

Isolation and characterization of probiotic lactic acid bacteria from human breast milk

Nur Kusmiyati^{1,✉}, Septian Tri Wicaksono², Ari Surya Sukarno³

¹Agricultural Product Technology Department, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

²Biology Department, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim, Malang, Indonesia

³Animal Product Technology Department, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia

✉ Corresponding author: kusmiy4tinur@gmail.com

Article info

Article history:

Received: 13th July 2021

Accepted: 28th February 2022

Keywords:

16S rRNA gene
human breast milk
lactic acid bacteria
probiotics

Abstract

The lactic acid bacteria (LAB) isolated from human breast milk is known as probiotics and comprises numerous health benefits. This study aims to select and determine the species name of LAB based on the 16S rRNA gene, which has the potential to be the best indigenous probiotic. The method used included analysis of LAB resistance at acidic pH 2.0 and bile salts (0.5 %), antimicrobial activity against pathogenic microorganisms, and determining the autoaggregation properties. LAB isolates with the best ability in the analysis were then identified using a partial sequence of the 16S rRNA gene. The isolation and purification revealed eight LAB isolates with different parameters named as L19A, L19B, L19C, L19D, L19E, L19F, L19G, and L19H. Isolates L19A, L19E, and L19H have good tolerance ability against acid pH and bile salts, compared to others. Meanwhile, the L19H isolate had the strongest antimicrobial activity against pathogenic microorganisms *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *C. albicans* ATCC 11778, while the L19A had the highest hydrophobicity, autoaggregation, and coaggregation ability. Based on the partial sequence analysis of the 16S rRNA gene, the L19A, L19E, and L19H have similar values with *L. casei*, *L. rhamnosus*, and *L. paracasei*, respectively. These isolates belong to the *L. casei* group (LCG) from human breast milk, which can be used as an indigenous probiotic.

Introduction

COVID-19 is a respiratory disease caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2). The signs of the virus range from mild to severe, such as acute respiratory disease syndrome (ARDS). Specifically, ARDS is caused by inflammatory cytokines, such as tumor necrosis

factor (TNF), interleukin (IL), and granulocyte colony-stimulating factor (G-CSF) (Schirmer *et al.* 2016). The inflammatory cytokines have a specific correlation with the gut microbiome, necessitating probiotic modulation of the gut microbiota (Yeoh *et al.* 2021). According to FAO/WHO (2006), probiotics are living microorganisms found in food with the ability to survive and thrive in the

digestive system, positively affecting the host. These microorganisms work by increasing macrophages in the host's immune system and releasing antibodies (Maldonado Galdeano *et al.* 2019). According to Ganjbakhsh *et al.* (2017), administering probiotics increases the activity of Natural Killer (NK) cells in humans. Furthermore, probiotics heighten the activity of phagocytes from macrophages and levels of immunoglobulin A (IgA) in mice model diarrhea (Rocha-Ramirez *et al.* 2017).

Jatmiko *et al.* (2017) stated that lactic acid bacteria (LAB) from the genus *Lactobacillus* and *Bifidobacterium* also act as probiotics. Studies on LAB probiotics from fermented products, digestive system, feces, milk, and human breast milk have been widely published. Some LABs found in human breast milk include *B. longum*, *L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. pentosus*, *L. plantarum*, *L. reuteri*, *L. fermentum*, *L. brevis*, *L. casei*, *L. gastricus*, and *L. salivarius* (Sinkiewicz and Ljunggren 2008; Jamyuang *et al.* 2019; Łubiech and Twarużek 2020). The isolation of LAB from different humans can affect the differences in species obtained and their abilities. In Algeria and China, LAB isolates, including *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. fermentum*, and *L. plantarum*, and, namely, *L. plantarum* were found in human breast milk. According to Jiang *et al.* (2016), LAB isolated from human breast milk is probiotic and contains various health benefits.

The LAB of human breast milk as a probiotic potential was assessed by following specific criteria and tested to determine low pH and bile salts resistance. Furthermore, it was analyzed to determine the antimicrobial properties, hydrophobicity, and aggregation, correlating with the ability to attach to the digestive tract (Li *et al.* 2020). During initial selection, an *in vitro* assessment of hydrophobicity and auto-aggregation properties was performed to assess LAB's capacity to bind the digestive tract. One of the important functions for inhibiting pathogenic bacteria colonization is its ability to co-aggregate with LAB.

A partial sequence of the 16S rRNA gene was used to identify the LAB with the best ability as an indigenous probiotic substance. The standard identification method and phylogenetic

relationships in a bacteria is 16S rRNA gene sequence analysis, consisting of highly conserved and hypervariable regions (Tilahun *et al.* 2018). Therefore, it can be used to determine phylogenetic relationships and the levels of kinship between microbes. Moreover, the 16S rRNA gene is constant and does not change for long, though there is a minute mutation rate (Johnson *et al.* 2019). Furthermore, its amplification and sequencing processes identify a bacterial isolate to the species level.

This study used universal primers to partially amplify LAB isolates from human breast milk to a base sequence of 1,500 bp in the V1-V9 regions of the 16S rRNA gene. According to Bukin *et al.* (2019) and Michel *et al.* (2016), the V1-V9 regions can differentiate between species. Regional sequences V1 and V3 were used to identify LAB up to strain level (Bin Masalam *et al.* 2018). Variations in subjects used to collect human breast milk samples affected the LAB skill obtained and the results of species recognition. This study aimed to obtain LAB species that can serve as an indigenous probiotic from human breast milk for health benefits.

Experimental

Sampling

Breast milk samples were collected from 20 mothers (aged 27 – 30) breastfeeding babies of 0 – 6 months. Participants were recruited from Malang Regional Unit Hospital, East Java, Indonesia, between September-October 2020. The sampling process involved using a sterilized breast pump. However, the initial 10 – 50 mL was discarded, while the subsequent 50 – 100 mL was put into a sterile bottle for utilization. The sample was kept frozen at 4 °C. The LAB isolation process was conducted (Kaškonienė *et al.* 2017). This study was approved for ethical clearance from LPPT UGM No 00154/08/LPPT/II/2020.

