



Comprehensive *in silico* analysis of lactic acid bacteria for the selection of desirable probiotics

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ABSTRACT

In this study, the probiotic properties of nine lactic acid bacteria (LAB) were evaluated by using an *in silico* analysis comprising principal component, heat map, and network analyses. Acid and bile resistances, bile salt hydrolase (BSH) activity, antimicrobial activity, auto- and co-aggregation, and hydrophobicity were analyzed in the strains. All tested LAB exhibited > 60% of survival percentage in acid and bile resistances. Five strains of LAB exhibited positive BSH activities and all LAB demonstrated antimicrobial activities against at least one pathogen except *L. acidophilus* IDCC3302. Following a time-dependent manner, *L. acidophilus* IDCC3302 and *L. plantarum* LP-K1791 exhibited the greatest auto- and co-aggregation percentages, respectively. The hydrophobicity of all LAB was satisfied with the minimum requirement of hydrophobicity (40%) as potential probiotics. Based on the *in silico* analysis, *L. casei* IDCC3451, *L. plantarum* LP-K1791, and *L. rhamnosus* IDCC3201 were selected as the most promising probiotics. This *in silico* analysis will be useful for the precise selection of probiotics for the development of functional foods and healthy dietary supplements in the food and pharmaceutical industries.

1. Introduction

In recent years, the health benefits and food application properties of lactic acid bacteria (LAB) have been increasingly studied. LAB are generally recognized as probiotics that can confer health and nutritional benefits to the host (Pinto, Barbosa, Albano, Isidro, & Teixeira, 2020; Yasmin et al., 2020). The role of LAB in human health and nutrition has been widely reported including studies on their potential therapeutic effects against respiratory and urogenital disorders, allergic reactions, and inflammatory bowel disease (Rokana, Mallappa, Batish, & Grover, 2017). As probiotics, they can be used as components of nutritional supplements for the elderly and for the prevention of hypercholesterolemia (Oh, Daliri, & Oh, 2018). In addition, they can help maintain the normal flora of the human gastrointestinal tract by reducing the presence of pathogens and stimulating the growth of beneficial microorganisms, as well as improve the human immune system

and condition of the skin, and prevent the development of influenza and cold (Chlebowska-Smigiel, Gniewosz, Kieliszek, & Bzducha-Wrobel, 2017; Chlebowska-Smigiel et al., 2019).

Some studies have also demonstrated the ability of LAB to inhibit the growth of several pathogens as well as their adherence to Caco-2 cells by displacing enteropathogens from the Caco-2 cell layer (Tejero-Sariñena, Barlow, Costabile, Gibson, & Rowland, 2012). Furthermore, through their bile salt hydrolase (BSH) activities, LAB have been reported to lower serum cholesterol levels in humans (Boricha, Sheikh, Pithva, Ambalam, & Vyas, 2019). At present, combinations of probiotics and natural polymers or prebiotic substances are increasingly studied in the food and pharmaceutical industries for the development of biopreservatives, healthy dietary supplements, and functional foods (Chlebowska-Smigiel et al., 2019). LAB produces conjugated linoleic acid, which is currently being investigated for the development of functional food products based on this beneficial bioactive lipid with

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health-promoting properties (Ribeiro, Stanton, Yang, Ross, & Silva, 2018).

In the food industries, LAB are being used in the production of fermented food products such as dairy products, vegetables, and meat, improving taste, flavor, texture, and dietary qualities, which provides greater health benefits. For example, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *L. acidophilus* are being used as starter cultures in the production of yogurt (Chlebowska-Śmigiel et al., 2019; Pinto et al., 2020). *L. brevis*, *L. plantarum*, *Pediococcus pentosaceus*, and *P. acidilactici* have been used in vegetable fermentation such as sauerkraut and cucumbers (Tamang et al., 2005). In addition, *L. lactis* and *Leuconostoc mesenteroides* are predominantly used in the production of sour cream (Yu et al., 2015).

To expand the use of LAB as probiotics, the careful selection of LAB strains is desired and required for industrial use. The properties required for their selection as probiotics include the ability to survive during transit in the gastrointestinal tract, the possession of cell surface properties such as aggregation and cell surface hydrophobicity, and the ability to tolerate bile salts and the acidic pH of the stomach. Furthermore, these potential probiotics should have functional properties such as antimicrobial, antioxidant, and cholesterol-reducing activities. In addition, potential probiotics should be screened for safety and the maintenance of their beneficiary properties under harsh conditions in the food process chain should be investigated (Pinto et al., 2020; Ribeiro et al., 2018; Yasmin et al., 2020). For these reasons, the similarities and differences of various probiotic strains should be screened to select the best probiotic strains for the next level of *in vivo* studies. Many studies have focused on the screening of various LAB strains as probiotics, however, the non-reproducible results of conventional clustering analyses have been a major setback for the precise identification of candidate probiotics (Nami, Panahi, Jalaly, Bakhshayesh, & Hejazi, 2020). To generate reproducible datasets, an *in silico* analysis comprising principal component analysis (PCA), heat map, and network analyses has been recently proposed as an alternative approach (Choudhary et al., 2019; Nami et al., 2020; Panahi, Mohammadi, Ruzicka, Holaso, & Mehrjerdi, 2019). Especially, network analysis based on similarity analysis can contribute to select promising probiotics among LAB by providing a reproducible and visual result. To the best of our knowledge, this is the first study to apply network analysis to investigate the relationship among LAB based on their probiotic properties for the precise selection of candidate probiotics for industrial use. Therefore, this study screened nine LAB to identify candidate probiotics using principal component, heat map, and network analyses as tools for achieving reproducible results. The selected probiotics are expected to be used in diverse food formulations, human nutrition, and for pharmaceutical product development.

2. Materials and methods

2.1. LAB and pathogenic bacterial strains

Seven LAB including *Lactococcus lactis* IDCC2301, *Lactobacillus rhamnosus* IDCC3201, *Lactobacillus acidophilus* IDCC3302, *Lactobacillus casei* IDCC3451, *Lactobacillus plantarum* IDCC3501, *Lactobacillus plantarum* BNH17, and *Lactobacillus helveticus* IDCC3801 were obtained from Ildong Pharmaceutical Co. (Gyeonggi-do, Korea). *Lactobacillus plantarum* LP-K1791 and *Lactobacillus rhamnosus* LR-K1461 were obtained from NewLife Healthcare Co. (Gyeonggi-do, Korea). The pathogenic bacterial strains with ATCC were obtained from the American Type Culture Collection and *Salmonella* Senftenberg was obtained from the School of Food Science and Biotechnology, Kyungpook National University (Daegu, Korea).

