and animal by-products such as honey (Endo and Okada 2008; Endo et al. 2011; Syed Yaacob et al. 2018).

Some species of FLAB, like *Lactobacillus apinorum*, *Lactobacillus kunkeei*, and *Fructobacillus fructosus*, have been isolated from the guts of honeybees and are reported to exhibit antibacterial activity against bacteria that cause diseases in honeybees (Endo and Salminen 2013; Olofsson et al. 2014; Maeno et al. 2017). Development on possible probiotic application of FLAB has advanced in recent years with the discovery of kunkecin A, a bacteriocin produced by *Apilactobacillus kunkeei* FF30-6 isolated from honeybees, which had effectively inhibited *Melissococcus plutonius*, which causes European foulbrood in honeybee larvae (Zendo et al. 2020). It is possible that other members of FLAB isolated from other sources with observed antimicrobial activity can be used as probiotics for other animals.

Probiotics are live microorganisms that confer health benefits when consumed in adequate amounts, such as restoration of normal gut microflora, prevention of colonization of pathogenic bacteria, prevention of viral infections, and promotion of oral health care by preventing or treating oral diseases (Reid 2002; Kang et al. 2019). The mechanism of action of probiotics includes the production of anti-adhering molecules to prevent the attachment of pathogenic bacteria to the epithelial lining of the gut; alteration of gut pH through the production of lactic acid and acetic acid; and modulation of the host's immune response by enhancing the expression of genes involved in the integrity of the intestinal barrier (Bermudez-Brito et al. 2012; Collins et al. 2019).

Currently, there are no published studies related to FLAB in the Philippines. *Fructobacillus tropaeoli*, the most recent species of the genus *Fructobacillus*, was isolated from the Nasturtium flower, *Tropaeolum majus* (Endo et al. 2011). Recent studies had also shown that other members of FLAB such as *L. kunkeei and F. durionis* were isolated from various fruit and flowers (Ruiz Rodriguez et al. 2019; Sakandar et al. 2019). It would be interesting to study the FLAB population present in the diverse flora found in the Philippines, especially the endemic floral species.

The objectives of this study were (1) to isolate presumptive FLAB from flowers in the Institute of Biology, University of the Philippines Diliman, (2) to characterize and screen the isolates for in vitro antimicrobial activity, and (3) to identify the isolates through DNA sequencing. The study aims to contribute information on FLAB by detecting their presence in local flowers and by screening for isolates for antimicrobial activity against selected bacteria associated with intestinal diseases.

MATERIALS AND METHODS

Sample collection

Fourteen flower species were aseptically collected from the Institute of Biology, University of the Philippines Diliman from 30 January 2019 to 8 February 2019. The samples were identified up to the genus and species level by the Jose Vera Santos Memorial Herbarium and were placed in sterile plastic bags before briefly storing in a refrigerator at 4°C prior to processing. Samples were dissected inside a biosafety cabinet, using sterile scalpel and forceps, to collect 1 g of floral nectaries (stamen and pistil), which were macerated inside a sterile 50 mL conical tube.

Isolation and purification of isolates

Enrichment, isolation, and purification methods were adapted and modified based on the study of Endo et al. (2009). Samples were enriched separately in 10 mL of Fructose Yeast Peptone Broth (FYPB) (HiMedia Laboratories, Mumbai, India), de Man, Rogosa and Sharpe Broth (MRSB) (HiMedia Laboratories, Mumbai, India), and Sobremisana, Artezuela and Penuliar Broth (SAPB), supplemented with 0.02% sodium azide and 0.01% nystatin as antifungal agent (ACME Laboratories, Dhaka, Bangladesh), and were incubated at room temperature (28°C) for 24 h.

A loopful of enrichment culture was streaked on Fructose Yeast Peptone Agar (FYPA), de Man, Rogosa and Sharpe Agar (MRSA), MRSA supplemented with 1% fructose, and Sobremisana, Artezuela and Penuliar Agar (SAPA) plates, each supplemented with 0.5% $CaCO_3$. The plates were incubated at room temperature (30°C) for 24–48 h, and isolated colonies with distinct zones of clearing were purified on their respective isolation media using the 3-quadrant streak method. Purification plates were incubated at room temperature (30°C) for 24–48 h.

Gram-staining

Gram-staining was performed based on a method adapted from the American Society for Microbiology (Smith and Hussey 2005). Briefly, one loopful of a 24-h old bacterial culture was smeared onto a glass slide with one drop of distilled water

and allowed to air dry. The smear was heat fixed by passing the glass slide over a flame three times. The slide was flooded with crystal violet for 30 s, rinsed with distilled water, then flooded with Gram's iodine for 1 min. The slide was then rinsed with 95% ethanol drop-wise until the runoffs were clear. The slide was flooded with safranin for 30 s and was rinsed with distilled water. The slide was then blotted dry and viewed using a microscope with 100x magnification. *Staphylococcus aureus* and *Escherichia coli* were used as positive and negative controls, respectively.

Catalase test

Approximately 3 mL of 3% hydrogen peroxide was dispensed in each slant of 24-h old cultures. The presence of effervescence indicated that the isolate was catalase-positive. *S. aureus* and *Streptococcus mutans* were used as positive and negative controls, respectively.

Polymerase chain reaction confirmation using LAB-specific primers

The DNA of the isolates were extracted using the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology), following the protocol for Gram-positive bacteria. A preliminary PCR amplification of the isolates was performed to confirm their identity as LAB. Each 10 µL reaction mixture contained 5 µL GoTaq® Master Mix (Promega, Wisconsin, USA) (with 50 units/mL of Taq DNA polymerase in a proprietary reaction buffer [pH 8.5], 400 µM each of dATP, dGTP, dCTP and dTTP, and 3 mM of $MgCl_2$), 2.4 µL nuclease-free water, 1 µL DNA template, and 0.8 µL each of forward (L15f, 5' GCTCAGGAYGAACGCYGG 3') and reverse (L687r, 5' CACCGCTACACATGRADTTC 3') primers (Hou et al. 2018). PCR was performed in a MyCycler™ Thermal Cycler System (Bio-Rad, California, USA), with the following cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were electrophoresed in a 1% agarose gel with 0.5 µg/mL ethidium bromide at 100 V for 24 min. The amplicons were visualized using a UV transilluminator.

Fructose osmotolerance test

The method used to determine the fructose tolerance of the isolates was adapted and modified from the work of Endo (Endo et al. 2009). Approximately 1.5 x 10^8 CFU/mL of 24-h old cultures were prepared in 0.9% saline, and 500 µL of each suspension was inoculated into scintillation vials containing 5 mL of FYPB with 30% (w/v) fructose. The vials were incubated at 37°C for 24 h under aerobic conditions on an orbital shaker (120 rpm) and observed for growth.