The isolation and purification of LAB

The isolation and purification of LAB from human breast milk were effected using the Kaškonienė *et al.* (2017) method. A sample of 10 mL was put into

a test tube and diluted to 10^{-5} by inserting 1 mL spacemen into 99 mL of equates. The inoculum was streaked using de Man Rogosa and Sharpe Agar (MRSA) medium alongside 1 % calcium carbonate. Thereafter, samples were incubated for 48 h at 37 °C with a clear zone around a single bacterial colony, indicating a positive result. The cultures were further purified using the streak plate method and incubated at 37 °C for 48 h on MRSA. Notably, the pure cultures were stored on MRSA slants at 4 – 10 °C.

Analysis of LAB resistance to acid pH and bile salts

pLAB resistance test from human breast milk to acidic pH 2.0 and bile salts was carried out by Shehata *et al.* (2016) with the same method used on the de Man Rogosa and Sharpe Broth (MRSB), which rejuvenated LAB culture for 24 h. A culture of 0.1 mL was inoculated into 10 mL of MRSB media (control) with pH 2.0 by adding 37 % HCl into the medium and incubated at 37 °C for 5 h. A total culture of 0.1 mL was inoculated into 10 ml MRSB (control) and added 0.3% bile salt, then incubated at 37 °C for 5 h. Afterward, calculations on the number of colonies on MRSA media using the plate count method were conducted. The resistance of LAB isolates to acidic pH bile salts was calculated based on the difference in log units of the number of colonies that grew in control. The smaller the difference grew, the more resistant the LAB culture was to acid pH and bile salts.

Hydrophobicity analysis of LAB from human breast milk

Activities from hydrophobicity analysis of bacteria were determined using the microbial adhesion to solvent (MATS) (Krausova *et al.* 2019). The analysis was conducted using solvents with different polarities, namely: xylene, ethyl acetate, and chloroform (all from Merck KGaA, Darmstadt, Germany). Adhesion of bacteria cells to xylene solvents describes hydrophobicity, while chloroform and ethyl acetate analyze cell surface properties. Bacteria were grown in MRSB at 37 °C for 16 – 18 h. The cells were harvested using the centrifugation method (Hermle Z 383 K, Hermle

AG, Gosheim, Germany) at a speed of 3,500 rpm for 20 min. The precipitates were washed using urea magnesium phosphate buffer (PUM) and suspended in the PUM buffer until the bacterial cell was collected at 10^8 CFU.mL⁻¹. Afterward, the original value was calculated at 600 nm wavelength (X_0) with a total cell suspension of 5 ml in the PUM buffer transferred to a new tube. Furthermore, 1 ml of solvents like xylene, ethyl acetate, and chloroform were applied and combined with the vortex in a test tube rapidly for a minute and left still for an hour at 37 °C for disillusion. The aqueous was carefully transferred using a sterile pipette alongside a spectrophotometer (UV-1800 UV-Vis, Shimadzu Corp., Kyoto, Japan) to measure its absorbance at 600 nm (X_1). Absorbance decrease value in the aqueous phase is expressed as adhesion value to the solvent (%) using Eq. 1.

$$\text{Adhesion (\%)} = \left(1 - \frac{X_0}{X_1}\right) A - 100 \quad (1)$$

X_0 = initial absorbance value at 600 nm

X_1 = final absorbance value at 600 nm

Autoaggregation analysis of LAB from human breast milk

The autoaggregation analysis of LAB was determined using the Krausova *et al.* (2019) method, with the isolates grown on MRSB for 18 h at 37 °C. The cells were then harvested using centrifugation at 3,500 rpm for 20 min. The residue was washed twice with phosphate buffer saline (PBS) and later suspended in PBS up to 10^8 CFU.mL⁻¹. Furthermore, autoaggregation was determined by measuring the initial (0 hours) alongside the final absorbance (5 h) after incubation at room temperature. This process was implemented by putting 0.1 mL of the upper suspension into 3.9 mL of PBS with a spectrophotometer at 600 nm, measuring the absorbance. The autoaggregation percentage is expressed in Eq. 2.

$$\text{Autoaggregation (\%)} = 1 - \left(\frac{A_t}{A_0}\right) A - 100 \quad (2)$$

A_t = absorbance at time t = 5

A_0 = absorbance at time t = 0

Coaggregation analysis of LAB from human breast milk

The coaggregation analysis conducted by Krausova *et al.* (2019) proposed this method. LAB isolates were grown on the MRSB and pathogenic bacteria on TSB for 18 h at 37 °C. Furthermore, the LAB and pathogenic cells were later harvested by centrifugation at 3,500 rpm for 20 min. Notably, the residue obtained was washed twice and suspended in PBS up to 10⁸ CFU mL⁻¹. The volume of 2 mL of each suspension was then mixed in a control tube containing 4 ml per bacteria. The coaggregation was determined by measuring the initial (0 h) and the final absorbance (5 h) after room temperature incubation. This measurement was determined by putting 0.1 mL of the upper suspension into 3.9 mL of PBS and measuring the absorbance using a spectrophotometer at 600 nm. The percentage of co-aggregation is expressed in Eq. 3.

$$\text{Coaggregation (\%)} = \frac{\left[\frac{M_x+M_y}{2} - M_{x+y}\right]}{M_x+M_y} A - 100 \quad (3)$$

M_x = absorbance of LAB isolate suspension

M_y = absorbance of pathogenic isolate suspension

M_{x+y} = absorbance of the mixture of LAB and pathogenic isolate suspensions

Antimicrobial activity of LAB from human breast milk

Antimicrobial activity was qualitatively analyzed using the proper diffusion method proposed by Le *et al.* (2019). The test microbes used were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *C. albicans* ATCC 11778. Also, 1 mL of test microbes were piped into a petri dish and poured in a sterilized medium before cooling to about 40 °C. The Tryptone Soy Agar (TSA) is left to solidify for 1 h after cooling, with a diameter of 0.5 cm at room temperature. The 1 mL test isolate was inoculated in the well and incubated at 37 °C for 2 d. After that, the inhibition zone diameter was measured from three different sides.

