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FULL PAPER

Identification and characterization of lactic acid bacteria isolated from some medicinal and/or edible Philippine plants

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Abstract

Probiotics research on lactic acid bacteria (LAB) continues to be paramount in the development of nutraceutical or functional foods. In this study, 47 selected Philippine plants having nutritional (edible such as vegetables) and/or medicinal values (therapeutic), were collected, from which selected fruit and leaves were subjected to LAB enumeration. Among these, 46.7% plant leaves reported to have strong antimicrobial property resulted in non-isolation of LAB while edible plant leaves with less or no antimicrobial properties generally gave numerous LAB isolates. Isolates coming from ripened guava (*Psidium guajava* L.), lobo-lobohan or cape gooseberry (*Physalis peruviana* L.) fruit, parsley (*Petroselinum crispum*), *pandan (Pandanus amaryllifolius)*, spinach (*Spinacea oleracea*), leek (*Allium ampeloprasum var. porrum*) and *niyog-niyogan* (*Quisqualis indica L.*) leaves were identified through partial 16S rRNA analysis and tested for probiotic properties. Overall, *Streptococcus luteciae* Lb17 from ripe gooseberry fruit exhibited the highest antimicrobial activities against *Staphylococcus aureus* BIOTECH 1526*, Escherichia coli* O157: H7 BIOTECH 10311 and *Bacillus cereus* BIOTECH 1509. On the other hand, *Enterococcus hirae* (H and S63) from stevia were susceptible to streptomycin at minimum inhibitory concentration or MIC of 128 ug/mL). *Lactobacillus plantarum* F39 and all the other strains tested, meanwhile, was susceptible to ampicillin at MIC of 2 ug/mL and 0.125 ug/mL. Results were lower or equal to the established cut off value indicating the absence of antibiotic resistance genes among the identified strains, except for *Pediococcus* (Par5 and NN39) which showed resistance against streptomycin. Further investigation is needed to rule out the possibility of transfer of antibiotic resistance to pathogens present in the gut. All isolates tested were able to survive at artificial gastric juice (pH 2), revived at the simulated intestinal fluid (pH 8), and exhibited minimal titratable acidity and diacetyl production. For genetic screening of plantaricin genes, F39 possesses both plantaricin EF and plantaricin J, while *Lactobacillus fermentum* F36 has plantaricin EF. Both isolates were subjected to DNA fingerprinting. Such findings on the local isolates' probiotic properties suggest the possibility of incorporating them into different plant-based probiotic foods.

1. Introduction

The application of probiotics continues to evolve due to its profound importance. This is particularly pertinent in terms of its use in developing health products.

Fuller (1989) defines probiotics, including lactic acid bacteria (LAB), as live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance. LAB remain to be a crucial topic for research as its link on health and nutritional benefits are far more relevant today with the increasing demand for plant-based probiotic foods and nutraceutical commodities.

It was at the beginning of the 20th century when Nobel Prize-winning Russian scientist Elie Metchnikoff first proposed the significance of *Lactobacillus,* a probiotic strain. He suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. Since then, many claims relating to probiotic properties have been made varying from the prevention of infectious diseases (Rolfe, 2000),

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curing of irritable bowel syndrome, alleviation of allergies, digestion of lactose and lowering of serum cholesterol levels (Anderson *et al.,* 2001) to the prevention of cancer (Gibson and MacFarlane, 1994). Today, aside from its inclusion to dairy products, probiotics have similarly been incorporated into non-dairy products including plants, plant parts or their extracts. This is in line with the increasing number of those shifting to vegetarianism and the large number of individuals who are lactose intolerant (Furtado Martins *et al.,* 2013). It is, therefore, crucial to continuously identify additional possible means to isolate other probiotic strains, particularly from medicinal and/or edible plants.

The Philippines is a tropical country endowed with very rich biodiversity including those plants having nutritional and/or medicinal use. A medicinal plant is defined by WHO, as any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for the synthesis of useful drugs (Gopal, et al. 2014). On the other hand, edible plants include plants with parts that are safely consumed as food by humans. Examples are the vegetables which refer to edible plants, commonly collected and/or cultivated for their nutritional value for humans (Kuete, 2017). Most often, the botanical definition of vegetables is the "edible part of the plant" (Agudo, 2005). Other obvious examples are the edible fruit-bearing trees.

Some plants are both edible, having nutritional properties and medicinal, having therapeutic purpose. Recent estimates suggest that over 9,000 plants have known medicinal applications in various cultures and countries (Farnsworth and Soejarto, 1991). Some of these medicinal plants are extensively studied, such as guava (*Psidium guajava* L.), moringa (*Moringa oleifera*) and lagundi (*Vitex negundo*).

It is with this high potential of determining other further uses of novel Philippine isolates that continued efforts on LAB isolation from fermented foods, animal gut, and herbage or plants (Saguibo and Elegado, 2012) have been done. This study, in particular, is aimed to isolate and identify potential LAB from different Philippine plants to determine their probiotic properties and assess the possibility of incorporating them into non-dairy plant-based functional products.

2. Materials and methods

2.1 Isolation and purification of lactic acid bacteria from herbal plants

A total of forty-seven plant samples were collected from different sites within the municipality of Los Baños, Laguna, Philippines. Plant parts such as leaves and fruit were washed with tap water and rinsed with sterile distilled water prior to fermentation in sterile flask covered with cotton plug. Fruits were chopped into small cubes (about 1 cm^3) using clean knife and chopping board while leaves were cut into small pieces (about 1 cm^2) using clean scissors. Samples weighed 10 g were blended with 90 mL sterile 0.1% peptone for 1 min. After 1-2 days incubation at 37°C, it was serially diluted and pour-plated on De Mann Rogosa and Sharpe (MRS), Pronadisa Madrid, Spain, agar containing 1% $CaCO_3$. The plates were incubated in an air-tight candle canister at 37°C for 48 hrs. Presumptive LAB colonies with clearing were purified by repeated streak plating using the same agar medium until pure colonies were obtained. Gram staining and catalase test were also performed.