Growth comparison in FYP broth and GYP broth

Growth of the isolates was compared using FYPB and Glucose, Yeast and Peptone Broth (GYPB) under microaerophilic conditions. GYPB has the same components as FYPB except fructose is replaced with glucose. Approximately 1.5 x 10^8 CFU/mL of 24-h old cultures were prepared in 0.9% saline and 500 µL of each suspension was inoculated into separate scintillation vials containing 5 mL of FYPB and GYPB. The vials were incubated at 37°C for 24 h under microaerophilic conditions. An aliquot of 200 µL of each culture was transferred into a 96-well plate, and the absorbance of the cultures was measured by spectrophotometry (BMG Labtech, Offenburg, Germany) using 600 nm wavelength in triplicate and using sterile media as blank. Isolates with higher absorbance readings in FYPB compared to GYPB were considered FLAB.

Agar plug diffusion assay

The agar plug diffusion assay was used as primary screening method for antimicrobial activity against the following test bacteria: *Acinetobacter baumannii* MML ESAB1701, *Campylobacter jejuni* ATCC 33560, *Enterococcus faecalis* BIOTECH 10348, *E. coli* BIOTECH 1634, *Klebsiella pneumoniae* BIOTECH 1754, *Proteus mirabilis* MML ESPM1701, *Pseudomonas aeruginosa* BIOTECH 1335, and *Salmonella* Typhimurium BIOTECH 1756. Approximately 1.5 x 10^8 CFU/mL of 24-h old cultures of the isolates were prepared in 0.9% saline and were lawned on their respective agar medium. The plates were incubated at 37 °C for 24–48 h until the lawns were confluent. Similarly, about 1.5 x 10^8 CFU/mL of 24-h old cultures of the test bacteria were prepared in 0.9% saline and were lawned on Mueller-Hinton Agar (MHA) plates, except for *C. jejuni*, which was prepared using MHA with 5% lysed horse blood. Agar plugs 4.5 mm in diameter were made from the FLAB lawns and were aseptically transferred onto lawns of the test bacteria. The plates were incubated at 37°C, and 42°C for *C. jejuni*, for 24 h. The assay was performed in duplicates. The diameters of zone of inhibition (ZOI) were measured using a caliper and were classified as weak (diameter ⩽ 3 mm), moderate (diameter ⩽ 6 mm) or strong inhibition (diameter > 6 mm) (Pan et al. 2009). Isolates that exhibited moderate to strong inhibition were selected for secondary screening.

Agar well diffusion assay

The agar well diffusion assay was used to screen the cell free supernatant (CFS) of the isolates for antimicrobial activity. Approximately 1.5 x 10^8 CFU/mL of 24-h old cultures were prepared in 0.9% saline and 1 mL of each suspension was inoculated

in a test tube containing 10 mL of their respective broth medium and incubated at 37°C for 24 h. An aliquot of 1 mL from each culture was transferred into separate 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm for 1 min. The supernatants were collected and transferred into new 1.5 mL microcentrifuge tubes. About 1.5 x 10^8 CFU/mL of 24-h old test bacteria cultures were prepared in 0.9% saline and lawned on MHA plates using cotton applicators. Agar wells with a diameter of 6 mm were made on the lawns of test bacteria, and 50 µL of CFS was added into the wells. The CFS was allowed to dry and the plates were incubated at 37°C, and 42°C for *C. jejuni*, for 24 h. The assay was performed in duplicates. The ZOI of the CFS were measured using a caliper and were classified as previously mentioned.

Identification of isolates by DNA sequence analysis

The DNA of isolates were extracted as previously described, and uniplex PCR amplification of the 16S rRNA gene was performed for DNA sequencing. Each 30 µL reaction mixture contained 15 µL GoTaq® Master Mix (Promega, Wisconsin, USA) (with 50 units/mL of Taq DNA polymerase in a proprietary reaction buffer [pH 8.5], 400 µM each of dATP, dGTP, dCTP and dTTP, and 3 mM of $MgCl_2$), 7.2 µL nuclease-free water, 3 µL DNA template, and 2.4 µL each of forward (27F, 5' AGAGTTTGATCCTGGCTCAG 3') and reverse (1492R, 5' TACGGYTACCTTGTTACGACTT 3') primers (Lane 1991). PCR was performed in a MyCycler™ Thermal Cycler System (Bio-Rad, California, USA) with the following cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 74°C for 1 min, and final extension at 74°C for 5 min. Agarose gel electrophoresis was performed as previously described.

Purified PCR products were sent to Macrogen (10F World Meridian Center, 60-24 Gasan-dong, Geumcheon-gu, Seoul 153-781, South Korea) for DNA sequencing. The consensus sequences were determined from the forward and reverse sequences using the Bioedit Sequence Alignment Editor 7.2.6.1 (Hall 1999), and were used to identify the isolates using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) Nucleotide database. The sequences were then deposited in GenBank.

Phylogenetic analysis

A phylogenetic tree was constructed to determine the relationship of the isolates to type strains of FLAB and LAB in GenBank. Full formats of 16S rRNA gene sequences of 43 type strains were downloaded from the nucleotide database of NCBI, including

the sequence of *Lactococcus lactis*, which was used as outgroup. Multiple alignments of the sequences were performed using MEGA X and CLUSTAL_W algorithm (Kumar et al. 2018). The Kimura two-parameter model with gamma distribution was used in calculating the distance matrices for the aligned sequences (Kimura 1980). The Neighbor-Joining (NJ) method (Saitou and Nei 1987) was used to construct the phylogenetic tree, and a bootstrap of 1000 replicates was used to estimate the robustness of each branch (Felsenstein 1985).

Statistical analysis

Significant differences in the growth of the isolates in FYPB and GYPB were determined using student's t-test (α=0.05) in Microsoft® Excel® for Microsoft 365 MSO.

RESULTS

Isolation of presumptive FLAB

Flowers from 14 plant species were collected in the Institute of Biology, University of the Philippines Diliman. The plants were identified up to the genus and species level with the assistance of the staff from the Jose Vera Santos Memorial Herbarium at the Institute of Biology. The plant species were as follows: *Acalypha indica*, *Begonia* sp., *Calotropis gigantea*, *Clerodendrum intermedium*, *Clerodendrum quadriloculare*, *Cosmos sulphureus*, *Hibiscus rosa-sinensis*, *Impatiens* sp., *Plumbago* sp., *Pseuderanthemum* sp., *Rosa* sp., *Ruellia* sp., *Tabernaemontana pandacaqui*, and *Thunbergia grandiflora*. Enrichment and plating of the samples in four isolation media produced colonies of different morphologies ranging from raised and convex elevations, entire and undulate margins, and smooth and glistening appearance. Presumptive FLAB isolates were isolated in all samples except in *T. grandiflora*. Twenty-eight colonies with distinct morphologies and clearing zones were selected for purification and characterization (Table 1).