Molecular identification

Direct PCR is the initial stage for 16S rRNA gene analysis. 16S rRNA gene is a common feature for best acid pH and bile salts, antimicrobial, and autoaggregation. The process of amplifying 16S rRNA gene partial sequence from lactic acid bacteria was conducted using direct PCR amplification as proposed by Woodman *et al.* (2008). One LAB colony with the potential of an indigenous probiotic was withdrawn from a petri dish to a tube. Thereafter, the PCR mix (50 µL) contained 2 µL DNA templates (100 ng/µL), 5 µL 10X buffer (Mg²⁺), 4 µL dNTP (10 mmol/L), 1.5 µL primer FA-27F (10 pmol/µL), 1.5 µL primer RA-1495R (10 pmol/µL), 0.5 µL Taq DNA polymerase (5 U/µL) and 35.5 µL tri-distilled water were added. The primers used to amplify lactic acid bacteria were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3').

Afterward, PCR amplification was carried out by denaturation of 94 °C for 5 minutes, to 30 denaturation cycles at 94 °C for 1 min, annealing at 58 °C for 1 min, to 72 °C for 2 min, and the final extension of 72 °C for 10 min and 4 °C for heat preservation (Wang *et al.* 2016). The PCR products were separated on 1 % gel agarose by electrophoresis process in 100 volts for 30 min and visualized using gel documentation (Gel Doc XR+ System, Bio-Rad Laboratories, Inc., Hercules, USA). The DNA marker BenchTop 1kb (Promega Corp., Madison, USA) was used as the molecular weight standard. Moreover, DNA sequencing was achieved using an automated DNA Sequencer. The results were analyzed by comparing the DNA sequences database from NCBI (<http://www.blast.ncbi.nlm.nih.gov>) with BLAST (Basic Local Alignment Search Tool). MEGA 5.0 used for phylogenetic tree analysis was achieved using the Neighbor-Joining (NJ) algorithm method with 1,000 Bootstrap repetitions based on p-distance.

Data analysis

The quantitative data group was analyzed by SPSS 16.0 and tested for normality and homogeneity using the Shapiro-Wilk and Levene Test. When the

results are normal and homogenous, the Analysis of Variance process is carried out by further DMRT test to determine the effect of differences between isolates.

Results

The ability of LAB at acidic pH and bile salts

A total of eight LAB isolates were collected with different macro and microscopic characters and

later purified with human breast milk. All isolates were analyzed and proved to function as probiotics, including low PH resistors. The LAB analysis with low pH and bile salts indicated that the L19A isolate had better resistance. However, L19E and L19H isolates performed better than L19B, L19C, L19D, L19F, and L19G, as shown in Fig. 1. The tolerance for bile acids and salts was characterized by survival in media containing a pH of 2.0 and a bile salt of 0.5 % with a difference below 2 logs.

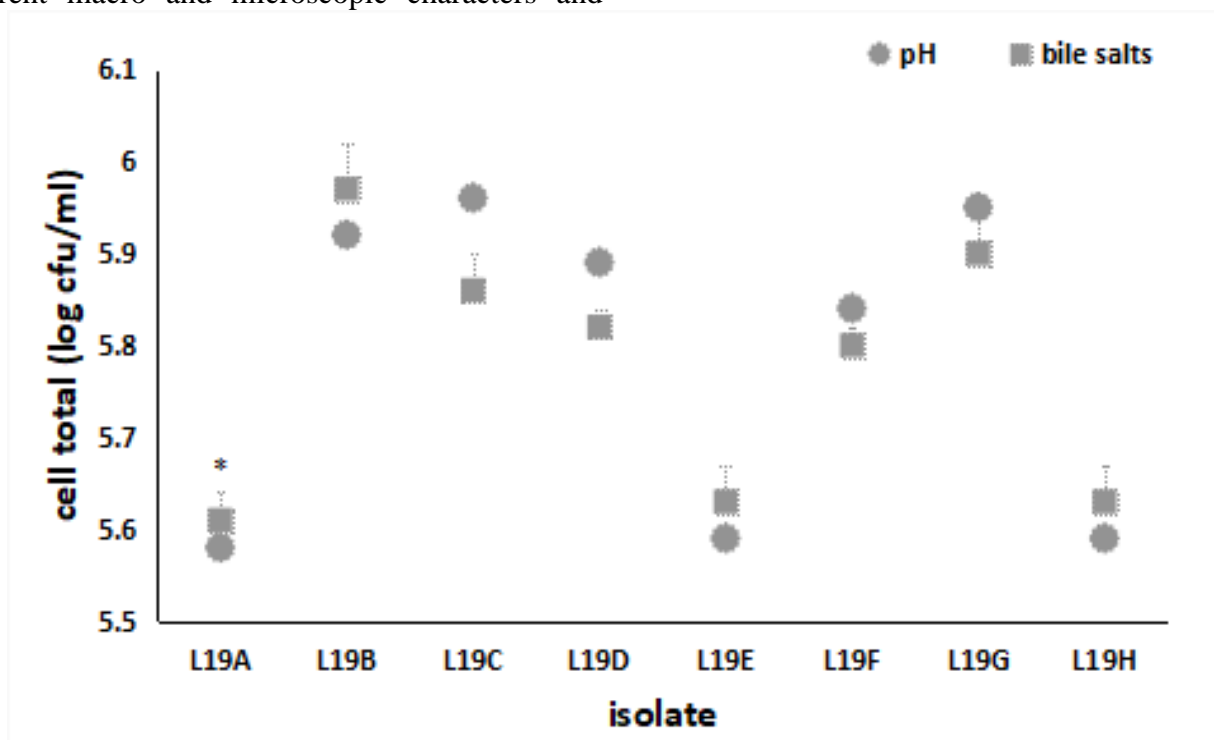


Fig. 1. Analysis of the resistance of LAB isolates to low pH and bile salts; asterisks indicate mean comparisons that differ significantly ($P < 0.05$).