2.2. Acid resistance

The acid resistance of each LAB was investigated using a previously

described method (Oh et al., 2018) with a slight modification. LAB was cultured in MRS (Difco Laboratories Inc., Sparks, MD, USA) broth at 37 °C for 24 h and harvested by centrifugation at 6000 × g for 15 min. The pellets were then resuspended in phosphate-buffered saline (PBS, pH 7.4) to prepare culture suspensions. Each culture suspension was adjusted to pH 2.5 and incubated at 37 °C for 4 h. The strains were serially diluted using 0.85% (w/v) sterile saline (NaCl) solution and plated on MRS agar before and after incubation. After incubation at 37 °C for 24 h, the surviving cells were enumerated to calculate the rate of survival as follows:

$$\text{Survival (\%)} = \frac{\text{Number of surviving cells after 4 h of incubation (CFU/ml)}}{\text{Initial number of cells prior to incubation (CFU/ml)}} \times 100$$

2.3. Bile resistance

The resistance of LAB to bile salts was evaluated as described previously (Oh et al., 2018) with a minor modification. One percent of the culture suspension (v/v) was inoculated into 0.3% (w/v) dehydrated fresh bile (Sigma-Aldrich Co., St. Louis, Missouri, USA). After serial dilution, the culture suspension was plated on MRS agar before and after incubation at 37 °C for 24 h. The surviving cells were enumerated and the survival percentage was calculated as stated in section 2.2.

2.4. BSH activity

The BSH activity of LAB was evaluated as previously described (Mallappa et al., 2019). Cultures of LAB were streaked onto MRS agar plates containing CaCl_2 (0.375 g l^{-1}) and 0.3% (w/v) bile salt (Sigma-Aldrich Co.) and incubated at 37 °C for 48 h. Either visible halos or white opaque colonies were determined to possess BSH-positive activity.

2.5. Antimicrobial activity

The agar well diffusion method described by Rokana et al. (2017) was used with a minor modification. The cell-free supernatant (CFS) of each LAB was collected after centrifuging the overnight culture at 6000 × g for 15 min and then filtered using 0.20-μm cellulose acetate filter. Each CFS was divided into two equal portions. One portion was unneutralized (pH 3.6–4.6) whereas the other portion was neutralized by adjusting the solution to pH 6.8 using 1 M NaOH. For the negative control, fresh MRS broth adjusted to pH 6.8 was used without any inoculated bacteria. Each pathogenic bacterium was grown in TSB (Difco Laboratories Inc.) at 37 °C for 18 h, centrifuged at 5000 × g for 10 min, and resuspended in PBS. A 200-μl aliquot of the pathogenic suspension was inoculated into 4 ml of soft TA agar (4 g l^{-1} agar, 8 g l^{-1} nutrient broth, 5 g l^{-1} NaCl, 0.2 g l^{-1} MgSO_4 , 0.05 g l^{-1} MnSO_4 , and 0.15 g l^{-1} CaCl_2) and poured onto solidified TSA agar plates. After solidification, 100 μl of the negative control, unneutralized CFS, and neutralized CFS were added into 7-mm diameter wells and incubated at 22 °C for 1 h to permit diffusion of the suspension into the agar. After incubation at 37 °C for 24 h, the zone of inhibition was measured in millimeters.

2.6. Auto-aggregation and co-aggregation properties

An auto-aggregation property of LAB was performed following the methods of Mallappa et al. (2019) with few modifications. Culture suspension of each LAB was adjusted with PBS to approximately 10^8 cfu/ml at 600 nm, and the initial absorbance (A_0) of each culture was measured. After incubation at 37 °C for 4 h (A_4), 18 h (A_{18}), and 24 h (A_{24}), the absorbance of the upper fraction of the incubated suspension was measured and the percentage of auto-aggregation was calculated depending on the incubation time (A_t):

$$\text{Auto-aggregation (\%)} = (1 - A_t/A_0) \times 100$$

For the co-aggregation with pathogens, suspensions of LAB and pathogenic bacteria were prepared following the same protocol of auto-aggregation (Oh et al., 2018; Rokana et al., 2017). The initial absorbances of LAB (A) and pathogenic bacterial suspensions (B) were measured at 600 nm. Then, a mixture of equal volume of the LAB and pathogen was vortexed for 10 s prior to incubation. After 4 h (AB₄), 18 h (AB₁₈), and 24 h (AB₂₄) of incubation at 37 °C, the absorbance of the mixture (AB_t) was again measured for the calculation of the percentage of co-aggregation:

$$\text{Co-aggregation (\%)} = [(A + B)/2 - AB_t] / [(A + B)/2] \times 100$$

2.7. Hydrophobicity

Culture suspension of each LAB was adjusted to approximately 10⁸ cfu/ml, and the initial absorbance (A₀) of each culture was measured. Three milliliters of each LAB suspension was mixed with 1 ml of xylene (Sigma-Aldrich Co.), and incubated at 37 °C for 10 min. The mixture was then vortexed and incubated for 3 h at 37 °C without agitation. Afterwards, the aqueous phase was removed and the absorbance (A_t) was measured at 600 nm. The cell surface hydrophobicity for each LAB was calculated as follows:

$$\text{Cell surface hydrophobicity (\%)} = (1 - A_t/A_0) \times 100$$

2.8. Statistical analysis

PCA, heat map, and network analyses were performed using R-script (v.3.5), ClustVis, and PAST (4.0) programs, respectively, to visualize the clustering of multivariate data. The experimental results were expressed as mean ± standard deviation (SD). All statistical analyses were performed using GraphPad and InStatV.3 programs (GraphPad, San Diego, CA, USA). The one-way analysis of variance (ANOVA) among more than two groups were performed for statistical comparison at p < 0.05.