2.2 Characterization of probiotic properties

2.2.1 Antimicrobial activity assay

The antimicrobial activity of presumptive LAB isolates was tested against standard indicators which are also common food pathogens, namely *Escherichia coli* Ec2*, Escherichia coli* O157: H7 BIOTECH 10311, *Salmonella enterica* serovar Typhimurium BIOTECH 1826*, Staphylococcus aureus* Sa16 BIOTECH 1526*, Bacillus cereus* BIOTECH 1509 and *Listeria innocua* NB5, using dual agar overlay method. Quantification of the zones of inhibition as a measurement of antimicrobial activity was conducted following the method of dual overlay agar by Muhialdin *et al.* (2012) with some modification. A known number of LAB cells were spotted onto MRS agar plate. After 18-24 hrs incubation at 37°C, it was overlaid with Nutrient Agar containing the test pathogen. Zones of inhibition of three replicates were thereafter measured and recorded. Antimicrobial activity (mm^2/cell) was mathematically expressed following the formula Πr^2/total spotted LAB count.

2.2.2 Antibiotic susceptibility test

Antibiotic susceptibility test was done by replica plating the LAB isolates in different concentrations of streptomycin (Boehringer Mannheim, Germany) and ampicillin (Boehringer Mannleim, Germany), following the method of Wiegand *et al.* (2008) but with modifications. Active overnight cultures grown in MRS broth were resuspended in 6 mL saline solution for turbidity assessment by OD reading at 625 nm (UV1800 Spectrophotometer, Shimadzu Corp., Kyoto, Japan). The absorption of each cell suspension was adjusted to the range of 0.08 to 0.13 which approximately yields $1x10^8$ CFU/mL. Dilutions of ten-fold of the cell suspensions were prepared by adding 10 µL in 90 µL 0.9% saline placed in the wells of a sterile 96-well microtiter plate. From the same bacterial suspension, 10 µL was obtained

for serial dilution for actual plating and another 10µl was added to microtiter plate.

The antibiotic-containing agar plates were carefully prepared by allotting 25 mL MRSA medium per plate and adding calculated volumes of the antibiotics to obtain the recommended range of concentrations (Final concentrations in agar were 0.125, 0.25, 0.5, 1, 2, 4, 8 µg/mL ampicillin and 2, 4, 8, 16, 32, 64, 128, 256 µg/mL streptomycin). A 48-pin replicator was alcohol and heat-sterilized and allowed to cool down on a fresh MRSA plate without antibiotic. Inoculations were done by dipping the pins in the diluted cell suspensions and transferring in the MRSA plates, first in the control plate without antibiotic then in plates containing antibiotic with the lowest concentration to the highest concentration. The inoculations were done in duplicates. The inoculum spots were allowed to dry inside the biosafety cabinet and the plates were incubated at 37°C for 40-48 hrs then observed for presence or absence of growth. The absence of growth indicates susceptibility of the tested LAB isolates to that particular antibiotic concentration.

2.2.3 Acid and bile tolerance

The acid and bile tolerance of LAB was determined using artificial gastric juice (pH 2.0) and simulated intestinal fluid (pH 8.0) based on the procedure by Fernandez, Boris and Barbes (2003). Colony counts were obtained at the initial time. After a three-hour exposure to each of the artificial fluids, these were incubated at 37°C for 24-48 hrs using MRS agar. The percent survival was thereafter computed by dividing the final over the initial colony count multiplied by 100. The highest percent survival was considered the most tolerable.

2.2.4 Titratable acidity

The LAB isolates were separately inoculated to 9 ml 10% skim milk and incubated at 37°C for 24 hours. Two to three drops of 1% phenolphthalein indicator were added. Samples were titrated using standardized 0.1N NaOH until a faint pink color was obtained. The volume of NaOH used was recorded and percent titratable acidity was computed as follows:

$$\%TA = \frac{\left[[N_{NaOH} x V_{NaOH}] \times \left[\frac{MW_{lactic\ acid}}{1000}\right] \times 100\right]}{V_{sample\ titrated}}$$

Where V_{NaOH} = Volume of NaOH used in titration; and $MW_{lactic\ acid}$ = molecular weight of lactic acid, 90 g/mol

2.2.5 Diacetyl test

An amount of 10 mg of creatine (Sigma Aldrich) and 2 mL of 40% NaOH was added to 2.5 mL test culture (18-24 hrs old). The mixture was allowed to stand until color formation was observed. Formation of red color at the surface indicated the presence of diacetyl and color intensity relative to the quantity of diacetyl compounds present in the sample.

2.2.6 Identification of isolates by partial 16s r-RNA gene sequencing

Amplification was carried out using universal primers FC27 sense (5' AGAGT TTGATCCTGGCTCAG 3') and RC1492 antisense (5' TACGGCTACCTTGTTACGACTT 3'), and using a thermocycler (PTC-100™, Programmable Thermal Controller, MJ Research, Inc., Watertown, MA 02172, USA) following the reaction mix and amplification parameters. The PCR amplicons obtained were purified using DNA purification kit, (PureLink, PCR purification kit Invitrogen, CA, USA) and sent to Macrogen Inc., Korea for DNA sequencing. Meanwhile, sequence similarity searches were performed using the Basic Local Alignment Search Tool Nucleotide (BLASTN) program from the National Center for Biotechnology Information (NCBI), USA GenBank database.

2.3 Genetic screening of plantaricin gene

Amplification of plantaricin genes were carried out in 25 µl reaction volume consisting of 1x PCR buffer buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4)], 2 mM $MgCl_2$, 200 µM each of dNTPs, 50 pmoles of each primer (plnEF-F 5'GGCATAGTTAAAATTCCCCCC 3'; plnEF-R 5'CAGGTTGCCGCAAAAA AAG 3'; plnJ -F 5'TAACGACGGATTGCTCTG 3'; plnJ-R 5'AATCAAGGAATTATCACATTA GTC 3'), 1U of Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA USA) and 50 ng of template DNA. The amplification parameters were as follows: 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 1 min at 53.2°C [pln EF] or 52°C [plnJ] and 1 min at 72°C, with a final extension of 10 mins at 72°C. Duplicate reactions were carried out for DNA sequencing.

Meanwhile, the amplified products were detected by gel electrophoresis in 1% agarose gels at 100 volts. The molecular weight standard 1 kb plus DNA ladder (Invitrogen Life Technologies Inc.) was included for size estimation of amplified fragments. The molecular weights of all amplicons generated were determined using Labworks Image Acquisition and Analysis software (UVP Inc., Upland, CA USA).