Table 1: Number of distinct colonies observed in each flower species

Specimen	Media			
	FYPA	MRSA	MRSA + 1% fructose	SAPA
Acalypha indica	0	1	1	0
Begonia sp.	0	1	1	1
Calotropis gigantea	0	0	0	1
Clerodendrum intermedium	0	0	1	1
Clerodendrum quadriloculare	1	1	1	0
Cosmos sulphureus	1	1	1	0
Hibiscus rosa-sinensis	0	1	1	1
Impatiens sp.	1	0	0	0
Plumbago sp.	1	0	0	1
Pseuderanthemum sp.	1	1	2	0
Rosa sp.	0	0	0	0
Ruellia sp.	1	1	1	0
Tabernaemontana pandacaqui	0	1	0	0
Thunbergia grandiflora	0	0	0	0
Subtotal	6	8	9	5
Total	28			

All 28 isolates were Gram-positive, but only 20 isolates were screened for catalase activity due to loss of viability in eight isolates. Eighteen isolates were catalase-negative, while two isolates exhibited catalase activity. All 20 isolates were confirmed as LAB using LAB-specific primers (Table 2, Figure 1). Seventeen isolates were fructose osmotolerant, while eight isolates (FACQ 9, FAIM 21, MAPS 4, MABE 25, MAFPS 4.1, MFRU 7.2, MAFAI 8, and MFHR 16) exhibited higher absorbance in FYPB compared to GYPB and were selected for in vitro antimicrobial activity screening.

Table 2: Characterization of the isolates based on flower origin, catalase test, amplification of LAB-specific primers, fructose osmotolerance and growth in FYPB and GYPB

Isolate	Flower source	Catalase test	PCR with LAB-specific primers	Fructose osmotolerance	Absorbance (600 nm)		
					FYPB	GYPB	p-value
FACS 2	*C. sulphureus*	Negative	Positive	Negative	-	-	-
FACQ 9	*C. quadriloculare*	Negative	Positive	Positive	0.649	0.505	0.074
FAIM 21	*Impatiens* sp.	Negative	Positive	Positive	0.310	0.237	0.007
FAPL 23	*Plumbago* sp.	Negative	Positive	-	-	-	-
MACS 1	*C. sulphureus*	Negative	Positive	Positive	0.530	0.767	0.303
MAPS 4	*Pseuderanthenum* sp.	Negative	Positive	Positive	0.553	0.446	0.059
MARU 7.1	*Ruellia* sp.	Negative	Positive	Positive	-	-	-
MAAI 8	*A. indica*	Negative	Positive	Positive	-	-	-
MACQ 10	*C. quadriloculare*	Negative	Positive	Positive	0.548	0.856	0.166
MATP 17	*T. pandacaqui*	Negative	Positive	Positive	0.138	0.169	0.048
MABE 25	*Begonia* sp.	Negative	Positive	Positive	0.354	0.286	0.355
MFCS 1	*C. sulphureus*	Negative	Positive	Positive	0.693	1.123	0.163
MFPS 4.1	*Pseuderanthenum* sp.	Negative	Positive	Positive	0.734	0.517	0.043
MFPS 4.2	*Pseuderanthenum* sp.	Negative	Positive	Positive	0.830	0.857	0.471
MFRU 7.2	*Ruellia* sp.	Negative	Positive	Positive	0.838	0.483	0.001
MFAI 8	*A. indica*	Negative	Positive	Positive	0.494	0.356	0.008
MFHR 16	*H. rosa-sinensis*	Negative	Positive	Positive	0.523	0.404	0.011
MFBE 25	*Begonia* sp.	Positive	Positive	Positive	0.034	0.113	0.051
SABE 30	*Begonia* sp.	Negative	Positive	Positive	0.018	0.020	0.378
SAHR 35	*H. rosa-sinensis*	Positive	Positive	-	-	-	-

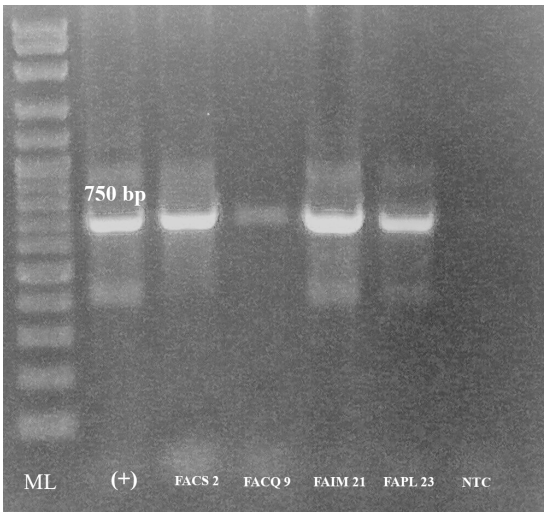

Figure 1: Representative polymerase chain reaction (PCR) results using LAB-specific primers through agarose gel electrophoresis (AGE). Expected amplicon size was around 750 base pairs.

In vitro antimicrobial activity of the isolates

Seven isolates exhibited medium to strong *in vitro* antimicrobial activity in at least three test bacteria in the primary screening (Figure 2 and Figure 3). The largest ZOI was observed in isolate MFPS 4.1 against *E. coli* (9.7 mm) and *Salmonella* (8.925 mm), although a few colonies were seen in the inhibition zone. The largest complete ZOI was observed in isolate MAPS 4 against *C. jejuni* (8.3 mm), and in isolate MFRU 7.2 against *E. faecalis* (7.4 mm). Inhibition of all test bacteria was observed in isolates MFPS 4.1, MFRU 7.2, MFAI 8, and MFHR 16.

Figure 2: Representative result of growth inhibition of isolate MFPS 4.1 against test bacterium *E. faecalis*. Measurements were done using a digital caliper and expressed in millimeter. Agar plug size was measured at 4.5 mm.