3. Results and discussion

3.1. Acid resistance

Because probiotics, after oral consumption, are usually exposed to gastrointestinal acidity which can damage the cells and reduce their growth and viability (Giles-Gómez et al., 2016), it is essential for potential probiotics to be able to tolerate and survive in acidic conditions (pH 2.0–3.0) (Giles-Gómez et al., 2016; Vinderola & Reinheimer, 2003). Among the nine LAB investigated in our study, *L. helveticus* IDCC3801 and *L. plantarum* BNH17 exhibited the best survival percentage [(92.83 ± 6.56)%] and [(90.50 ± 1.23)%], respectively, which were significantly greater than those of the other five strains (p < 0.05) (Fig. 1A). The survival (%) of *L. plantarum* IDCC3501 and *L. rhamnosus* LR-K1461 were slightly lower (7–9%) than those of *L. helveticus* IDCC3801 and *L. plantarum* BNH17. However, there was no significant difference observed among them (p < 0.05). Conversely, *L. rhamnosus* IDCC3201 had the lowest survival percentage (59.56 ± 4.01) (p < 0.05). Overall, all tested LAB exhibited at least 60% of survival after exposure to acidic conditions (pH 2.5) for 3 h.

Yasmin et al. (2020) also reported that more than 60% of isolated *Bifidobacterium* strains survived in low pH (pH 2 and 3) during 2-h incubation. Another study (Oh et al., 2018) found that the survival percentages of 29 LAB tested at pH 2.5 were determined to be more than 50%. Furthermore, our survival percentage for *L. plantarum* BNH17 [(90.50 ± 1.23)%], *L. plantarum* IDCC3501 [(83.71 ± 2.25)%], and even *L. plantarum* LP-K1791 [(68.52 ± 7.13)%] were much greater than those (50%) of *L. plantarum* reported by Fossi et al. (2015). Large variations in acid resistance among LAB have been reported, and this

variation is dependent on the strain specificity (Castorena-Alba, Vázquez-Rodríguez, López-Cabanillas Lomelí, & González-Martínez, 2018). The excellent acid resistances of these tested LAB at acidic pH supported their potential use as industrial probiotics.

3.2. Bile resistance

Since probiotics must be able to survive in the presence of bile salts in the small intestine, the bile resistance ability is a crucial criterion when selecting probiotics for conferring beneficial effects in the gastrointestinal tract (Margolles, García, Sánchez, Gueimonde, & de los Reyes-Gavilán, 2003). The nine LAB in this study exhibited variable levels of resistance to 0.3% bile salts (Fig. 1B). Among the nine strains, *L. rhamnosus* LR-K1461 and *L. plantarum* LP-K1791 exhibited the best and very similar survival rates (75.59 ± 7.09)% and (75.29 ± 24.79)%, respectively, which were significantly greater than those of the other five strains (p < 0.05). In addition, *L. helveticus* IDCC3801 and *L. lactis* IDCC2301 also provided the competitive survival percentage, although the survival percentage of *L. lactis* IDCC2301 was reduced by approximately 8%. Finally, this study demonstrated that all LAB had survival percentages of approximately 60% or higher when exposed to bile salts.

Yasmin et al. (2020) studied the bile resistances of *Bifidobacterium* and LAB strains and recorded survival percentages exceeding 50% after 3 h of incubation with 0.3% bile salts, whereas minimal survival rates were observed in the presence of 1% bile salts. They also found that higher bile salt concentrations decreased the survival percentage of *Bifidobacterium* and LAB by 5%. In addition, Oh et al. (2018) reported that the bile salt resistances of their LAB exceeded 50%, which was supported by our findings. However, another study (Ribeiro et al., 2018) reported that *L. plantarum* could survive in the presence of 0.3% bile salts with the survival rate of 97–99% for 3 h. Although there could be survival variations depending on the type of strains and conditions, the most important point was that all the nine LAB demonstrated relatively excellent survival rates in the presence of 0.3% bile salts for their survival in the small intestine.

3.3. BSH activity

Conjugated forms of bile acid, which are originally produced in the liver, are converted into deconjugated forms by LAB possessing BSH activity. This property is beneficial for reducing serum cholesterol levels (Bustos, Saavedra, de Valdez, Raya, & Taranto, 2012; Patel, Singhania, Pandey, & Chincholkar, 2010). From the qualitative result of BSH activity in this study, *L. rhamnosus* IDCC3201, *L. casei* IDCC3451, *L. plantarum* IDCC3501, *L. plantarum* BNH17, and *L. plantarum* LP-K1791 exhibited the BSH activity (Table 1). Conversely, BSH activity was absent in *L. lactis* IDCC2301, *L. acidophilus* IDCC3302, *L. helveticus* IDCC3801, and *L. rhamnosus* LR-K1461. Similarly, Boricha et al. (2019) found that *L. plantarum* and *L. rhamnosus* exhibited positive BSH activity. In addition, Ru, Zhang, Yuan, Yue, and Guo (2019) reported positive BSH activity in *L. casei* and *L. plantarum*, which was agreed with our results. However, our result for *L. casei* IDCC3451 contradicted the study of Mallappa et al. (2019). Overall, five strains could be used as therapeutic agents for the reduction of serum cholesterol level.

3.4. Antimicrobial activity

Antimicrobial activity of LAB is another important probiotic property for excluding or inhibiting harmful pathogens in the intestine. Several studies have found the inhibitory effect of LAB was derived from the production of antimicrobial substances such as organic acid, hydrogen peroxide, and bacteriocins (Fei et al., 2018; Servin, 2004). In our study, nine LAB were screened for their antimicrobial activities against nine selected pathogens (Fig. 2). All eight pathogens, except for *B. cereus* ATCC 13061, were inhibited by at least one of unneutralized CFS of LAB with

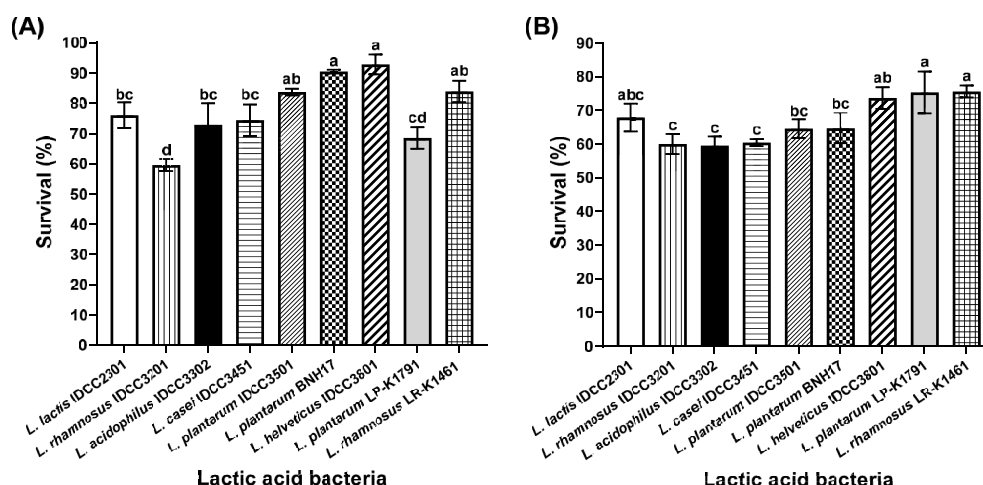


Fig. 1. (A) Acid resistance and (B) bile resistance of lactic acid bacteria.