3. Results

3.1 Isolation and purification of lactic acid bacteria from herbal and plants

LAB is naturally found in dairy products, grain products, meat and fish products, water, fruit and fruit

juices, pickled vegetables and sour dough. By nature, all plant surfaces contain lactobacilli in low numbers along with other LAB strains in many decaying plant materials, especially decayed fruits. Prominently isolated species from different plant sources are *Lb. plantarum, Lb. brevis, Lb. coryniformis, Lb. casei, Lb. curvatus, Lb. sake, Lb. fermentum* (Kandler, 1984). Despite the reported antimicrobial properties of some leaf extracts against common pathogens such as *Escherichia coli*, *Salmonella enterica* serovar Typhi, *Staphylococcus aureus*, *Proteus mirabilis*, *Shigella dysenteria*, *Streptococcus* spp., *Sarcina lutea* and *Mycobacterium phlei* (Joseph and Priya, 2011), it is interesting to note that some LAB strains have profound resistance against leaf extract antimicrobials. The isolation of lactics *Lb. fermentum* F36 and *Lb. plantarum* F39 have already been reported by Saguibo *et al*. (2012). Based on several experiments conducted, a total of 47 Philippine plants and herbs were used to isolate LAB in this study, from which 46.7% resulted in non-isolation of LAB. These plants, shown in Table 1, are reported to have antimicrobial properties. On the other hand, plants which are edible or possess less antimicrobial property resulted in the isolation of LAB (Table 2).

Table 1. Selected Philippine plant leaves without isolated lactic acid bacteria.

Ashetaba (*Angelica keiskei)*
Acapulko (*Cassia alata)*
Ampalaya (*Momordica charantia)*
Aratiles (*Muntingia calabura)*
Atis (*Annona squamosa Linn.)*
Avocado (*Persea americana)*
Banaba (*Lagerstroemia speciosa)*
Cape gooseberry (*Physalis peruviana)*
Cassava (*Manihot esculenta)*
Duhat (*Syzygium cumini)*
Garem-garem (*Achyranthes aspera)*
Guyabano *(Annona muricata)*
Guava *(Psidium guajava L)*
Ikmo *(Piper betle)*
Kakawate *(Gliricidia sepium)*
Lagundi *(Vitex negundo)*
Lobo-lobohan (*Physalis peruviana L.)*
Malunggay *(Moringa oleifera)*
Mango *(Mangifera indica)*
Sambong *(Blumea balsamifera)*
Takip-kuhol *(Centella asiatica L.)*
Tawa-tawa *(Euphorbia hirta)*
Ulasimang bato or pansit-pansitan (*Peperomia pellucida Linn.)*

LAB strains that are innately present on the surface of the plant do not go away easily by washing as that of the transient bacteria. Given appropriate medium, both the innate and some of the left transient bacteria and also yeast will grow together. The use of MRSA supplemented with calcium carbonate will differentiate lactic acid bacteria.

Table 2. Selected Philippine plant leaves with isolated lactic acid bacteria.

Alugbati (*Basella alba L.)*
Calamansi (*Citrofortunella microcarpa)*
Celery (*Apium graveolens)*
Gabi (*Colocasia esculenta)*
Kamaria (*Artemisia vulgaris Linn.)*
Kangkong (*Ipomoea aquatica)*
Kintsay (*Apium graveolens Linn.)*
Leek (*Allium ampeloprasum var. porrum)*
Lemon grass (*Cymbopogon citratus)*
Mustasa (*Sinapsis integrifolia)*
Niyog-niyogan (*Quisqualis indica L.)*
Okra (*Abelmoschus esculentus)*
Oregano (*Coleus aromaticus Benth.)*
Pakô (*Athyrium esculentum)*
Pandan (*Pandanus amaryllifolius)*
Papaya (*Carica papaya)*
Parsley (*Petroselinum crispum)*
Pechay (*Brassica rapa)*
Saluyot (*Corchurus olitorious)*
Sampa-sampalukan (*Phyllantus nimuri)*
Sili (*Capsicum frutescens)*
Spinach (*Spinacea oleracea)*
Stevia (*Stevia rebaudiana L)*
Sweet potato (*Ipomoea batatas)*
Tsaang-gubat (*Carmona retusa)*

Findings of the study showed that the isolation of LAB from 25 plant leaves samples yielded 650 isolates that exhibited clearing on MRS agar with $CaCO_3$ after repeated serial dilutions and pour plating. Calcium carbonate was used as an indicator for acid-producing strains since it gets degraded with interaction to acids forming a clear zone around the colony. However, only 362 isolates were identified as LAB based on being catalase negative and Gram-positive. From these, 65 representative isolates were randomly selected in which 20 were rod-shaped, ten were cocci, and 35 were coccoid or ovoid.

Five LAB isolates (Lb6, Lb17, Lb21, Lb24 and Lb29) from ripe cape gooseberry fruit and two LAB isolates from ripe guava fruit were also included in this study.

3.2 Characterization of probiotic properties

LAB produce antimicrobial compounds, such as bacteriocins, organic acids, hydrogen peroxide and diacetyl, that inhibit the growth of pathogenic and spoilage bacteria. Some LAB also possesses antibiotic resistance to certain levels of antibiotics. There are likewise possibilities on the transfer of antibiotic resistance genes from beneficial microorganisms to pathogens through bacterial conjugation or transposition

of insertion elements when the antibiotic resistance markers are plasmid-borne.

3.2.1 Antimicrobial activity assay

Figure 1 shows zones of inhibition indicating antimicrobial activity against the lawn of pathogen. Figure 2 meanwhile shows that the highest antimicrobial activity was exhibited by cape gooseberry or lobo-lobohan ripened fruit isolates, followed by the stevia leaf isolates. The isolate from guava fruit exhibited resistance against guava leaf extract (Saguibo *et al.,* 2012). In general, Lb17 (identified as *Streptococcus luteciae* in the latter part of the study) from ripe gooseberry fruit exhibited the highest antimicrobial activities against *Staphylococcus aureus, Escherichia coli* O157: H7 and *Bacillus cereus*.

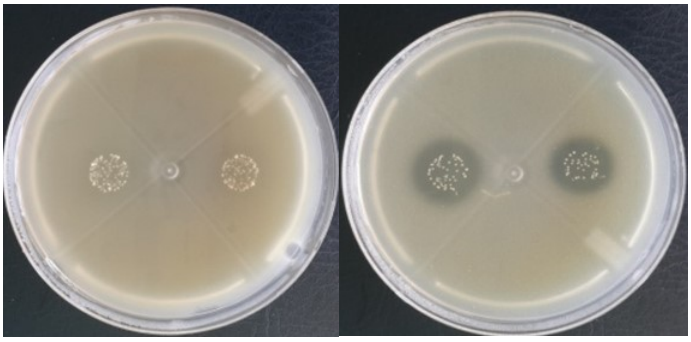

Figure 1. Antimicrobial assay by dual overlay agar method showing zones of inhibition. No zones of inhibition (left) indicate no antimicrobial activity while presence of zones of inhibition (right) indicate antimicrobial activity of the test LAB against the lawn of pathogen added in the basal medium.