Hydrophobicity analysis of LAB from human breast milk

The hydrophobicity nature affects its auto-aggregate and adhesiveness to various surfaces. Some isolates indicated a higher affiliation to chloroform than for ethyl acetate, as shown in Table 1. L19A had the strongest hydrophobicity properties based on xylene solvent affinity and was significantly unique. The high xylene affinity is indicated by L19E and L19H, and those above 50 % are declared strong hydrophobic.

Table 1. Adhesion (%) of LAB isolates from human breast milk with various solvents.

| BAL isolate | Adhesion [%] | | |
|-------------|----------------------------|---------------------------|----------------------------|
| | Xylene | Eethyl acetate | Chloroform |
| L19A | 71.01 ± 3.17 ^e | 77.21 ± 3.54 ^f | 52.01 ± 2.20 ^d |
| L19B | 28.21 ± 2.50 ^a | 10.68 ± 5.47 ^a | 16.23 ± 1.33 ^a |
| L19C | 37.05 ± 0.67 ^b | 17.47 ± 2.13 ^b | 13.89 ± 2.22 ^a |
| L19D | 38.18 ± 4.80 ^b | 22.32 ± 3.81 ^b | 18.05 ± 2.82 ^a |
| L19E | 66.02 ± 1.85 ^{de} | 65.33 ± 6.06 ^e | 45.56 ± 2.50 ^c |
| L19F | 38.45 ± 3.70 ^b | 32.44 ± 4.40 ^c | 19.44 ± 3.80 ^{ab} |
| L19G | 48.13 ± 3.63 ^c | 44.52 ± 4.66 ^d | 20.69 ± 2.95 ^b |
| L19H | 55.09 ± 0.71 ^{de} | 69.41 ± 1.45 ^e | 47.26 ± 1.44 ^c |

Note: Asterisks indicate mean comparisons that differ significantly ($P < 0.05$).

Autoaggregation analysis of LAB from human breast milk

LAB isolates from human breast milk showed auto-aggregation of over 10 %. However, six exceeded 40 %, as shown in Fig. 2. L19A, L19E, and L19H isolates had better auto-aggregation than others, while L19A performed best. Each LAB isolate has

a unique probiotic auto-aggregation ability determining its colonization and survival in the digestive tract. Based on the analyses, L19A, L19E, and L19H isolates have the best values, despite the statistical analysis indicating varying analysis tests. Therefore, the three isolates ought to analyze the 16S rRNA gene to determine the species' name to be used as a candidate for probiotics.

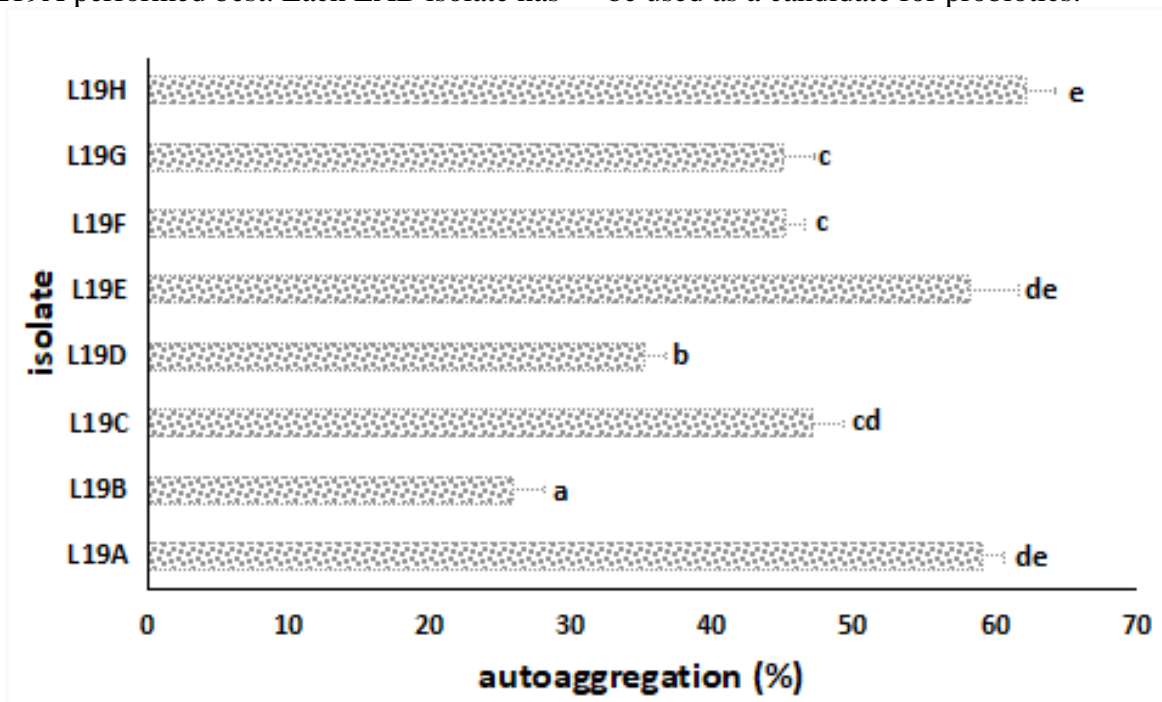


Fig. 2. The ability of lactic acid bacteria to adhere to the small intestinal mucosa; different superscripts showed a different mean comparison significantly ($P < 0.05$).

Coaggregation analysis of LAB from human breast milk

This study conducted co-aggregation testing between LAB isolates and two pathogenic bacteria with Gram-positive and negative characteristics, as shown in Table 2. The co-aggregation activity against *S. aureus* exceeded *E. coli* because the cell wall arrangement of Gram-positive bacteria contains more peptidoglycan and polysaccharides, with less lipid. The co-aggregation results indicated that isolate L19A had the highest ability with the five pathogenic bacteria. In the following table, the auto-aggregation test correlates with antimicrobial activity, where L19A has the strongest antimicrobial properties. Notably, the differences in species and strains strongly influence the co-aggregation ability. Out of eight isolates tests, only three exceeded 30 % co-aggregation values.

Meanwhile, the results of this study showed that L19A, L19E, and L19H had a co-aggregation value of 31.05 – 38.08 %, while the rest were below 30 %.