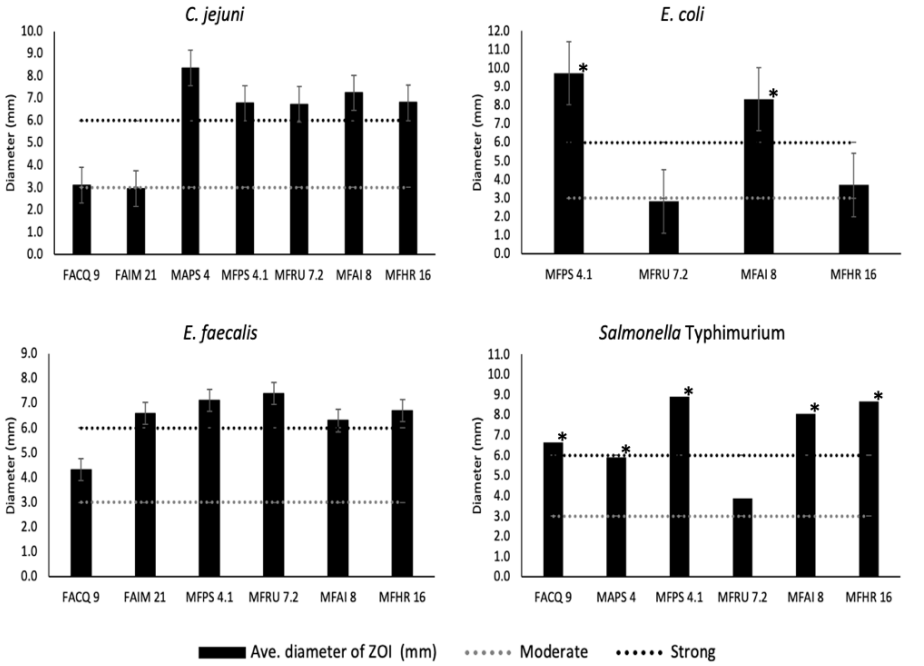

Figure 3: Results of the primary screening of the isolates against four test bacteria. The grey dotted line represents the 3 mm diameter zone of inhibition (ZOI) threshold for isolates with moderate inhibition and the black dotted line represents the 6 mm diameter threshold for isolates with strong inhibition. Isolates that exhibited partial inhibition are marked with an asterisk (*). Isolates that did not exhibit inhibition are not included. Error bars represent standard errors.

Only four isolates had inhibitory activity in at least two test bacteria in the secondary screening (Figure 4). The largest inhibition was observed against *C. jejuni* by CFS from isolates MFPS 4.1 (6.7 mm) and MAPS 4 (6.2 mm), although the CFS from isolate MFRU 7.2 also gave moderate to strong inhibition against *C. jejuni* (4.4 mm) and *E. faecalis* (3.5 mm).

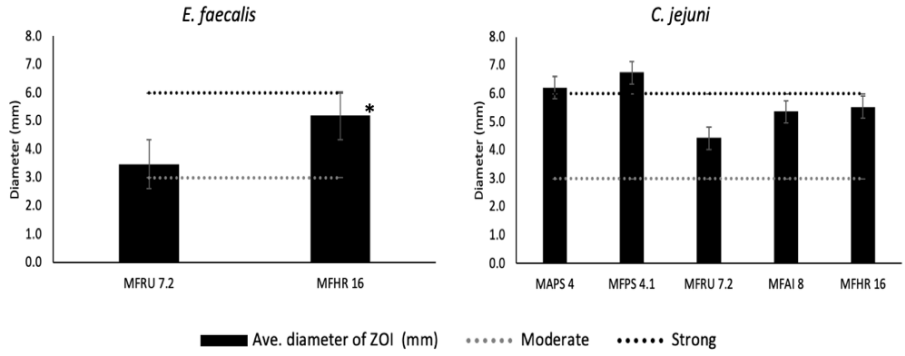

Figure 4: Results of the secondary screening of the isolates against test bacteria. The grey dotted line represents the 3 mm diameter zone of inhibition (ZOI) threshold for isolates with moderate inhibition and the black dotted line represents the 6 mm diameter threshold for isolates with strong inhibition. Isolates that exhibited partial inhibition are marked with asterisk (*). Isolates that did not exhibit inhibition are not included. Error bars represent standard errors.

Identification of the isolates

Only two isolates were selected for molecular identification based on their in vitro antimicrobial activity. DNA sequencing and BLAST identified isolates MFPS 4.1 and MFRU 7.2 as *Weissella cibaria,* although the sequence similarity with the top hit was less than 97%, 95.6% for MFPS 4.1 and 92.2% for MFRU 7.2. The two isolates formed a sub-cluster with *W. cibaria* and *W. confusa* with 95% and 100% bootstrap support (Figure 5).

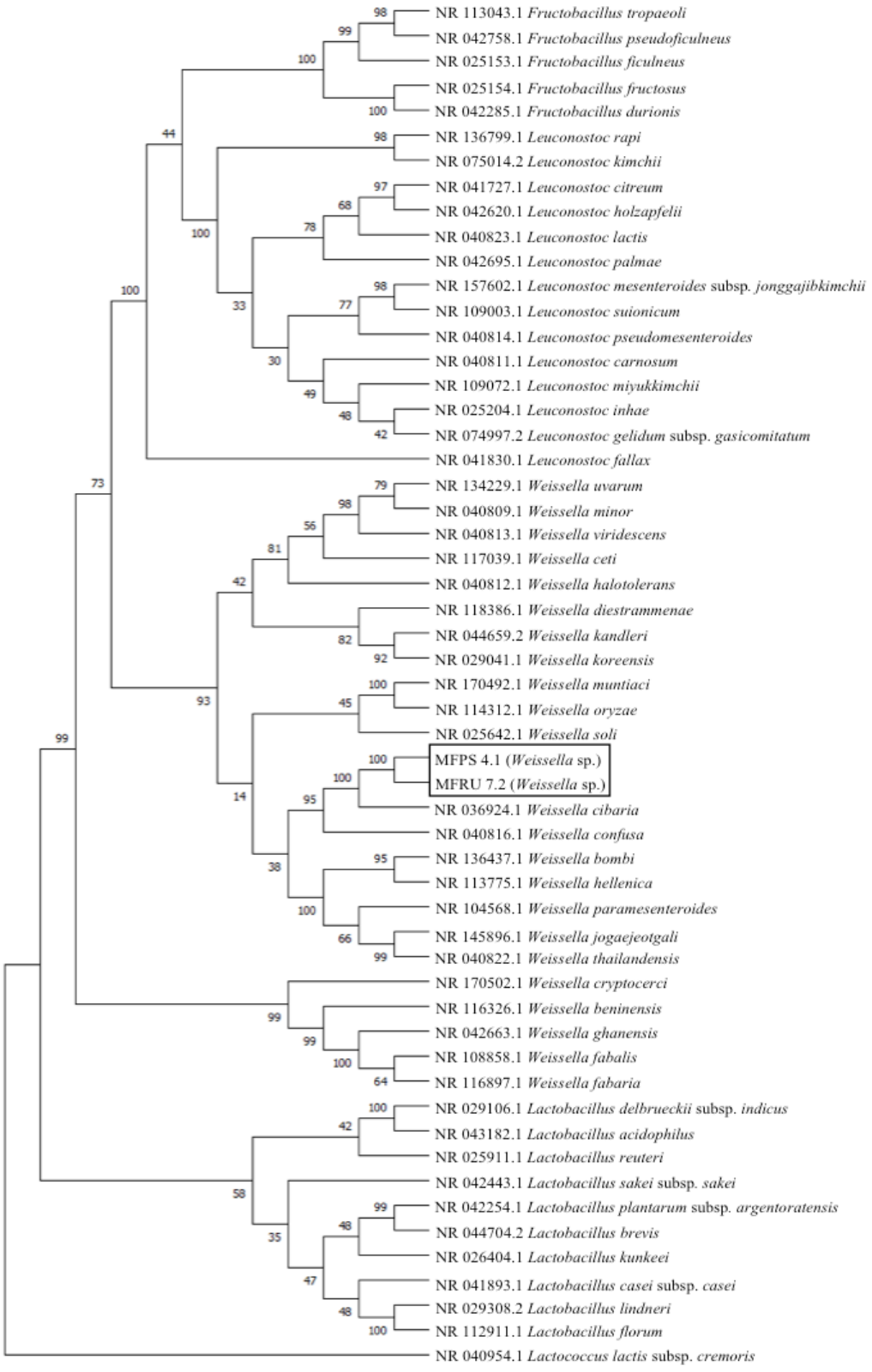

Figure 5: Phylogenetic position of the isolates MFPS 4.1 and MFRU 7.2. The neighbor-joining tree based on the 16S rRNA genes of the isolates and type strains of FLAB and LAB confirms the identification of the isolates as members of the genus *Weissella*.