As depicted in Figure 2, the different LAB isolates demonstrated varying degrees of antimicrobial activity based on the diameter of zones of inhibition against the growth of the different pathogenic microorganisms. LAB isolate Lb17 showed the highest antimicrobial activities against Gram-positive *S. aureus* (34.61 mm^2/cell), *B. cereus* (27.49 mm^2/cell) and *L. innocua* (9.42 mm^2/cell). On the other hand, LAB isolates Lb21 showed best antimicrobial activities against Gram-negative *E. coli* (15.56 mm^2/cell), *E. coli* O157: H7 (16.33 mm^2/cell, although Lb17 had 16.75 mm^2/cell zone of inhibition) and *S. enterica* serovar Typhimurium (11.34 mm^2/cell). This result suggests the preservative effect of Lb17 and Lb21 when used in food fermentation because of their ability to inhibit food-borne pathogens.

3.2.2 Antibiotic susceptibility test

The minimum inhibitory concentration (MIC) is defined as the lowest concentration (in mg/L) of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions. According to EFSA (2015), strains can be categorised as susceptible to antimicrobials, when it is inhibited at a concentration of a specific antimicrobial equal or lower than the established cut-off value (S ≤ x mg/L) or resistant to antimicrobials, when it is not inhibited at a concentration of a specific antimicrobial higher than the established cut -off value (R > x mg/L). The established cut-off for ampicillin and streptomycin in *Lactobacillus* strains is varied ranging from 1-4 mg/L and 16-64 mg/L, respectively. For other LAB genera, a similar range is tabulated for ampicillin, but a higher range of 32-128 mg/L for streptomycin.

Figure 3a presents the MIC of lactic acid bacteria isolates against ampicillin. L*actobacillus fermentum* F36 had MIC of 2 ug/mL ampicillin which conforms to the established cut-off value. Thus, F36 and all the other lactic acid bacteria tested are considered susceptible because they are inhibited at equal or lower concentrations than the established cut-off value.

Figure 3b presents the MIC of lactic acid bacteria isolates against streptomycin. *Enterococcus hirae* (H and S63) from stevia, *Pediococcus pentosaceous* (Par5 and NN39) and the unidentified SP5 and SP6 had the MIC of 128 µg/mL streptomycin. This value is still considered susceptible for *Enterococcu*s but resistance is observed with *Pediococcus* (Par5 and NN39). High susceptibility is observed for *Streptococcus luteciae* Lb17 because they were inhibited at lower concentration (32 µg/mL) than the established cut-off value (64 µg/mL). Also, for *Enterococcus hirae* A which were inhibited at lower concentration (32 µg/mL) than the established cut-off value (128 µg/mL).

Higher concentrations were set for ampicillin than for streptomycin. LAB is Gram-positive and possess a cell wall that is more susceptible to antibiotics. This is due to a virtually none lipopolysaccharide (LPS) content, low lipid and lipoprotein content, and absence of outer membrane and periplasmic space.

Ampicillin, which is almost similar to amoxicillin, belongs to the penicillin group of beta-lactam antibiotics and is part of the aminopenicillin family. It has an amino group, present on both ampicillin and amoxicillin, that helps these antibiotics pass through the pores of the outer membrane of Gram-negative bacteria, such as *E. coli*, *Proteus mirabilis*, *Salmonella enterica*, and *Shigella* (Keeton and Gould, 1993).

Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make the cell wall. Its bacteriolytic action results from inhibition of the third and final stage of bacterial cell wall synthesis in binary fission (Gallagher, 2011).

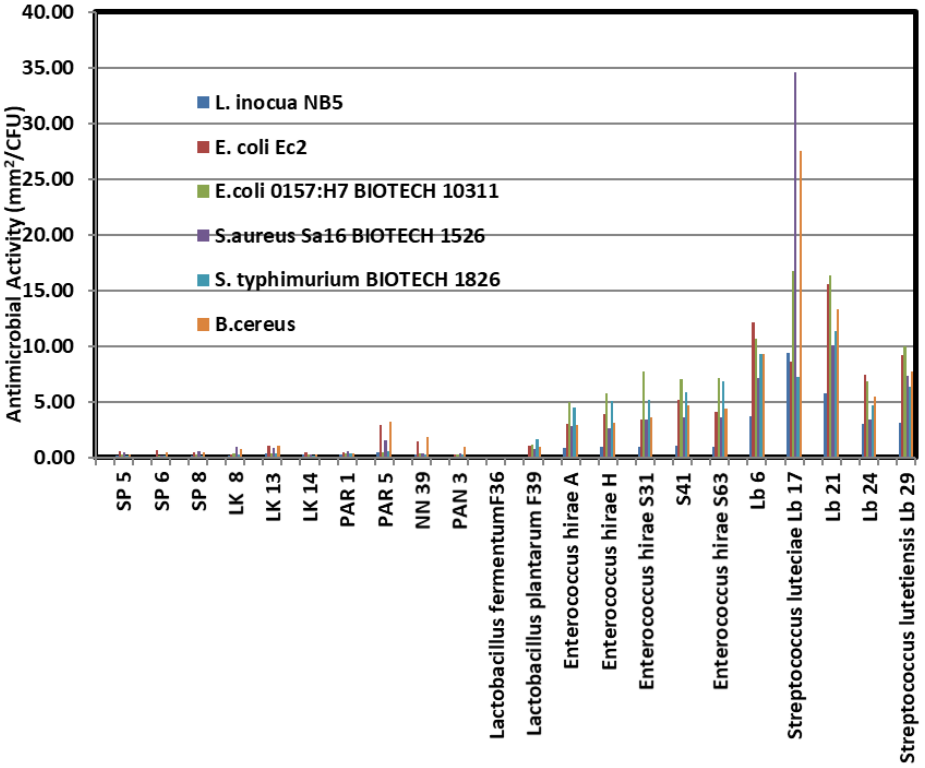

Figure 2. Antimicrobial activity of lactic acid bacteria isolates.