Table 2. Coaggregation ability (%) of LAB isolates from human breast milk on Gram-positive and negative pathogenic bacteria.

| BAL isolate | Coaggregation [%] | |
|-------------|------------------------------|--------------------------------|
| | <i>E. coli</i> ATCC 25922 | <i>S. aureus</i> ATCC 25923 |
| L19A | 19.00 ± 1.02 ^e | 38.08 ± 1.17 ^c |
| L19B | 2.86 ± 1.29 ^a | 11.46 ± 2.11 ^a |
| L19C | 7.76 ± 4.26 ^{ab} | 11.70 ± 1.11 ^a |
| L19D | 6.06 ± 1.06 ^{ab} | 16.36 ± 0.29 ^b |
| L19E | 15.69 ± 2.72 ^c | 31.05 ± 0.20 ^d |
| L19F | 12.85 ± 1.04 ^c | 17.83 ± 0.48 ^b |
| L19G | 1.62 ± 1.80 ^a | 19.16 ± 1.27 ^c |
| L19H | 17.31 ± 2.74 ^{cd} | 31.82 ± 0.61 ^d |

Note: Asterisks indicate mean comparisons that differ significantly ($P < 0.05$).

Antimicrobial activity of LAB from human breast milk

The ability of LAB to inhibit pathogenic microorganisms was analyzed based on the presence of hindrance of the zone marked. A clear part appeared around the isolated colonies after 48 hours combination, both in the cultures of *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans* ATCC 11778S. This indicated that LAB

isolated from human breast milk inhibited pathogenic microorganisms. However, the diameter of the inhibition zone formed by LAB isolates against *S. aureus*, *E. coli*, and *C. albicans* varied, as shown in Fig. 3. Quantitative analysis of the inhibition zone of the antimicrobial test of LAB isolates against pathogenic microbes showed that L19A, L19E, and L19H had higher values than L19B, L19C, L19D, L19F, and L19G. However, the L19H isolate had the best ability.

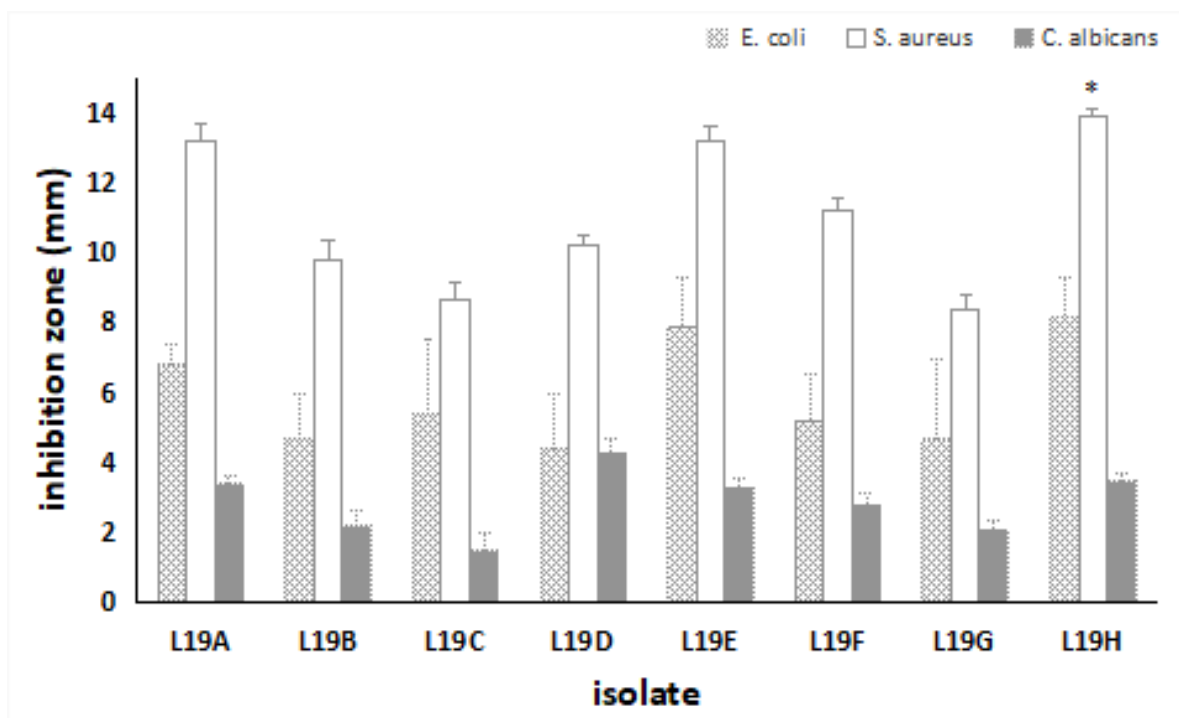


Fig. 3. Inhibition zone of LAB isolates antimicrobial test against pathogenic microbes; asterisks indicate mean comparisons that differ significantly ($P < 0.05$).

Direct PCR product analyzing

Based on resistance at low pH and bile salts, hydrophobicity, autoaggregation, co-aggregation, and inhibiting pathogenic microorganism’s properties, L19A, L19E, and L19H were the best indigenous probiotic candidates. However, the 16S rRNA gene sequence amplified in direct PCR LAB products obtained amplicons with a size of $\pm 1,585$ bp, as shown in Fig. 4. Molecular analysis of LAB isolates from human breast milk was carried out using partial sequences of the 16S rRNA. The partial sequences were suitable for analysis of bacteria down to the species level.

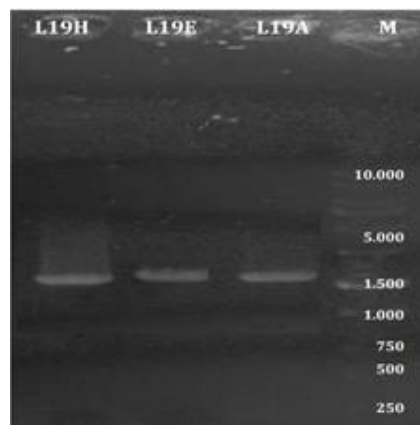


Fig. 4. The amplicon of partial sequences of 16S rRNA BAL gene from human breast milk; M – marker.