DISCUSSION

The Institute of Biology, University of the Philippines Diliman was chosen as the sampling site for the flower collection as it is deemed a suitable starting point because it is one of the few remaining green spaces in Metro Manila and also because it houses several native and endemic flowering plant species (Cabel 2019; Rodriguez 2019). Flowers possess nectaries that can produce nectars that are composed of varying concentration of sugars, most commonly a combination of sucrose, glucose and fructose, making flowers a fructose-rich niche (Chalcoff et al. 2006). Fructose-rich niches are probable sources of FLAB since fructose is the preferred carbohydrate source of FLAB over glucose in the absence of external electron acceptors (Endo and Okada 2008).

Among the four different culture media used, both MRSA and MRSA with 1% fructose medium had more isolates observed than both FYPA and SAPA medium (Table 1). A possible explanation might be that formulated media like MRSA would be a better growth media than media created by mixing separate components like FYPA and SAPA. A similar study yielded the same results wherein MRSA with fructose medium was able to isolate both LAB and FLAB (Ruiz Rodriguez et al. 2019) while FYPA was able to isolate strictly FLAB species (Endo et al. 2009). Isolation of FLAB from fructose-rich niches requires enrichment with a medium that contains 1% - 2% (w/v) fructose or, alternatively, conventional medium for LAB isolation, such as MRSA, with fructose supplementation (Endo and Salminen 2013; Olofsson et al. 2014; Endo 2019). Optimum temperatures for isolation range from 30–35°C, depending on the sample used, to simulate the environment where the sample was taken, while the optimum pH for growth is pH 6.5, which was achieved by placing the isolation setup at room temperature (30°C) during the enrichment step (Endo and Okada 2008; Endo et al. 2011; Syed Yaacob et al. 2018; Olofsson et al. 2014). The addition of sodium azide to the enrichment medium inhibits the growth of Gram-negative bacteria, while the addition of nystatin inhibits the growth of yeasts (Snyder and Herman 1940; Barney et al. 2003).

Two out of 14 flower species have the greatest number of distinct isolates. These are *C. quadriloculare* and *Pseuderanthemun* sp., which have four distinct isolates each. Both flower species have deep corolla tubes. A study conducted in temperate forests in South America stated that flowers with deep corolla tubes have low viscosity nectars due to low evaporation rates compared to more open flowers (Chalcoff et al. 2006). This might explain the number of distinct isolates obtained from the two flower species and agrees with the absence of isolates from the

T. grandiflora, which are relatively open flowers with short corolla tubes, although further studies are needed to establish the correlation.

All 28 isolates were Gram-positive and 18 out of 20 isolates tested were catalase-negative, which are typical characteristics of FLAB (Endo and Okada 2008; Endo et al. 2012). Similar results can be found in a study conducted by Endo et al. (2009) wherein isolates identified as *Fructobacillus* sp. were catalase-negative while other LAB isolated exhibited catalase-like activity. Members of FLAB are expected to have higher absorbance values when cultured in FYPB compared to GYPB under microaerophilic conditions, as observed in this study and particularly seen in eight isolates (FACQ 9, FAIM 21, MAPS 4, MABE 25, MAFPS 4.1, MFRU 7.2, MAFAI 8, and MFHR 16). However, two of the isolates (MAPS 4 and MABE 25) did not have significant difference when analyzed using t-test ($p>0.05$). Another study, however, reported that all their presumptive FLAB isolates were isolated using FYPB and FYPA, and had better growth in FYPB than GYPB (Endo et al. 2010). In contrast, the isolates in the current study were isolated using MRSA, MRSA with fructose, and FYPA. Members of FLAB are xerotolerant and can survive in an environment with low water activity (Endo and Okada 2008). Increasing the fructose content to 30% (w/v) in FYPB results in an environment with high osmotic pressure and low water activity, which is a condition conducive for FLAB selection (Endo 2012; Endo 2019). All eight isolates still proceeded to the next step based on the higher absorbance observed in FYPB compared to GYPB and to test more isolates for antimicrobial activity.

Although not currently recognized as FLAB, our isolates that were identified as *Weissella* spp. were obtained using standard methods for isolating FLAB. *Weissella* is a genus of Gram-positive, catalase-negative, and non-spore forming LAB, with some species reported as osmotolerant. *Weissella* spp. have been isolated from fermented food, vegetation, and silage. Many of its species are heterofermentative LAB that utilize a wide variety of carbohydrate sources, including glucose and fructose, due to their numerous phosphotransferase systems (Fusco et al. 2015; Assamoi et al. 2016; Säde and Björkroth 2019).

Antimicrobial activity of FLAB isolates

Seven test bacteria that cause several gut related diseases, such as a variety of infections, diarrhea, campylobacteriosis, and salmonellosis, were chosen for the study. Several studies also included some of the test bacteria in determining the antimicrobial activity of LAB isolated from various sources particularly against *E. coli* and *Salmonella* sp. (Gao et al. 2019; Sakandar et al. 2019; Lakra et al. 2020).

Numerous members of LAB have been used as probiotics in recent years, particularly *W. cibaria*, which had been studied for its possible growth inhibitory activity against different pathogens. Both isolates MFPS 4.1 and MFRU 7.2, identified as *W. cibaria*, exhibited strong antagonistic activity against *C. jejuni*, which causes campylobacteriosis. *W. cibaria* JW15 was shown to have inhibitory effects on the growth of *C. jejuni* in the study conducted by Yu et al. (2019). The same study also showed that *W. cibaria* JW15 inhibited the growth of *E. coli* and *Salmonella* Typhimurium, which was also observed on isolates MFPS 4.1 and MFRU 7.2, respectively. Based on the study, *W. cibaria* JW15 was shown to have an increased acetic acid production compared to other LAB, which explains its ability to inhibit the growth of several pathogens. Another study also observed that *W. cibaria* was able to inhibit the growth of *E. coli* (Lakra et al. 2020). In the study, it was concluded that acid production in the supernatant of *W. cibaria* inhibited the growth of *E. coli*, and loss of inhibitory activity was observed when the pH was neutralized. To date, studies on specific inhibition of *W. cibaria* against *E. faecalis* are scarce but other members of LAB, such as *Lactococcus* sp., were able to inhibit the growth of said test pathogen (González et al. 2007). Similar published studies on the antimicrobial activity of FLAB from the Philippines are also scarce, indicating that the current study might be the first one.