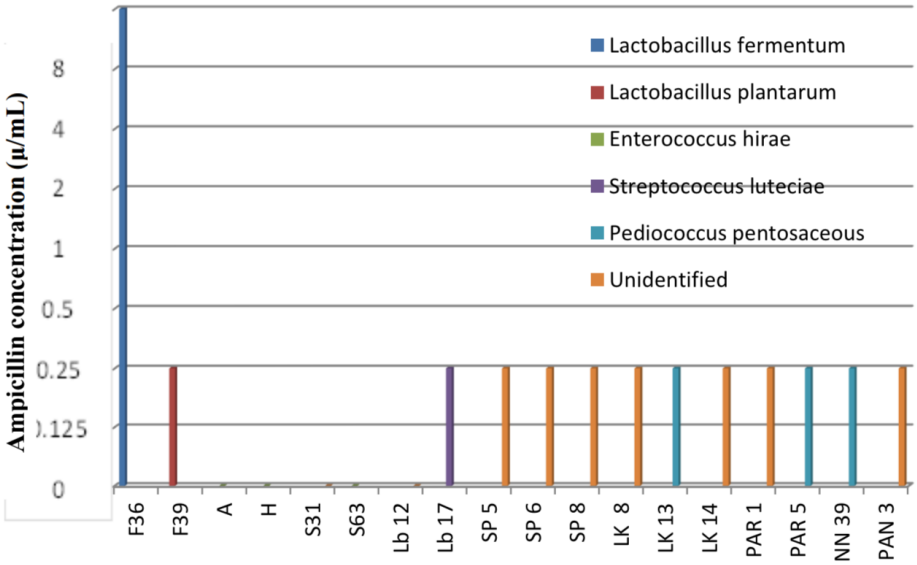

Figure 3a. Susceptibility of lactic acid bacteria isolates against ampicillin.

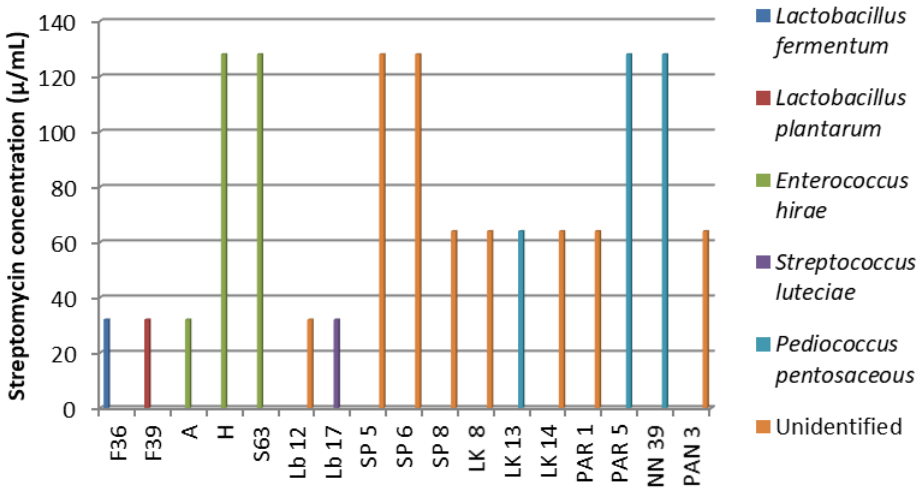

Figure 3b. Susceptibility of lactic acid bacteria isolates against streptomycin.

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On the other hand, streptomycin is an antibiotic drug of the class aminoglycosides, derived from the actinobacterium *Streptomyces griseus*. It stops bacterial growth by damaging cell membranes and inhibiting protein synthesis. Specifically, it binds to the 16S rRNA of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This prevents the initiation of protein synthesis (Gallagher, 2011).

The antibiotic resistance of some LAB may be advantageous to patients who are undergoing antibiotic therapy while taking probiotics to boost their immune response. However, there is speculation that antibiotic resistance genes that are plasmid-borne could be transferred from beneficial microorganisms to pathogens through bacterial conjugation or transposition of insertion elements. It is therefore relevant to further investigate the extent of antimicrobial activity and antibiotic susceptibility of LAB strains isolated from traditional fermented food and local herbage or plants. It is also recommended to conduct transconjugation experiments. Relevant studies on this subject include the susceptibility of *Lactobacillus* species to cell wall synthesis inhibitors such as ampicillin and penicillin which have been reported by Danielsen and Wind (2003). Coppola *et al.* (2005), Sharma *et al*. (2018) and Rozos *et al*. (2018) also observed resistance against various antibiotics by some lactobacilli isolated from cheese and commercial probiotic products. In a study by Huys *et al*. (2002), the resistance patterns were significantly affected by the medium such as MRS agar.

3.2.3 Acid and bile tolerance

Probiotics possess intestinal absorption ability and tolerance for gastric acid (a thin, almost colorless liquid secreted by the glands in the lining of the stomach that is strongly acidic with pH varying from 1 to 3), enzymes and bile (yellowish brown fluid, comprising salts (basic), pigments, lecithin and cholesterol, which aids in the process of digestion). These facilitate the probiotics' survival up to the gut system to promote significant health benefits to the host. The findings of this study show that all LAB isolates decreased in number but were able to survive in artificial gastric juice (pH 2) and then revived upon exposure to simulated intestinal fluid (pH 8), with 50-80% survival.

3.2.4 Titratable acidity and diacetyl test

Titratable acidity is defined as the amount of titrant needed to react stoichiometrically with LAB in milk (Wehr and Frank, 2004). Titration measures all of the acids in milk including its apparent acidity. Any increase in acidity due to the formation of lactic acid, known as the major organic acid produced during the process, is also identified through titration. According to Lindgren and Dobrogosz (1990), the levels and types of organic acids produced during the fermentation process depending on the LAB species or strains, culture composition and growth conditions. The reduction of pH is relative to the antimicrobial effect of organic acids, as well as the undissociated form of the molecules.

On the other hand, diacetyl or 2,3-butanedione is the most important flavor compound in lactic cultures, cultured buttermilk, butter and others (Jay, 2000). Its presence may be detected although little change in total acidity or pH has been noted.

Results of this study show that all LAB isolates tested had titratable acidity of about 0.3 - 0.5% and very distinct diacetyl formation. Figure 4 shows the top 5 lactic acid producer, F39 and LK8 being the highest.