Values represent mean \pm standard deviation of results from three replicate experiments.

The letters over the bars indicate that the mean values differ significantly by one-way ANOVA test ($p < 0.05$).

Table 1

BSH activity of lactic acid bacteria.

Lactic acid bacteria	BSH activity
<i>L. lactis</i> IDCC2301	-
<i>L. rhamnosus</i> IDCC3201	+
<i>L. acidophilus</i> IDCC3302	-
<i>L. casei</i> IDCC3451	+
<i>L. plantarum</i> IDCC3501	+
<i>L. plantarum</i> BNH17	+
<i>L. helveticus</i> IDCC3801	-
<i>L. plantarum</i> LP-K1791	+
<i>L. rhamnosus</i> LR-K1461	-

variable inhibition zone sizes. More importantly, the unneutralized CFS of *L. rhamnosus* IDCC3201 significantly inhibited eight pathogens. Interestingly, *Shigella sonnei* was inhibited by eight LAB excluding *L. acidophilus* IDCC3302 with a relatively bigger size of inhibition. Next, *E. coli* O157:H7

and *Salmonella* Senftenberg were also inhibited by seven strains, except for *L. lactis* and *L. acidophilus*. As expected, the neutralized CFS of LAB could not inhibit the growth of any of the nine pathogens (data was not provided), which indicated that organic acids might be the major antimicrobial compound for potential probiotics (Fei et al., 2018).

Similar to our results, Prabhurajeshwar and Chandrakanth (2019) found that *Lactobacillus* isolates were able to inhibit *E. coli* and *Shigella*. Furthermore, Halder, Mandal, Chatterjee, Pal, and Mandal (2017) reported that the growths of *E. coli*, and *Salmonella* Typhi were inhibited by *L. rhamnosus* LMEM9. The contradictory results obtained from three strains of *B. cereus* suggest that there is room for further investigation. In addition, a further study is required to understand the antimicrobial activity of LAB against inclusive and exclusive bacterial strains. Overall, our result demonstrated that LAB showed more effective antimicrobial activity against the three strains (*E. coli* O157:H7, *S. Senftenberg*, and *S. sonnei*) as compared to the other strains (three strains of *B. cereus*, *S. Typhimurium*, and *S. Enteritidis*).

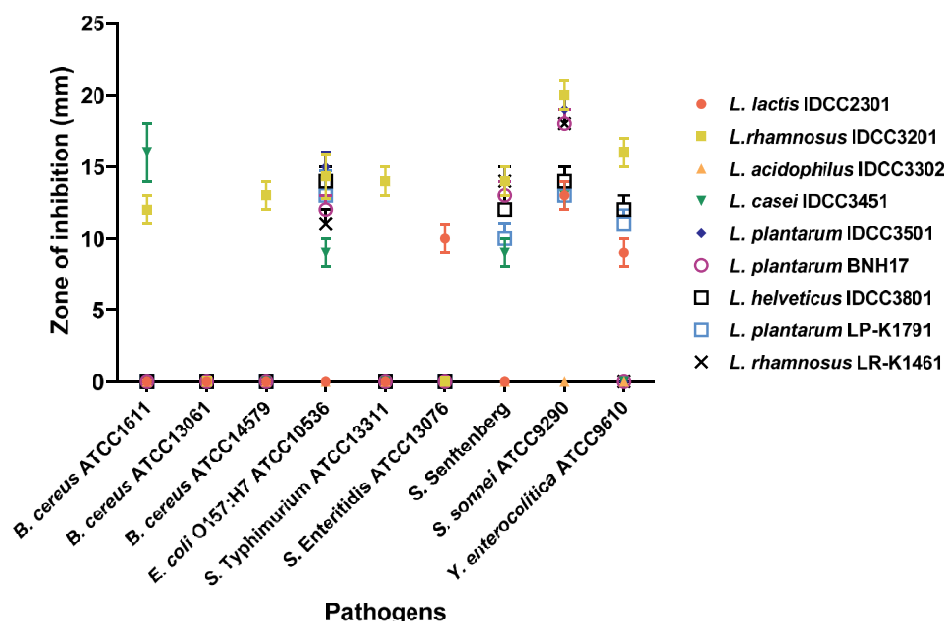


Fig. 2. Antimicrobial activity of unneutralized cell-free supernatant of lactic acid bacteria against selected pathogens.

Values represent mean \pm standard deviation of results from three replicate experiments.

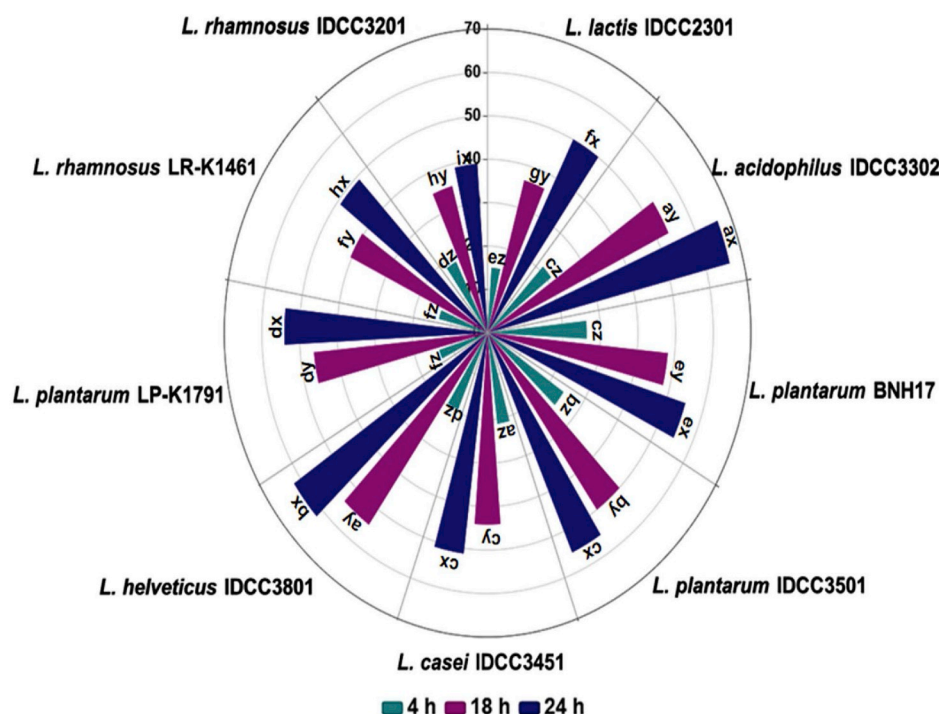


Fig. 3. Auto-aggregation percentage of lactic acid bacteria determined at different incubation times.