Data from sequences of L19A, L19E, and L19H isolates alongside those in the NCBI were analyzed using the BLAST program, as shown in Table 3. Therefore, this analysis indicates that the L19A isolate is similar to *L. casei* NWAUFU1544, while L19E and L19H are equivalent to *L. rhamnosus* JCM 8649 and *L. paracasei* 2281.

Table 3. BLAST results from isolates L19A, L19E, and L19H from human breast milk.

| Isolate | BLAST | | |
|---------|------------------------------|----------------|------------|
| | Species | Similarity [%] | Seq. Id. |
| L19A | <i>L. casei</i> NWAUFU1544 | 99 | MG551218.1 |
| L19E | <i>L. rhamnosus</i> JCM 8649 | 99 | AB690234.1 |
| L19H | <i>L. paracasei</i> 2281 | 99 | MT604755.1 |

Phylogenetic analysis

Phylogenetic tree reconstruction based on 16S rRNA gene sequences indicated that L19A, L19E, and L19H isolates forms two clades that can be separated from the out-group *Bacillus subtilis* (Fig. 5). L19A isolate has similarities with *L. casei* NWAUFU1544 up to 99 % with a genetic distance of 0.021. Also, the L19E isolate has a similarity value with *L. rhamnosus* JCM 8649 with a genetic distance of 0.00, meaning both are identical. Similarly, the L19H isolate has 99 % resemblance with *L. paracasei* 2281 with a genetic distance of 0.011. The bootstrap value for branching between isolates is 99 %, indicating stability.

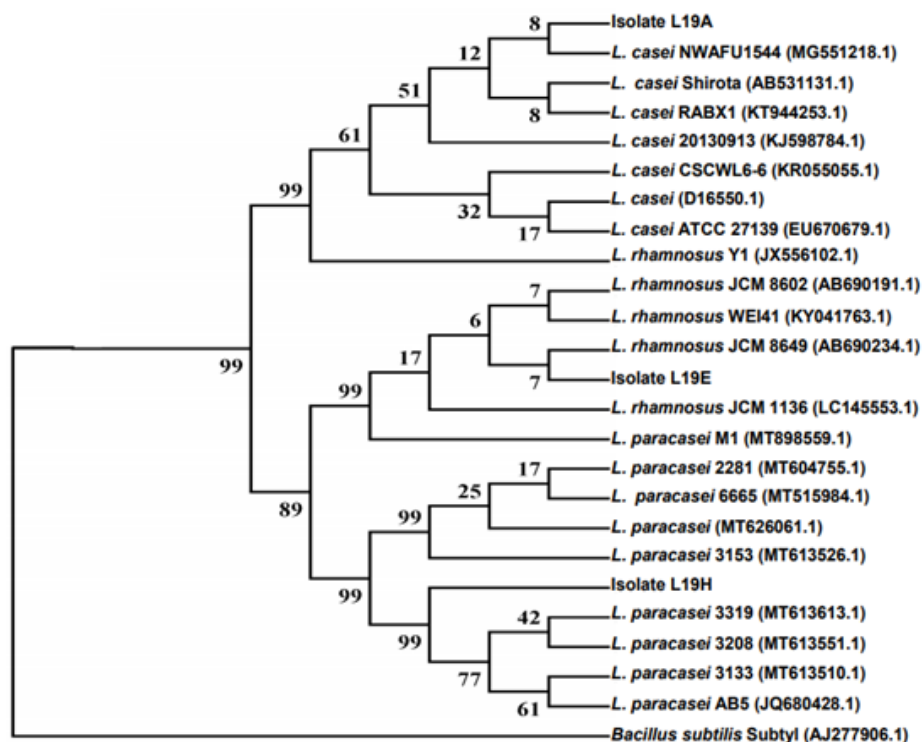


Fig. 5. Phylogenetic tree reconstruction of L19A, L19E, and L19H isolates with reference species based on the neighbor-joining method, Bootstrap 1,000x.

Discussion

The study aimed to investigate whether human breast milk can be the best indigenous probiotics. Several supporting methods were considered, including the ability of LAB at acidic pH and bile salts resistance, hydrophobic analysis, autoaggregation, coaggregation analysis,

antimicrobial, and gene analyses based on 16S rRNA. The initial analysis was conducted to determine whether the LAB isolates obtained could survive and grow in acid and bile salt conditions. The eight isolates showed positive results following Li *et al.* (2020) and Shehata *et al.* (2016). Some of the main requirements for microbes that function as probiotics include the resistance to low pH, ability

to grow on bile salts, the capability to colonize and possess antimicrobial activity. Tolerance of acidic pH and bile salts is an important characteristic of probiotic bacteria since its a requirement to pass through the digestive tract to reach the colon. LAB isolates adapt to low pH due to their ability to balance the internal cell pH, carried out by removing a proton (H^+) from the cell through the hydrolysis process of ATP (H^+ -ATPase). Also, the LAB has histidine decarboxylase and arginine deaminase, an important condition for acidic survival (Chen *et al.* 2019). Some lactic acid bacteria comprise of enzyme Bile Salt Hydrolase (BSH) to hydrolyze, which changes physicochemical properties to non-toxic.

Hydrophobicity is a significant supporting characteristic for probiotics. The nature of hydrophobicity determines the ability to adhere and auto-aggregate to various surfaces. According to Krausova *et al.* (2019), the hydrophobic nature of the LAB cell surface is demonstrated by its adhesion or affinity to xylene. Specifically, the hydrophobic tests help comprehend the surface properties of the bacterial cells critical for adhesion. In an acidic solvent, an affinity for chloroform indicates that the cell surface is an electron donor. Aggressiveness and hydrophobicity are among the components responsible for adhesion because the attachment mechanism is complex (Li *et al.* 2020). Based on the outcomes, a few isolates have a high affinity for xylene exceeding 50 %. However, it is considered moderate between 20 – 30 %, while below 20 % is indicated as negative (Guan *et al.* 2020). Moreover, a few isolates had a poor affinity for xylene, indicating a more hydrophilic surface with cell wall components such as phospholipids and polysaccharides (Krausova *et al.* 2019). The bacterial cell wall component is attached to the host, forming hydrophobic interactions. According to Li *et al.* (2020), hydrophobic properties result from protein on the cell surface and lipoteichoic acid, while polysaccharides produce hydrophilic properties. Bacteria are hydrophobic because their cell surface is negatively charged and associated with bacterial adhesion properties, which vary depending on the strain. Also, it is influenced by the plant medium, the age of the bacteria, and the bacterial cell's surface structure.