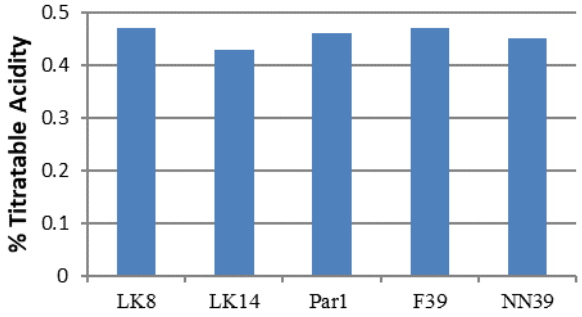

Figure 4. Titratable acidity of the top 5 LAB (two replications).

LAB, the most widely used as starter culture in food fermentation (Hwanhlem *et al.,* 2011), owe their desirable properties to their ability to produce organic acids (predominantly lactic acid and other antimicrobial metabolites such as ethanol, hydrogen peroxide, diacetyl (an aroma compound) and bacteriocins (Diop *et al.,* 2010). Desirable properties of LAB could be improved by the use of mixed starter culture, depending on the food application.

3.2.5 Genetic screening of plantaricin gene

LAB produce antimicrobial peptides, called bacteriocins, which are potent at pico- to nanomolar concentrations. These bacteriocins are divided into two main classes: the class I lantibiotics that contain post-translationally modified lanthionine residues and the class II non-lantibiotics that do not contain extensive modifications. The class II bacteriocins may be further divided into four subclasses: the class IIa pediocin-like bacteriocins that have similar amino acid sequences, the class IIb two-peptide bacteriocins that consist of two different peptides, the class IIc cyclic bacteriocins and the class IId noncyclic one peptide non-pediocin-like

bacteriocins (Ekblad, 2016).

Plantaricin EF is a two-peptide bacteriocin that depends on the complementary action of two different peptides, PlnE and PlnF, to function (Ekblad *et al.,* 2016). On the other hand, plnJ is part of the three-dimensional structure of the two-peptide bacteriocin pln JK (Rogne, 2009).

Together with the other *Lb. plantarum* isolates, *Lactobacillus plantarum* F39 and *Lb. fermentum* F36 amplified a 450 bp product with pln EF primers indicative of the presence of the structural EF gene. *Lb. fermentum* F36 was PCR positive for the pln J gene (data not shown).

For verification of the genes, the EF and J gene amplicons were submitted to 1st BASE Pte Ltd., Singapore for DNA sequencing. Determination of DNA homologies using BLAST showed that the F39 plnEF and plnJ amplicons had 100% and 99% identification with the plantaricin gene locus of *Lb. plantarum* subsp. plantarum strain YM-4-3. Likewise, F36 plnEF and plnJ amplicons had 100% homology with the plantaricin gene locus of *Lb. plantarum* subsp. plantarum strain YM-4-3. Alignment of the F39 and F36 translated sequences with the plantaricin locus of *Lb. plantarum* subsp. *plantarum* strain YM-4-3 indicated identical peptide sequences of the partial plnE gene and plnF genes of the three strains (data not shown.). With the plnJ peptide sequence, F39 differed with YM-4-3 by a single amino acid, having a threonine (T) instead of an isoleucin (I) (data not shown). On the other hand, the F36 plnJ peptide sequence was found to be identical with that of YM-4-3 (data not shown).

3.2.6 Identification and fingerprinting

Table 3 summarizes the sources, identity and fingerprint of selected LAB isolated from plants. The repetitive sequence-based PCR of *Lb. plantarum* and *Lb. fermentum* strains generated DNA markers for identification at the species level and for strain differentiation. Distinct strain-specific markers for *Lb. fermentum* F36 was obtained in its repetitive Extragenic Palindromic (REP) sequences and BOX profiles. However, *Lb. plantarum* F39 did not show specific marker in its REP and BOX profiles. *Lb. plantarum* strains showed specific markers in ERIC PCR and BOX A1R sequences which could serve as markers (Perez *et al.,* 2013; Perez *et al.,* 2014).

LAB isolated from plants is very diverse. However, most of these are soil residents that are probably from animal wastes. Probiotic LAB is normal microflora of the gut of humans and other animals especially birds and insects, which also feed on the fruits of the plants. Although enterococci are implicated in nosocomial infections, recent findings show their probiotic potential particularly in the treatment of diarrhea (Franz *et al.,* 2011). In this study, *Streptococcus hirae* and *Streptococcus lutetiensis* were isolated from stevia leaves and ripe cape gooseberry fruit. Further study is recommended to determine their safety and probiotic potentials.

4. Discussion

4.1 Isolation of LAB from non-dairy sources

LAB comprise groups of Gram-positive bacteria with low-GC content, acid-tolerant, generally non-sporulating, non-respiring, occasionally non-motile rod or cocci, either in pairs or in chains that are associated by their common metabolic and physiological

Table 3. Summary of sources, identity and fingerprint of lactic acid bacteria.

Lactic Acid Bacteria	Source	Identity by 16S rRNA (% Homology)	Fingerprint
F36	Guava fruit (Psidium guajava L)	*Lactobacillus fermentum* (100%)	Yes
F39	Guava fruit *(Psidium guajava L)*	*Lactobacillus plantarum* (100%)	Yes
PAR 5	Parsley (*Petroselinum crispum)*	*Pediococcus pentosaceous ATCC 25745* (99%)	No
LK 13	Leek (*Allium ampeloprasum var. porrum)*	*Pediococcus pentosaceous ATCC 25745* (96%)	No
NN39	Niyog-niyogan (*Quisqualis indica L.)*	*Pediococcus pentosaceous ATCC 25745* (99%)	No
A	Stevia (*Stevia rebaudiana L)*	*Enterococcus hirae* (98%)	No
H	Stevia (*Stevia rebaudiana L)*	*Enterococcus hirae* (98%)	No
S31	Stevia (*Stevia rebaudiana L)*	*Enterococcus hirae* (96%)	No
S41	Stevia (*Stevia rebaudiana L)*	Not yet identified	No
S63	Stevia (*Stevia rebaudiana L)*	*Enterococcus hirae* (88%)	No
Lb6	Cape goose-berry fruit (*Physalis peruviana)*	Not yet identified	No
Lb17	Cape goose-berry fruit(*Physalis peruviana)*	*Streptococcus luteciae* (98%)	No
Lb 21	Cape gooseberry fruit(*Physalis peruviana)*	Not yet identified	No
Lb 24	Cape goose-berry fruit(*Physalis peruviana)*	Not yet identified	No
Lb 29	Cape goose-berry fruit (*Physalis peruviana)*	*Streptococcus lutetiensis* (96%)	No

characteristics. Some LAB is commonly known as probiotics due to their properties as living microorganisms which upon ingestion by the host in certain numbers exert health effects beyond inherent basic nutrition. They are considered generally regarded as safe or GRAS microorganisms. LAB is presently classified to the following five core genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus* (Hutkins, 2006).