Values represent mean \pm standard deviation of results from three replicate experiments.

The letters (a - i and x - z) over the bars indicate the significant differences among the lactic acid bacteria strains and incubation time, respectively, by one-way ANOVA test ($p < 0.05$).

3.5. Auto-aggregation and co-aggregation

The ability of LAB to adhere to the mucosal surface and epithelial cells is another important probiotic characteristic and this ability may depend on the contact between the cell membrane and interacting surfaces (Kos et al., 2003; Vinderola & Reinheimer, 2003). Many studies have suggested that aggregation properties are related to this adherence ability, which could help probiotics survive in the gastrointestinal tract (Boris, Suarez, & Barbes, 1997; Ocaña & Nader-Macias, 2002). In our study, nine LAB were screened for auto-aggregation properties at different incubation times (Fig. 3). The auto-aggregation percentages of all tested LAB followed a time-dependent manner, which was significantly different among the incubation times within each strain ($p < 0.05$). After 4 h, the significantly greatest percentage of auto-aggregation was observed for *L. casei* IDCC3451 [(26.37 \pm 0.52)%] whereas the significantly lowest percentage of auto-aggregation was found in *L. rhamnosus* LR-K1461 [(13.31 \pm 0.44)%] and *L. plantarum* LP-K1791 [(13.41 \pm 0.58)%] ($p < 0.05$). During an 18-h incubation period, *L. helveticus* IDCC3801 [(54.28 \pm 0.40)%] and *L. acidophilus* IDCC3302 [(53.32 \pm 0.45)%] exhibited significantly greater percentages of auto-aggregation among others ($p < 0.05$). At 24 h, *L. acidophilus* IDCC3302 exhibited maximal auto-aggregation [(66.32 \pm 0.45)%] with significant differences among others, whereas *L. rhamnosus* IDCC3201 exhibited the significantly lowest level of auto-aggregation [(39.04 \pm 0.06)%]. Overall, *L. helveticus* IDCC3801 and *L. acidophilus* IDCC3302 exhibited the relatively greater auto-aggregation percentages than the others after 18-h incubation. Ramos, Thorsen, Schwan, and Jespersen (2013) also reported similar auto-aggregation rates ranging from 12.17 to 61.89% among various *Lactobacillus* strains during 5-h incubation. In addition, Kos et al. (2003) reported that *L. acidophilus* M92 exhibited an auto-aggregation percentage of 69% for 5-h incubation.

The ability of probiotic strains to co-aggregate with potential pathogens may confer additional advantages to them as potential probiotics due to their role as the barrier for preventing the colonization of pathogens (Bao et al., 2010; Rokana et al., 2017). The pattern of co-

aggregation percentages (Fig. 4) followed a time-dependent manner, which indicated that most of the LAB at 24-h incubation were significantly greater than those at both 4-h and 18-h incubations ($p < 0.05$) (Table 1S). The co-aggregation percentages at 4 h, 18 h, and 24 h were in the ranges of ~3–30%, ~22–68%, and ~34–71%, respectively. Although some of the LAB did not provide the maximum percentage of co-aggregation at 24 h, the overall co-aggregation percentages of each LAB with pathogens were compared in Fig. 4 using 24-h incubation data. *L. plantarum* LP-K1791 exhibited the greatest co-aggregation percentage with a range of 25.1–71.4% against eight pathogens (except for *S. Enteritidis*), followed by *L. rhamnosus* LR-K1461 ($p < 0.05$). Meanwhile, *L. lactis* IDCC2301 and *L. rhamnosus* IDCC3201 demonstrated nearly the lowest percentage of co-aggregation against nine pathogens with the range of 27.6–57.3%. All LAB used in our study exhibited co-aggregation activities with all of the selected pathogens, although the co-aggregation percentages varied depending on not only the target strain but incubation time. Campana, van Hemert, and Baffone (2017) reported that co-aggregation properties are strain-specific and that it is a beneficial mechanism. Oh et al. (2018) reported that LAB could be co-aggregated more strongly with gram-negative pathogens (*E. coli* and *Salmonella enterica*) rather than with gram-positive ones (*Listeria monocytogenes*, *Staphylococcus aureus*, and *B. cereus*), which were consistent with our result. Our finding on the diverse aggregation capacity of LAB was substantiated from some previous studies, which clearly indicated that their aggregation properties were related to the composition and structure of bacterial surface components (Castagliuolo et al., 2005; Rokana et al., 2017).

3.6. Hydrophobicity

Cell hydrophobicity helps maintain bacterial survival in the gastrointestinal tract (Kos et al., 2003). Because adhesion and interaction with host cells are influenced by hydrophobicity, a minimum hydrophobicity of 40% is necessary for selecting candidate probiotics (Del Re, Sgorbati, Miglioli, & Palenzona, 2000). All LAB were screened for hydrophobicity in xylene, a polar solvent, that exhibits both