Several studies also reported the production of other antimicrobial metabolites by *Weissella* sp. The bacteriocin Weissellicin 110 from *W. cibaria* inhibits growth of other Gram-positive bacteria, while weissellin A from *Weissella paramesenteroides* has been reported to inhibit food spoilage bacteria and *Listeria* (Srionnual et al. 2007; Papagianni and Papamichael 2011). Production of hydrogen peroxide by *W. cibaria* was found to inhibit the growth of *Fusobacterium nucleatum* (Kang et al. 2006). This was supported by another study that reported that *W. cibaria* CMU, an oral care probiotic, inhibits the growth of common cariogenic bacteria such as *F. nucleatum* and *Porphyromonas gingivalis*. The same strain had also effectively inhibited the biofilm formation of *Streptococcus mutans* (Jang et al. 2016). As mentioned in the previous studies, isolates MFPS 4.1 and MFRU 7.2, which were identified as *W. cibaria*, can have potential use in the food industry as food additive to prevent food spoilage and in the medical field as a probiotic.

The growth inhibition exhibited by the isolates against the test bacteria in the primary screening were either reduced or absent in the secondary screening. Several studies have shown that the presence of stress in a microbial environment plays an important role in the production of antimicrobial substances. Cell-to-cell contact of the isolates with the test bacteria in the primary screening may

be interpreted as stress in the form of competitive exclusion, thus the production of antimicrobial substance. However, the same stress was not present in the preparation of CFS since the isolates were grown in optimized culturing condition in broth media, therefore production of antimicrobial substance may have been reduced or limited. Assuming that the inhibitory substances produced by the isolates were bacteriocin or bacteriolysins, a possible explanation might be that the inhibitory substances produced were stress-induced. It has been reported that *L. kunkeei* produces bacteriolysin in the presence of microbial stressors, such as peptidoglycan, lipopolysaccharides, and lipoteichoic acid (Butler et al. 2013). This is in accordance with another study where the gene promoter for the production of bacteriocin, bactofencin A from *L. salivarius*, observed an increase in activity when cells were subjected to microbial stress such as exposure to bile salts, gastric fluids, and target microbiota (Guinane et al. 2015). Addition of sodium acetate in *Weissella cibaria* culture has been reported to produce an increased amount of H_2O_2, which have inhibitory activity against *F. nucleatum* (Kang et al. 2006; [b]Endo et al. 2009).

Another possible explanation for the lack of antimicrobial activity of the CFS is that the conditions, like temperature, pH, amount of carbohydrate source, and oxygen requirements, for the optimized production of either bacteriocin, bacteriolysin or organic acids were not met when the isolates were grown in broth cultures. CFS used in the assay were collected from cultures that were grown in optimized conditions for growth rather than for optimized bacteriocin, bacteriolysin or organic acid production. Several studies, however, used similar culture conditions for optimized bacterial growth and CFS preparation for antimicrobial assays (Pan et al. 2009; Endo and Salminen 2013; Syed Yaacob et al. 2018). A study on the production of bacteriocin-like inhibitory substance (BLIS) produced by *Pediococcus acidilactici*, for example, had different growth conditions when grown for the optimized BLIS production compared to conditions optimized for cell growth. In the study, 37 °C was used as the optimal temperature and the culture was grown for 24 h for the growth of *P. acidilactici*, while 28.5 °C was used as optimal temperature and the culture was grown for 28 h for optimized BLIS production (Md Sidek et al. 2018). Another study on *Carnobacterium divergens* reported different culturing conditions for optimized growth and for optimized bacteriocin production. In the study, optimized condition for growth of *C. divergens* were 15 °C, pH 6.5–8, and the amount of carbohydrate source was 2% (w/v) while the optimized condition for its bacteriocin activity were 15 °C, pH 6.5, and the amount of carbohydrate source ranged from 0.2% - 2% (Brilletviel et al. 2016).

Phylogenetic tree analysis

The phylogenetic tree has 93% bootstrap support for the isolates clustering with the genus *Weissella*, and 95% and 100% bootstrap support for the isolates forming a sub-cluster with *W. cibaria* and *W. confusa*, respectively, which is consistent with the BLAST identification of the isolates based on their 16S rRNA gene sequences. The high support for the phylogenetic positions of the isolates confirms the identity of the isolates as belonging to genus *Weissella*.

With regard to the species identification of the isolates, it has been reported that the recommended range of 16s rRNA gene sequence similarity for species differentiation is 98.9% - 99.0% (Stackerbrandt and Ebers 2006). It is therefore possible that isolates MFPS 4.1 and MFRU 7.2 are new species under the genus *Weissella*, or new species that are closely related to *W. cibaria*. However, this needs to be confirmed by further studies on the molecular and physiological level, which should include an extensive study of their cell morphology using scanning electron microscope (SEM), assessment of their carbohydrate fermentation capabilities and antimicrobial susceptibility, and additional phylogenetic analyses using multiple housekeeping genes to definitively determine the taxonomic positions of the isolates.

CONCLUSIONS

This work describes the first attempt to isolate FLAB from flowers in the Philippines. The results show that eight presumptive FLAB isolates were isolated and two *Weissella* spp. were identified from flowers from the University of the Philippines Diliman based on the antimicrobial activity. Isolates MFPS 4.1 and MFRU 7.2 displayed FLAB features such as fructose osmotolerance and preferential growth in FYPB, and exhibited *in vitro* antimicrobial activity against several enteric pathogens, particularly *C. jejuni* and *E. faecalis,* which can be studied further for possible applications in both the food industry and the medical field, such as as probiotics.

Data Availability

The sequences of the isolates described in the study have been deposited in GenBank with the following accession numbers: MW836947.1, MW836946.1, MW836945.1, MW836944.1, and OL629482.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Gilbert S. Sobremisana, Ma. Socorro Edden P. Subejano and Jann Eldy L. Daquioag. This work was supported by the UP Diliman Research Load Credit/Creative Work Load Credit (RLC/CWLC) and the UP Diliman Office of the Vice-Chancellor for Research and Development, with project code IB-PFGMP-2020-01.