The lactic acid group of bacteria occur on plants with some degree of constancy, but not of consistency, and seldom at high levels of the population. Their role on the surfaces of plants is unknown, and apparently passive, for no functional, concrete role in an intimate bacterium to plant relationship has been detected (Mundt, 1970). Their recent applications on fiber fermentation such as in silage, organic decomposition such as in manure and sewage treatment and as plant growth promoter when used for soil humus formation, reflect their ability to produce various important enzymes necessary to digest plant components.

The utilization of LAB in food preservation, including plant-based food products, has long been practiced since time immemorial. Their occurrence was reported in raw plant food material (Mundt, 1970), from plants and other vegetable matrices (Anacarso *et al.,* 2015) or fermented plant beverage and pickles (Duangjitcharoen *et al.,* 2008). It was likewise found not only on leaves but also on fruits and flowers (Endo 2012) of various plants including medicinal herbs (Cakir, 2010) and even in extreme environments of mountains, desert and coastal regions (Kuda *et al.,* 2016). The discovery of new potential strains is important in fermentation and preservation of food (Rhee *et al.,* 2011; Admassie, 2018).

Plants synthesize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites. Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: (i) flavonoids and allied phenolic and polyphenolic compounds, (ii) terpenoids and (iii) nitrogen-containing alkaloids and sulphur-containing compounds (Crozier, 2007).

Tannins are naturally occurring plant polyphenols, whose main function are binding and precipitating proteins. Tannins are common in fruits (grapes, persimmon, blueberry, etc.), tea, and chocolate. It can also be found in legume forages (trefoil, etc.), legume trees (*Acacia* spp., *Sesbania* spp., etc.), and in grasses (sorghum, corn, etc.). Discussion on Cornell University's website about poisonous plants also states that tannins are "responsible for the astringent taste we experience when we partake wine or unripe fruits, and for the enchanting colors seen in flowers and in autumn leaves" (Cannas, 2018).

Isolation and utilization of LAB in the fermentation of high tannin-containing plants were already reported by several researchers. Okada (2002) noted that only *Lb. plantarum* equipped with DAP-peptidoglycan in cell wall participate in the fermentation of tea leaves because of their ability to survive from tannic acids contained in the leaves. However, LAB equipped with Lys-peptidoglycan in cell wall were unable to survive. Jakesevic (2011) meanwhile conducted a study on probiotics and berry-associated polyphenols, its catabolism, and antioxidative effects. There is also evidence that fermentation with LAB decreased the concentration of polyphenols and significantly changed the polyphenols profile in the fermented buckwheat flours (Wiczkowski *et al*., 2016). Tannase activity was also observed in *Xuan mugua* fruits fermented with *Lb. plantarum* (Shang *et al,* 2019). This was also evident in Lactobacillus strains isolated from grape must and wine (Vaquero *et al.,* 2004). Also on *Lb. plantarum* and *Lb. pentosus* in tea extracts (Chaikaew, 2018) and *Lb. brevis* isolated from fermented foods (Kivanci and Temel, 2018).

4.2 Antibiotic resistance

Antibiotic resistance is one of the biggest threats to global health. It occurs naturally, but misuse of antibiotics in humans and animals accelerates the process. A growing number of infections such as pneumonia, tuberculosis, gonorrhoea and salmonellosis, are becoming harder to treat as the antibiotics used for treatment become less effective. This scenario results in longer hospitalization, higher medical costs and increased mortality (WHO, 2018).

Because of the growing concern of antibiotic resistance, substantial pressure to reduce antibiotic use has necessitated the development of antibiotic alternatives. Mathur *et al.* (2017) reviewed studies in which bacteriocins were found to be effective in combination with other antimicrobials. The authors particularly discussed the combination of lantibiotics with antibiotics against clinical and veterinary pathogens, sanctibiotics and other groups of bacteriocins in combination with antimicrobials against clinical pathogens, and effective bacteriocin-antimicrobial combinations against biofilms. Other combinations studied included effects of antimicrobial combinations involving bacteriocins against food-borne pathogens, combinations of bacteriocins with essential oils, and naturally-derived compounds and preservatives against gram-positive food-borne pathogens. Also discussed

were bacteriocins in combination with other stressors against Gram-negative food-borne pathogens, bacteriocins in combination with antibiotics against food -borne pathogens, and other strategies such as bacteriocin in combination with phages/endolysins. A bacteriocin active against multi-resistant gram-negative bacteria in nosocomial infections was also reported (Ghodhbane, 2015).

Bacteria, in general, are very adaptable creatures and capable of continuous evolution in order to survive stressors present in their environments such as bacteriophages, antibacterial metals, minerals, and nanoparticles, organic acids, essential oils, and probiotics. The development of antibiotic alternatives necessitates extra caution because of the possibility to develop resistance to these alternatives. There are opportunities to optimize antibiotic alternative effectiveness as well as to minimize the development of resistance mechanisms (Willing *et al.,* 2018). Many investigators have speculated that commensal bacteria including LAB may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens. Genes conferring resistance to tetracycline, erythromycin and vancomycin have been detected and characterized in *Lactococcus lactis*, *Enterococci* and in *Lactobacillus* species isolated from fermented meat and milk products (Mathur and Singh, 2005).

4.3 Ensuring safety of probiotics

Since most probiotics are marketed as foodstuff or drugs, thorough consideration of its safety is therefore of utmost importance. Most probiotics used in humans are commonly isolated from dairy food. Increasingly, probiotics from sources other than milk products are being selected for use in people who are lactose intolerant. These sources are non-dairy fermented foods and beverages, non-dairy and non-fermented foods such as fresh fruits and vegetables, feces of breast-fed infants and human breast milk (Sornplang and Piyadeatsoontorn, 2016).