The hydrophobicity of probiotics depends on auto-aggregation. All LAB isolates samples from human breast milk indicated autoaggregation ability, shown by the results exceeding 10 %. According to Li *et al.* (2020), bacteria with autoaggregation ability below 10% are non-autoaggregation, important for probiotics in intestinal epithelial cells attachment. Subsequently, the autoaggregation nature of probiotic strains is important for attaching the intestinal epithelial cells (Krausova *et al.* 2019). Li *et al.* (2020) stated that cellular aggregation depends on species and the environment because aggregates stick to the mucosal surface, supporting probiotic bacteria survival in the intestine. Aggregates also hinder pathogenic bacteria from sticking to the intestine's surface, hence it is easy to extract from the digestive tract. The cellular aggregation mechanism is a complex interaction between cell surface components and secreted factors, such as protein, glycoprotein, teichoic acid, and lipoteichoic acid on the bacterial cell's surface, affecting the adhesion autoaggregation hydrophobicity (Krausova *et al.* 2019). However, the mechanism of these compounds in influencing adherence, autoaggregation, and hydrophobicity has not been clearly described. According to Krausova *et al.* (2019), the autoaggregation nature of a bacterium is influenced by the cell surface hydrophobicity.

The co-aggregation analysis of a probiotic bacterium correlates with its ability to form a barrier capable of preventing the colonization of pathogenic bacteria in the digestive tract (Li *et al.* 2020). Therefore, LAB that aggregates with the pathogenic bacteria is a beneficial trait as a probiotic (Campana *et al.* 2017). The co-aggregation activity against *S. aureus* was higher than *E. coli* because the cell wall arrangement of Gram-positive bacteria contains peptidoglycan, less lipid, and more polysaccharides. Teichoic acid is a water-soluble polymer that serves as positive ion transport, which demonstrating-positive bacterium that makes it easier to penetrate polar peptidoglycan than non-polar ones (Septiani *et al.* 2017). Co-aggregation testing is a suitable method to evaluate the interaction between LAB and pathogenic bacteria. LAB aggregates with pathogens expose antimicrobial compounds directly to pathogenic bacteria (Li *et al.* 2020). Therefore, LAB controls the surrounding pathogens

(Gao *et al.* 2019). To produce antimicrobial compounds, a mechanism for inhibiting the colonialization of pathogens in the digestive tract involves co-aggregation (Campana *et al.* 2017).

Antimicrobial tests supported the co-aggregation properties of inhibiting pathogenic microbial colonies based on the clear zone diameter formed. According to Li *et al.* (2015), differences in LAB antimicrobial activity against several tested microbes are based on differences in the structure of the cell walls of assessed microbes and the ability of concentrations variation of antimicrobial compounds to produce different inhibition zones. This inhibitory activity occurs due to the accumulation of primary metabolites in lactic acid, ethanol, and carbon dioxide. According to Kumar *et al.* (2016), this also occurred due to the presence of secondary metabolites in the form of hydrogen peroxide and bacteriocins. Amarantini *et al.* (2019) added that clear zones around LAB isolates are formed due to the activity of bactericidal antimicrobial compounds in the form of organic acids., which affect the cytoplasm of pathogenic microbial cells to become acidic and inhibit the transmembrane potential along with their substrate transport. According to Le *et al.* (2019), the antibacterial activity was determined based on the diameter of the bacterial inhibition zone against pathogenic microorganisms. Inhibition zone diameters ranging from 0 mm to 3 mm have weak antibacterial activity. The ones from 3 mm to 6 mm and over 6 mm contain good and strong antibacterial activities. These results are in line with Ren *et al.* (2018), which stated that LAB isolates inhibit *Escherichia coli* bacteria with an inhibition zone diameter of 7 – 14.3 mm.

Species analysis was performed for the top 3 isolates based on previous tests. Furthermore, molecular analysis of LAB isolates from human breast milk was carried out using partial sequences of the 16S rRNA. The partial sequences were suitable for analysis of bacteria down to the species level. This is in line with Bukin *et al.* (2019), which stated that the 16S rRNA gene's partial sequences could be used as a Universal Barcode of LAB. Isolates with the same 16S rRNA gene sequence above 97 % represent similar species (Edgar 2018). Wullur *et al.* (2020) stated that isolates are considered the same species when they have a maximum identity of 99 %. The bootstrap value for

branching between isolates is 99 %, indicating stability. According to (Tilahun *et al.* 2018), a branching value of 70 – 100 indicates that phylogenetic trees remain unchanged. Comparative sequencing analysis of the 16S rRNA gene is currently a common pathway for identifying and classifying bacteria. Since 1994, there have been strains with more than 97 % similarity of the 16S rRNA gene sequence, considered the same species. In 2018, the percentage level boundary for species was evaluated to be 98.65 % (Beye *et al.* 2018). However, in some cases, there were several adjacents in species, including the *Lactobacillus buchneri* group (*L. buchneri*, *L. kefir*, *L. parabuchneri*, and *L. parakefir*), *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*), *Lactobacillus plantarum* group (*L. fabifermentans*, *L. plantarum*, *L. paraplantarum*, and *L. pentosus*) and *Lactobacillus sakei* group (*L. curvatus*, *L. graminis*, and *L. sakei*). All these groups were indistinguishable using 16S rRNA gene sequencing due to a high degree of similarity up to 99 % between species (Fontana *et al.* 2018).