REFERENCES

Assamoi AA, Krabi ER, Ehon AF, N'guessan GA, Niamké LS, Thonart P. 2016. Isolation and screening of *Weissella* strains for their potential use as starter during attiéké production. Biotechnol Agron Soc Environ [Internet]. [cited 2020 May 6]; 20(3):355–362. http://doi.org/10.25518/1780-4507.13117

Barney M, Barbero F, Barbero I, Bates S, Bendiak D, Castañé FX, Conlin H, Dickerson P, Garcia M, Helber J, et al. 2003. Evaluation of nystatin as an alternative to cycloheximide in selective culture media (ICM). J Am Soc Brew Chem [Internet]. [cited 2020 May 6]; 61(4):235–238. http://doi.org/10.1094/asbcj-61-0235

Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. 2012. Probiotic mechanisms of action. Ann Nutr Metab [Internet]. [cited 2020 May 6]; 61(2):160–174. http://doi.org/10.1094/ASBCJ-61-0235

Brillet-viel A, Pilet M, Courcoux P, Prévost H. 2016. Optimization of growth and bacteriocin activity of the food bioprotective *Carnobacterium divergens* V41 in an animal origin protein free medium. Front Mar Sci [Internet]. [cited 2020 May 10]; 3(128):1–13. http://doi.org/10.3389/fmars.2016.00128

Butler È, Alsterfjord M, Olofsson TC, Karlsson C, Malmström J, Vásquez A. 2013. Proteins of novel lactic acid bacteria from *Apis mellifera mellifera*: an insight into the production of known extra-cellular proteins during microbial stress. BMC Microbiol [Internet]. [cited 2020 May 8]; 13(1):1–11. http://doi.org/10.1186/1471-2180-13-235

Cabel IC. 2019 April 8. Walk and learn to save PH native tree species [Internet]. Philippine Daily Inquirer. [cited 2021 Nov 15]. http://newsinfo.inquirer.net/1104179/walk-and-learn-to-save-ph-native-tree-species

Chalcoff VR, Aizen MA, Galetto L. 2006. Nectar concentration and composition of 26 species from the temperate forest of South America. Ann Bot [Internet]. [cited 2021 Nov 17]; 97(3):413–421. doi:10.1093/aob/mcj043.

Collins FWJ, Rea MC, Hill C, Ross RP. 2019. Antibacterials from lactic acid bacteria and their potential applications. In: Vinderola G, Ouwehand A, Salminen S, von Wright A, editors. Lactic acid bacteria: microbiological and functional aspects. 5th ed. Boca Raton (FL): CRC Press. p. 151–174.

Endo A, Okada S. 2008. Reclassification of the genus *Leuconostoc* and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. Int J Syst Evol Microbiol [Internet]. [cited 2020 April 5]; 58(9):2195–2205. http://doi.org/10.1099/ijs.0.65609-0

[a]Endo A, Futagawa-Endo Y, Dicks LMT. 2009. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. Syst Appl Microbiol [Internet]. [cited 2020 April 5]; 32(8):593–600. http://doi.org/10.1016/j.syapm.2009.08.002

[b]Endo A, Futagawa-Endo Y, Kawasaki S, Dicks LMT, Niimura Y, Okada S. 2009. Sodium acetate enhances hydrogen peroxide production in *Weissella cibaria*. Lett Appl Microbiol [Internet]. [cited 2020 April 5]; 49(1):136–141. http://doi.org/10.1099/ijs.0.65609-0

Endo A, Futagawa-Endo Y, Sakamoto M, Kitahara M, Dicks LMT. 2010. *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. Int J Syst Evol Microbiol [Internet]. [cited 2021 Nov 19]; 60:2478–2482. doi: 10.1099/ijs.0.019067-0

Endo A, Irisawa T, Futagawa-Endo Y, Sonomoto K, Itoh K, Takano K, Okada S, Dicks LM T. 2011. *Fructobacillus tropaeoli* sp. nov., a fructophilic lactic acid bacterium isolated from a flower. Int J Syst Evol Microbiol [Internet]. [cited 2020 April 5]; 61(4):898–902. http://doi.org/10.1099/ijs.0.023838-0

Endo A, Irisawa T, Futagawa-Endo Y, Takano K, du Toit M, Okada S, Dicks, LMT. 2012. Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium. Int J Syst Evol Microbiol [Internet]. [cited 2020 April 5]; 62:500–504. http://doi.org/10.1099/ijs.0.031054-0

Endo A. 2012. Fructophilic lactic acid bacteria inhabit fructose-rich niches in nature. Microb Ecol Health Dis [Internet]. [cited 2020 April 5]; 23:6–10. http://doi.org/10.3402/mehd.v23i0.18563

Endo A, Salminen S. 2013. Honeybees and beehives are rich sources for fructophilic lactic acid bacteria. Syst Appl Microbiol [Internet]. [cited 2020 April 5]; 36(6):444–448. http://doi.org/10.1016/j.syapm.2013.06.002

Endo A. 2019. Fructophilic lactic acid bacteria: an overview of their unique properties. In: Vinderola G, Ouwehand A, Salminen S, von Wright A, editors. Lactic acid bacteria: microbiological and functional aspects. 5th ed. Boca Raton (FL): CRC Press. p. 57–63.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution [Internet]. [cited 2020 May 1]; 39(4):783–791. http://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Fusco V, Quero GM, Cho GS, Kabisch J, Meske D, Neve H, Bockelmann W, Franz CMAP. 2015. The genus *Weissella*: taxonomy, ecology and biotechnological potential. Front Microbiol [Internet]. [cited 2020 March 16]; 6:1–22. https://doi.org/10.3389/fmicb.2015.00155

Gao Z, Daliri EBM, Wang JUN, Liu D, Chen S, Ye X, Ding T. 2019. Inhibitory effect of lactic acid bacteria on foodborne pathogens: a review. J Food Prot [Internet]. [cited 2021 Nov 18]; 82(3):441–453. doi:10.4315/0362-028X.JFP-18-303.

González L, Sandoval H, Sacristán N, Castro JM, Fresno JM, Tornadijo ME. 2007. Identification of lactic acid bacteria isolated from Genestoso cheese throughout ripening and study of their antimicrobial activity. Food Control [Internet]. [cited 2021 Nov 18]; 18(6):716–722. http://doi.org/10.1016/j.foodcont.2006.03.008

Guinane CM, Piper C, Draper LA, O'Connor PM, Hill C, Paul Ross R, Cotter PD. 2015. Impact of environmental factors on bacteriocin promoter activity in gut-derived *Lactobacillus salivarius*. Appl Environ Microbiol [Internet]. [cited 2020 May 10]; 81(22):7851–7859. http://doi.org/10.1128/AEM.02339-15

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95–98.

Hou Q, Bai X, Li W, Gao X, Zhang F, Sun Z, Zhang H. 2018. Design of primers for evaluation of lactic acid bacteria populations in complex biological samples. Front Microbiol [Internet]. [cited 2020 May 10]; 9:1–10. http://doi.org/10.3389/fmicb.2018.02045

Jang HJ, Kang MS, Yi SH, Hong JY, Hong SP. 2016. Comparative study on the characteristics of Weissella cibaria CMU and probiotic strains for oral care. Molecules [Internet]. [cited 2021 Nov 18]; 21(12). doi:10.3390/molecules21121752.