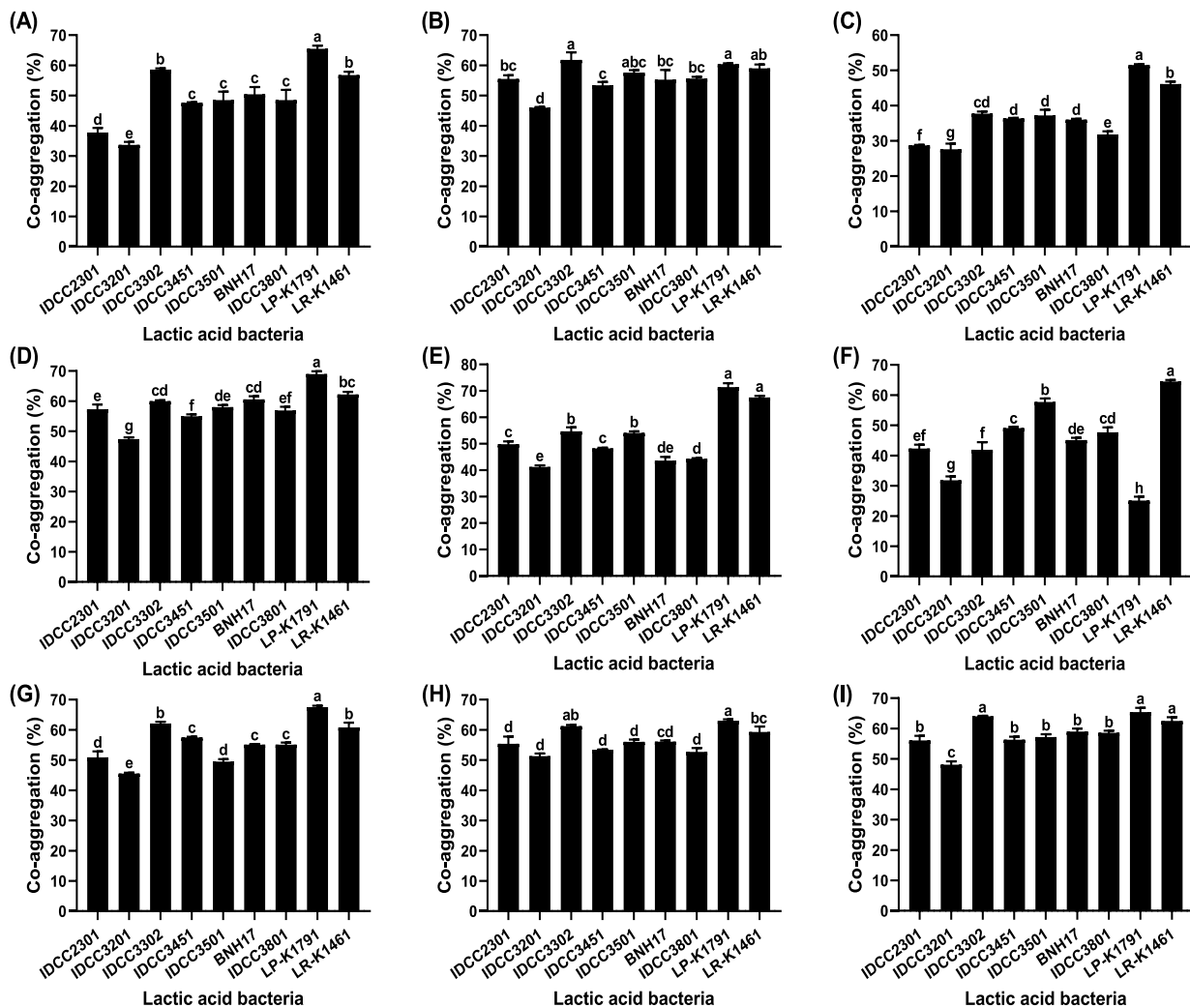


Fig. 4. Co-aggregation percentage of lactic acid bacteria with (A) *B. cereus* ATCC1611, (B) *B. cereus* ATCC13061, (C) *B. cereus* ATCC14579, (D) *E. coli* O157:H7 ATCC10536, (E) *S. Typhimurium* ATCC13311, (F) *S. Enteritidis* ATCC13076, (G) *S. Senftenberg*, (H) *S. sonnei* ATCC9290 and (I) *Y. enterocolitica* ATCC9610 after 24-h incubation.

The letters over the bars indicate that the mean values differ significantly by one-way ANOVA test ($p < 0.05$).

2301: *L. lactis* IDCC2301, 3201: *L. rhamnosus* IDCC3201, 3302: *L. acidophilus* IDCC3302, 3451: *L. casei* IDCC3451, 3501: *L. plantarum* IDCC3501, BNH17: *L. plantarum* BNH17, 3801: *L. helveticus* IDCC3801, LP-K1791: *L. plantarum* LP-K1791, LR-K1461: *L. rhamnosus* LR-K1461.

Table 2
Hydrophobicity (%) of lactic acid bacteria in xylene.

Lactic acid bacteria	Hydrophobicity (%)
<i>L. lactis</i> IDCC2301	55.53 \pm 0.19 ^{bc}
<i>L. rhamnosus</i> IDCC3201	50.96 \pm 1.36 ^d
<i>L. acidophilus</i> IDCC3302	51.18 \pm 0.42 ^d
<i>L. casei</i> IDCC3451	53.12 \pm 0.17 ^{cd}
<i>L. plantarum</i> IDCC3501	39.18 \pm 1.67 ^e
<i>L. plantarum</i> BNH17	64.98 \pm 0.01 ^a
<i>L. helveticus</i> IDCC3801	49.66 \pm 0.47 ^d
<i>L. plantarum</i> LP-K1791	50.80 \pm 0.27 ^d
<i>L. rhamnosus</i> LR-K1461	57.31 \pm 1.85 ^b

Values represent mean \pm standard deviation of results from three replicate experiments.

The letters (a-e) indicate that the mean values in the column differ significantly by one-way ANOVA test ($p < 0.05$).

hydrophobicity and hydrophilicity (Kos et al., 2003). Of the tested LAB, the significantly greatest hydrophobicity was observed for *L. plantarum* BNH17 [(64.98 \pm 0.01)%], whereas the significantly lowest hydrophobicity was observed for *L. plantarum* IDCC3501 [(39.18 \pm 1.67)%] ($p < 0.05$) (Table 2).

Yasmin et al. (2020) reported that their *Bifidobacterium* isolates exhibited a hydrophobicity range of 60.8–78.9%. In addition, Xu, Jeong, Lee, and Ahn (2009) reported hydrophobicity values of 53.6% for *Bifidobacterium longum* B6 and 46.5% for *L. rhamnosus* GG. Furthermore, Kos et al. (2003) reported 70% hydrophobicity for *L. acidophilus*, which exceeded our results. Although variable results were obtained from all tested LAB in this study, the overall hydrophobicity of LAB for further studies (*in vitro* and *in vivo*) were satisfied with the minimum requirement of hydrophobicity (40%) as potential probiotics (Del Re et al., 2000).