The selection criteria for probiotics can be microbiological, technological and clinical. For microbiological, LAB strains must be accurately identified, nonpathogenic, acid and bile stable, produce important enzymes and genetically stable during storage (Holzapfel *et al.,* 2001, Tannock 2001; Ishibashi and Yamazaki, 2001). Under the technological criterion, selected probiotics can be cultured in industrial scale, viable and able to survive throughout manufacturing and storage. In Japan, a standard was developed by the Fermented Milk and Lactic Acid Bacteria Beverage Association stipulating that a product must contain $\geq 1x10^7$ viable bifidobacteria/g or ml product to be considered as probiotic food with a proposed therapeutic minimum dose of $1x10^5$ viable cell/g or ml product (Stanton, 2001). Microencapsulation or immobilization and incorporation of prebiotics are performed to ensure viability (Charalampopoulos *et al.,* 2002). Generally, the viability of some strains is dependent on their resistance to the conditions for production. Other criteria are the availability of suitable carrier/fermenting substance, acceptable product quality and production of diacetyl compounds responsible for flavor, as well as mild acidity throughout storage. The clinical criterion, on the other hand, states that probiotics must adhere and colonize the human gut and produce antimicrobial substances such as lactic acid, H_2O_2, bacteriocins (Dunne *et al.,* 2001). Prior to food incorporation, determination of probiotic properties, as well as comparison to known probiotics for that particular food, are deemed important (Nomura *et al.,* 2006).

4.4 Health benefits of probiotics

Claims relating to probiotic properties vary from the prevention of infectious diseases (Rolfe, 2000), curing of irritable bowel syndrome, alleviation of allergies, digestion of lactose and lowering of serum cholesterol levels (Anderson *et al.,* 2001) to the prevention of cancer (Gibson and MacFarlane, 1994). Dicks and Botes (2010) published a comprehensive review on health benefits, safety and mode of action of probiotic LAB in gastrointestinal tract. Similarly, Fijan (2014) published an overview of recent literature on microorganisms with claimed probiotic properties which include *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus, Leuconostoc*, *Bacillus*, *Escherichia coli*. Probiotics have been found to promote good health for persons with autism (PWA) as well (Adams *et al.,* 2011; Gilbert *et al.,* 2013; Kerns *et al.,* 2017).

In assessing the quality and efficacy of the health benefits of probiotic products, a statistically acceptable clinical trial is recommended in addition to the validation studies conducted in animal models (Tamayo, 2008; Degnan, 2012). Reid *et al.* (2003) reviewed the numerous potential uses of probiotics in clinical practices. Several clinical trials have already been reported on glycemic control (Ruan *et al.,* 2015; Li *et al.,* 2016) in patients with cirrhosis (Horvath *et al.,* 2016).

However, the use of various probiotics for immunocompromised patients or patients with a leaky gut has also yielded infections, sepsis, fungemia, bacteraemia. Although the vast majority of probiotics that are used today are generally regarded as safe and beneficial for healthy individuals, caution in selecting and monitoring of probiotics for patients is needed and

complete consideration of risk-benefit ratio before prescribing is recommended (Fijan, 2014).

4.5 Probiotics and functional beverage

Due to growing vegetarianism and the large number of individuals who are lactose intolerant or prescribed with cholesterol-restricted diets, the development of non-dairy probiotic products, including food matrices based on fruit, vegetables and cereals, has been widely studied (Furtado Martins *et al.,* 2013). Non-dairy probiotic beverages, whether fermented or non-fermented, can also play an important role in probiotic delivery (Ranadheera *et al.,* 2017).

In fact, consumption of lactic acid fermented vegetable juice is now becoming more popular in many countries, including those in Europe, China, Japan and Korea. Generally, fermentation is carried out to enhance taste, aroma, shelf-life, texture, nutritional value and other properties of food. Lactic acid fermented vegetable juices can be produced by fermenting the vegetable the usual way and then processing it by pressing the juice or by first, processing the vegetable to mash or raw juice before fermentation. Fermentation of vegetable juices can be through spontaneous by natural microflora, or addition of starter cultures and fermentation of heat-treated or pasteurized materials by starter cultures at a concentration of 10^5 to 10^7 CFU/mL. For fermentation of juices, the most important LAB belong to genus *Lactobacillus, Pediococcus* and *Bifidobacteria* and the yeast, *Saccharomyces cerevisiae* boulardii (Karovicova and Kohajdova, 2005).

In general, fermentation improves the nutritional value of a particular food by affecting its digestibility and nutrient content through enhancing its nutrient density and increasing the amount and the bioavailability of nutrients (Svanberg and Lorri, 1997). Lactic acid fermentation leads to a decrease in the level of carbohydrates as well as some non-digestible poly- and oligosaccharides (Friend and Shahani, 1984). The latter reduces side effects such as abdominal distension and flatulence (Nout and Motarjemi, 1997). It also increases the utilization of iron from food by the breakaway of inorganic iron from complex substances under the influence of vitamin C (Siegenberg, 1991; Venkatesh, 1998). In addition, acid-fermented vegetables are important sources of vitamins and minerals (Lee, 1987). Vitamin C is better preserved in lactic acid-fermented vegetable products, compared to those processed by alternative methods (Nout and Motarjemi, 1997). Researchers similarly concluded that fermented vegetable juice combines the benefits of vegetable-based products besides functional roles occurring from the existence of viable probiotics in a proper amount during the shelf life (Profir *et al.,* 2015). Beverages are by far the most active functional food category because of convenience and possibility to meet consumer demands for container contents, size, shape, and appearance, as well as ease of distribution and storage for refrigerated and shelf-stable products. Moreover, they are an excellent delivering means for nutrients and bioactive compounds including vitamins, minerals, antioxidants, ω -3 fatty acids, plant extracts, fiber, prebiotics, and probiotics (Corbo *et al.,* 2014).

Tamang *et al*. (2016) meanwhile reviewed the various functional properties of microorganisms in fermented food, such as anti-oxidant activity, peptide production, enzyme production, increase in isoflavones and saponins and production of polyglutamic acid (PGA), degradation of anti-nutritive compounds, and synthesis of nutrient which has direct or indirect contributing effects on health.

5. Conclusion

Plants, besides other sources, are also good sources of probiotic LAB. Therefore, plants could have inherent characteristics that are advantageous in product development. The thorough characterization of the probiotic properties of these locally isolated probiotic LAB would dictate their industrial relevance particularly in the development of plant-based nutraceutical or functional foods. Further study is recommended to determine their safety and understand deeply their probiotic potentials. The demand for healthier alternatives and healthier food products remains a challenge for researchers. This underscores the importance of engaging in similar probiotics and LAB research analysis.

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