Kang MS, Kim BG, Chung J, Lee HC, Oh JS. 2006. Inhibitory effect of *Weissella cibaria* isolates on the productionof volatile sulphur compounds. J Clin Periodontol [Internet]. [cited 2021 Nov 18]; 33:226–232. doi: 10.1111/j.1600-051X.2006.00893.x

Kang MS, Yeu JE, Hong SP. 2019. Safety evaluation of oral care probiotics Weissella cibaria CMU and CMS1 by phenotypic land genotypic analysis. Int J Mol Sci [Internet]. [cited 2021 Nov 18]; 20(11). doi:10.3390/ijms20112693

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol [Internet]. [cited 2020 May 1]; 16(2):111–120. http://doi.org/10.1007/BF01731581

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol [Internet]. [cited 2020 April 29]; 35(6):1547–1549. http://doi.org/10.1093/molbev/msy096

Lakra AK, Domdi L, Hanjon G, Tilwani YM, Arul V. 2020. Some probiotic potential of Weissella confusa MD1 and Weissella cibaria MD2 isolated from fermented batter. LWT [Internet]. [cited 2021 Nov 18]; 125. doi:10.1016/j.lwt.2020.109261.

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. Chichester: John Wiley and Sons. p. 115–147.

Maeno S, Dicks L, Nakagawa J, Endo A. 2017. *Lactobacillus apinorum* belongs to the fructophilic lactic acid bacteria. Biosci Microbiota Food Health [Internet]. [cited 2020 May 6]; 36(4):147–149. http://doi.org/10.12938/bmfh.17-008

Md Sidek NL, Halim M, Tan JS, Abbasiliasi S, Mustafa S, Ariff AB. 2018. Stability of bacteriocin-like inhibitory substance (BLIS) produced by *Pediococcus acidilactici* kp10 at different extreme conditions. Biomed Res Int [Internet]. [cited 2020 May 10]; 2018; 1–11. http://doi.org/10.1155/2018/5973484

Olofsson TC, Alsterfjord M, Nilson B, Butler È, Vásquez A. 2014. *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isol. Int J Syst Evol Microbiol [Internet]. [cited 2020 April 5]; 64:3109–3119. https://doi.org/10.1099/ijs.0.059600-0

Pan X, Chen F, Wu T, Tang H, Zhao Z. 2009. The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. Food Control [Internet]. [cited 2020 April 5]; 20(6):598–602. https://doi.org/10.1016/j.foodcont.2008.08.019

Papagianni M, Papamichael EM. 2011. Bioresource technology purification, amino acid sequence and characterization of the class IIa bacteriocin weissellin A, produced by *Weissella paramesenteroides* DX. Bioresour Technol [Internet]. [cited 2020 April 5]; 102(12):6730–6734. http://doi.org/10.1099/ijs.0.059600-0

Rodriguez M. 13 Sep 2019. Ten green spaces in Metro Manila for a quick escape [Internet]. Spot.ph. [cited 2021 Nov 15]. http://spot.ph/newsfeatures/the-latest-news-features/79079/10-green-pockets-in-metro-manila-a4362-20190913

Ruiz Rodriguez LG, Mohamed F, Bleckwedel J, Medina R, De Vuyst L, Herber EM, Mozzi F. 2019. Diversity and functional properties of lactic acid bacteria isolated fromwild fruits and flowers present in Northern Argentina. Front Microbiol [Internet]. [cited 2021 Nov 17]; 10(1091):1–26. doi: 10.3389/fmicb.2019.01091

Reid G, Burton J. 2002. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microbes Infec [Internet]. [cited 2020 April 5]; 4(3):319–324. http://doi.org/10.1016/S1286-4579(02)01544-7

Sakandar HA, Kubow S, Sadiq FA. 2019. Isolation and in-vitro probiotic characterization of fructophilic lactic acid bacteria from Chinese fruits and flowers. LWT [Internet]. [cited 2021 Nov 17]; 104:70–75. doi:10.1016/j.lwt.2019.01.038

Säde E, Björkroth J. 2019. Introduction to the genera *Pediococcus*, *Leuconostoc*, *Weissella*, and *Carnobacterium*. In: Vinderola G, Ouwehand A, Salminen S, von Wright A, editors. Lactic acid bacteria: microbiological and functional aspects. 5th ed. Boca Raton (FL): CRC Press. p. 65–85.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol [Internet]. [cited 2020 May 1]; 4(4):406–425. http://doi.org/10.1093/oxfordjournals.molbev.a040454

Smith AC, Hussey MA. 2005. Gram stain protocols [Internet]. Atlanta (GA): American Society for Microbiology; [cited 2020 April 21]. http://asm.org/getattachment/5c95a063-326b-4b2f-98ce-001de9a5ece3/gram-stain-protocol-2886.pdf

Snyder ML, Herman LC. 1940. Sodium azide as an inhibiting substance for Gram-negative bacteria. J Infec Dis. 67(2):113–115.

Srionnual S, Yanagida F, Lin LH, Hsiao KN, Chen YS. 2007. Weissellicin 110, a newly discovered bacteriocin from *Weissella cibaria* 110, isolated from plaa-som, a fermented fish product from Thailand. Appl Environ Microbiol [Internet]. [cited 2020 May 8]; 73(7):2247–2250. http://doi.org/10.1128/AEM.02484-06

Stackerbrandt E, Ebers J. 2006 November. Taxonomic parameters revisited: tarnished gold standards. Microbiology Today. 33:152–155.

Syed Yaacob SN, Huyop F, Kamarulzaman Raja Ibrahim, R, Wahab RA. 2018. Identification of *Lactobacillus* spp. and *Fructobacillus* spp. isolated from fresh *Heterotrigona itama* honey and their antagonistic activities against clinical pathogenic bacteria. J Apic Science [Internet]. [cited 2020 April 5]; 57(3):395–405. http://doi.org/10.1080/00218839.2018.1428047

Yu HS, Lee NK, Choi AJ, Choe JS, Bae CH, Paik HD. 2019. Antagonistic and antioxidant effect of probiotic Weissella cibaria JW15. Food Sci Biotechnol [Internet]. [cited 2021 Nov 18]; 28(3):851–855. doi:10.1007/s10068-018-0519-6.

Zendo T, Ohashi C, Maeno S, Piao X, Salminen S, Sonomoto K, Endo A. 2020. Kunkecin A, a new nisin variant bacteriocin produced by the fructophilic lactic acid bacterium, *Apilactobacillus kunkeei* FF30-6 isolated from honey bees. Front Microbiol [Internet]. [cited 2021 Nov 18]; 11. doi:10.3389/fmicb.2020.571903.

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Renz Joseph R. Artezuela is a graduate of the B.S. in Biology program of the Institute of Biology. He was the first undergraduate student of the Medical Microbiology Laboratory to work on fructophilic lactic acid bacteria..

Gil M. Penuliar <gmpenuliar@up.edu.ph> is an Associate Professor of the Institute of Biology. He has a Ph.D. in Medical Science and is the current Principal Investigator of the Medical Microbiology Laboratory.