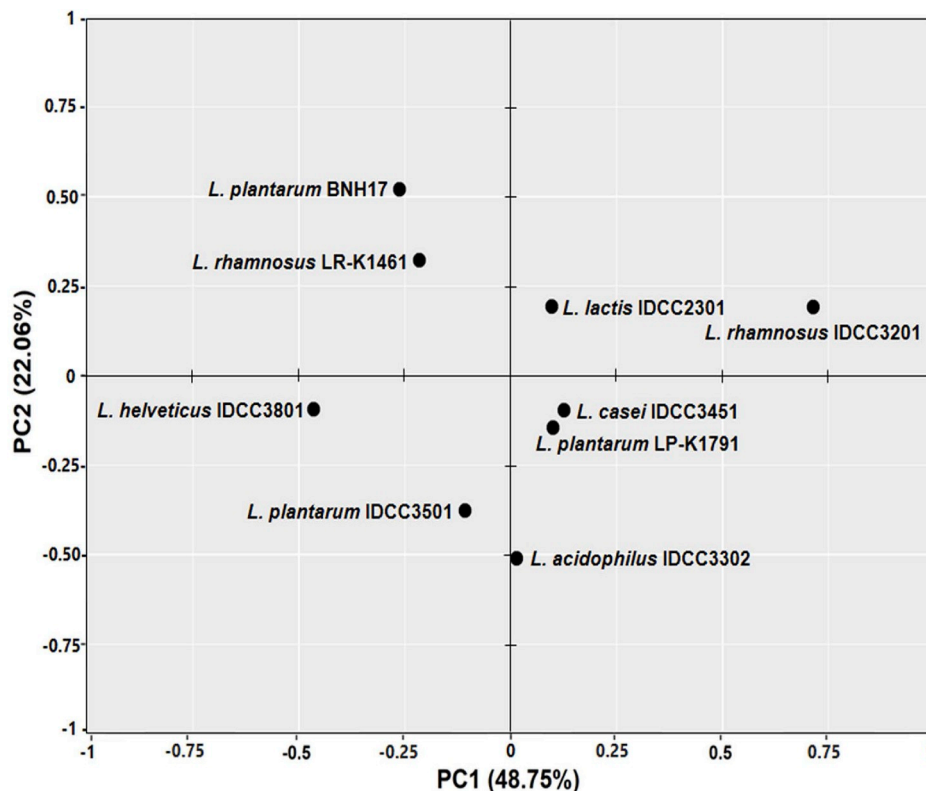


Fig. 5. Principal component analysis of probiotic properties (bile and acid resistances, auto- and co-aggregation, and hydrophobicity) of nine lactic acid bacteria.

3.7. Selection of probiotics

In our study, PCA was performed by five properties of acid and bile resistances, cell surface hydrophobicity, and auto- and co-aggregation properties for the selection of probiotic candidates. The PC1 covers maximum variation in the property data while PC2, which is orthogonal to the first, covers the remaining variation (Choudhary et al., 2019). As shown in Fig. 5, two principal components (PC1 and PC2) obtained from the property resulted in 70.81% total variance, where PC1 and PC2 accounted for 48.75% and 22.06% of the five properties, respectively. The projections of the nine LAB in the PCA plot was differentiated into four quadrants. *L. rhamnosus* LR-K1461 and *L. plantarum* BNH17 were placed in quadrant I, *L. lactis* IDCC2301 and *L. rhamnosus* IDCC3201 in quadrant II, *L. helveticus* IDCC3801 and *L. plantarum* IDCC3501 in quadrant III, and *L. casei* IDCC3451, *L. plantarum* LP-K1791 and *L. acidophilus* IDCC3302 in quadrant IV. The LAB in quadrants II and IV had greater correlation among their properties with respect to PC1 than other LAB. Therefore from the PCA analysis, *L. rhamnosus* IDCC3201, *L. lactis* IDCC2301, *L. casei* IDCC3451, and *L. plantarum* LP-K1791 were selected as the most promising probiotics among others.

Unlike PCA analysis, the heat map (Fig. 6) visualizes the clustering of two-dimensional representation of PCA data by using different colors. When the percentage value increases, the color or the intensity of the color varies (from red to green). The heat map provides the clusters of LAB with similar properties when compared with other strains. In this study, LAB were classified into four clusters indicated as A, B, C, and D, except for *L. acidophilus* IDCC3302, which indicated no similarity with any other strains. Both *L. casei* IDCC3451 and *L. rhamnosus* IDCC3201 in cluster C were selected as the most promising due to the almost fully green color and intensity. Then, *L. plantarum* LP-K1791 was selected from cluster D in comparison to *L. lactis* IDCC2301 due to the greener color and intensity. Lastly, *L. helveticus* IDCC3801 and *L. plantarum* IDCC3501 were selected from cluster A and B, respectively, which was contradictory to the result of PCA. Based on the overall

results of heat map, three LAB including *L. casei* IDCC3451, *L. rhamnosus* IDCC3201, and *L. plantarum* LP-K1791 could be selected as probiotic candidates.

The network analysis helps to confirm the similarity of LAB properties by visualizing the confidence of similarity and the node color. Usually, the strains to be studied are depicted as nodes and the size of each of the nodes is proportional to the number of connections. In addition, the density of the connecting lines indicates the extent of their similarities. In Fig. 7, all the LAB strains were connected in some ways as network pathways instead of clusters. *L. casei* IDCC3451, *L. rhamnosus* IDCC3201, and *L. plantarum* LP-K1791 shared stronger association with the other LAB as well as among each other (same node color). In addition, *L. lactis* IDCC2301 exhibited comparatively stronger association with the other LAB including *L. helveticus* IDCC3801, *L. rhamnosus* LR-K1461, *L. plantarum* BNH17, *L. plantarum* LP-K1791, *L. rhamnosus* IDCC3201 as well as *L. casei* IDCC3451. However, *L. helveticus* IDCC3801 and *L. plantarum* IDCC3501 did not prove strong association with other LAB, which were different from *L. casei* IDCC3451, *L. rhamnosus* IDCC3201, and *L. plantarum* LP-K1791. From the network analysis, *L. casei* IDCC3451, *L. rhamnosus* IDCC3201, *L. plantarum* LP-K1791, and *L. lactis* IDCC2301 were selected as the most promising probiotics.

Each of the three analyses (PCA, heat map, and network analyses) provided similar and contradictory results for the selection of candidate probiotics. This similar and contradictory result obtained from PCA and heat map analyses agreed with the findings of Mallapa et al. (2019) in that they were able to select 9 *Lactobacillus* isolates from among 14 probiotics isolates by using heat map analysis rather than PCA analysis. From our *in silico* analysis, the result of three analyses was objectively compared and evaluated. Thus, *L. casei* IDCC3451, *L. rhamnosus* IDCC3201, and *L. plantarum* LP-K1791 were finally selected as the most promising probiotics. This study confirmed that the network analysis helped to achieve a reproducible result and precisely select *L. casei* IDCC3451, *L. rhamnosus* IDCC3201, and *L. plantarum* LP-K1791 as probiotics.

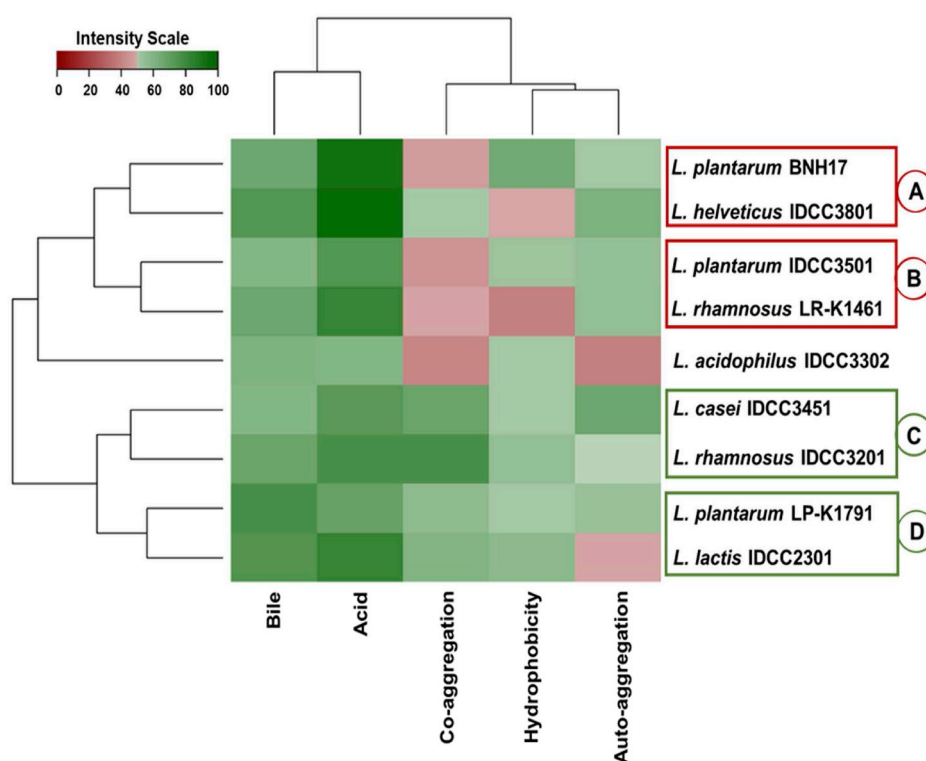


Fig. 6. Heat map plot of probiotic properties (bile and acid resistances, auto- and co-aggregation, and hydrophobicity) of nine lactic acid bacteria.

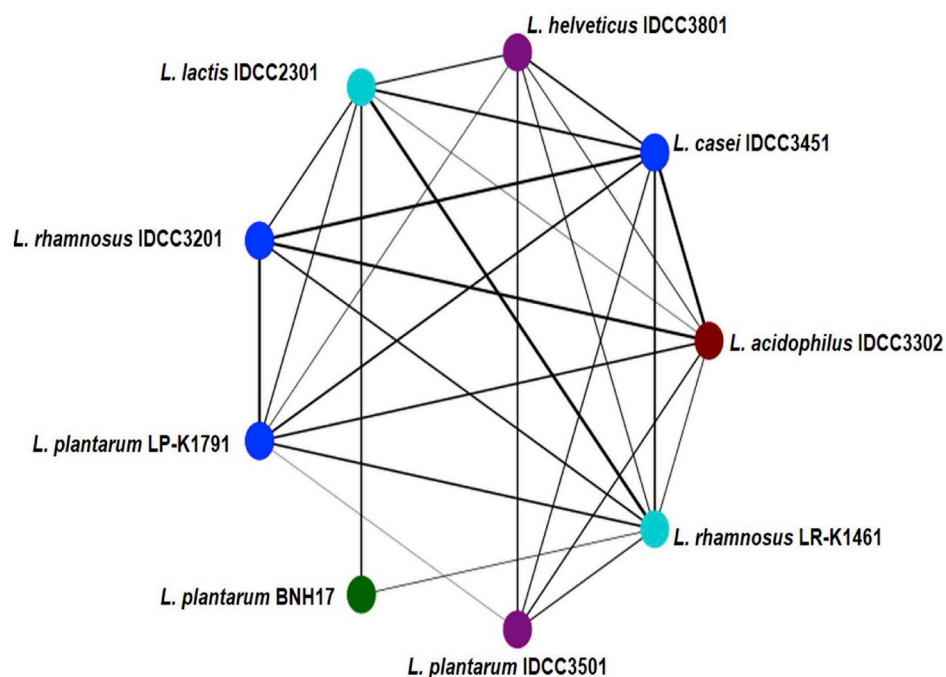


Fig. 7. Network analysis plot showing the correlation among nine lactic acid bacteria. Uniform node color represents the lactic acid bacteria sharing similar probiotic properties.

4. Conclusions

All tested LAB exhibited probiotic characteristics on various levels. They exhibited the range of acid resistance of 59.56–92.83%, bile resistance of 59.55–75.59%, hydrophobicity of 39.18–64.98%, auto-aggregation of 39.04–66.32%, and co-aggregation of 25.1–71.4%. To test for additional functional activity of LAB, BSH activity and the antimicrobial activity were also investigated. In order to select the most promising

probiotics from multivariate results, *in silico* analysis including principal component, heat map, and network analyses were performed and introduced in this study. *L. casei* IDCC3451, *L. plantarum* LP-K1791, and *L. rhamnosus* IDCC3201 were selected as the most promising probiotics. The results of this study are evidence of the importance of *in silico* analysis in the precise identification of candidate probiotics based on their various properties. This novel approach provides a robust method for the selection of potential probiotics for *in vitro* and *in vivo* studies.

CRediT authorship contribution statement

Selvakumar Vijayalakshmi: Methodology, Data curation, Writing - original draft, Writing - review & editing. **Damilare Emmanuel Adeyemi:** Methodology, Data curation, Writing - original draft, Writing - review & editing. **In Young Choi:** Software, Data curation, Writing - review & editing. **Ghazala Sultan:** Software, Data curation, Writing - original draft. **Inamul Hasan Madar:** Software, Data curation, Writing - original draft. **Mi-Kyung Park:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declarations of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2020.109617>.

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