Lactobacillus casei, *paracasei*, and *rhamnosus* have close phylogenetic and phenotypic relationships considered to be in the *L. casei* group. According to Hill *et al.* (2018), members of this group are facultative heterofermentative, have a G+C DNA content of 45 – 47 %, with identical peptidoglycan types (L-Lys-DAsp). The widely recognized probiotic strains belonging to this group, such as *L. casei* strain Shirota and *L. rhamnosus* GG are used worldwide in fermented milk products or dietary supplements to improve the host's health (Orlando *et al.* 2016). Although this group comprises many strains of commercial value, their taxonomic status is still debatable. Also, strains of commercial value as probiotics, the taxonomic status, and nomenclature of the *L. casei* are still debatable due to the difficulty in identifying these three species using the most frequently used genotypic methodology of 16S rRNA gene sequencing (Huang *et al.* 2018).

Strain *L. casei* and *L. paracasei* are widely used as probiotics or symbiotic supplementation to improve clinical outcomes. The results of several clinical trials indicate that *L. casei* and *L. paracasei* improve patients' condition from various digestive diseases, chronic infections, obesity, and depressive disorders. Oral administration of probiotics

shortens the duration of acute diarrheal disease in children by about 1 day. The prevention of acute diarrhea in adults and children, using *L. rhamnosus* GG and *L. casei*, has proven effective in several specific doses (Lai *et al.* 2019; Li *et al.* 2019). According to Andriani *et al.* (2020), *L. casei* and *L. rhamnosus* probiotics are effective as a substitute for Antibiotic Growth Promoter (AGP) against total cholesterol, Low-Density Lipoprotein (LDL), and High-Density Lipoprotein (HDL).

The *L. casei* group remains in demand as a probiotic and is widely used. In recent years, many studies have focused on its application to health promotion or prevention of several digestive-related ailments and disorders. LCG could be used prophylactically or therapeutically in diseases associated with the gut microbiota. This group has been researched extensively on the stress response, essential to their survival, therefore applied as a probiotic. LCG from human breast milk is an indigenous probiotic candidate that needs further investigation to determine other abilities, physiology, and metabolite products.

Conclusion

Lactic acid bacteria from human breast milk have the ability of an indigenous probiotic. Subsequently, isolates L19A, L19E, and L19H had the best ability to withstand acidic pH and bile salts, hydrophobicity, autoaggregation, coaggregation, and inhibit pathogenic microorganisms. The 16S rRNA gene analysis showed that the three isolates belonged to the *L. casei* (LCG) group. L19A isolate was similar to *L. casei*, L19E was similar to *L. rhamnosus*, and L19H was similar to *L. paracasei*. These isolates can be used as indigenous probiotics.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Amarantini C, Satwika D, Budiarto TY, Yunita ER, Laheba EA (2019) Screening of antimicrobial-producing lactic acid bacteria isolated from traditional fish fermentation against pathogenic bacteria. *J. Phys. Conf. Ser.* 1397: 012045.

- Andriani AD, Lokapirnasari WP, Karimah B, Hidanah S, Al-Arif MA, Soeharsono S, Harijani N (2020) Efektifitas probiotik *Lactobacillus casei* dan *Lactobacillus rhamnosus* sebagai pengganti antibiotik growth promoter terhadap total kolesterol, low density lipoprotein dan high density lipoprotein Ayam Broiler. *J. Med. Vet.* 3: 114.
- Beye M, Fahsi N, Raoult D, Fournier PE (2018) Careful use of 16S rRNA gene sequence similarity values for the identification of Mycobacterium species. *New Microbes New Infect.* 22: 24-29.
- Bin Masalam MS, Bahieldin A, Alharbi MG, Al-Masaudi S, Al-Jaouni SK, Harakeh SM, Al-Hindi RR (2018) Isolation, molecular characterization and probiotic potential of lactic acid bacteria in Saudi Raw and fermented milk. *Evid.-based Complement. Altern. Med.* 2018: 7970463.
- Bukin YS, Galachyants YP, Morozov IV, Bukin SV, Zakharenko AS, Zemskaya TI (2019) The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Sci. Data* 6: 1-14.
- Campana R, Van Hemert S, Baffone W (2017) Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut Pathog.* 9: 1-12.
- Chen CC, Lai CC, Huang HL, Huang WY, Toh HS, Weng TC, Chuang YC, Lu YC, Tang HJ (2019) Antimicrobial activity of *Lactobacillus* species against carbapenem-resistant enterobacteriaceae. *Front. Microbiol.* 10: 1-10.
- Edgar RC (2018) Updating the 97 % identity threshold for 16S ribosomal RNA OTUs. *Bioinf.* 34: 2371-2375.
- FAO/WHO (2006) Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Probiotics in food: Health and nutritional properties and guidelines for evaluation: Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacte, Rome, Italy.
- Fontana A, Zacconi, C, & Morelli L (2018) Genetic signatures of dairy *Lactobacillus casei* group. *Front. Microbiol.* 9: 1-9.
- Ganjbaksh SE (2017) The effect of probiotics on immune system. *J. Bacteriol. Mycol.* 5: 319-320.
- 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.* 82: 441-453.
- Guan C, Chen X, Jiang X, Zhao R, Yuan Y, Chen D, Zhang C, Lu M, Lu Z, Gu R (2020) *In vitro* studies of adhesion properties of six lactic acid bacteria isolated from the longevous population of China. *RSC Adv.* 10: 24234-24240.
- Hill D, Sugrue I, Tobin C, Hill C, Stanton C, Ross RP (2018) The *Lactobacillus casei* group: History and health related applications. *Front. Microbiol.* 9: 1-12.
- Huang CH, Li SW, Huang L, Watanabe K (2018) Identification and classification for the *Lactobacillus casei* group. *Front. Microbiol.* 9: 1-13.
- Jamyuang C, Phoonlapdacha P, Chongviriyaphan N, Chanput W, Nitisinprasert S, Nakphaichit M (2019)

- Characterization and probiotic properties of Lactobacilli from human breast milk. *3 Biotech* 9: 398.
- Jatmiko YD, Howarth GS, Barton MD (2017) Assessment of probiotic properties of lactic acid bacteria isolated from Indonesian naturally fermented milk. *AIP Conf. Proc.* 1908: 1-14.
- Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, Sodergren E, Weinstock GM (2019) Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat. Commun.* 10: 1-11.
- Kaškonienė V, Stankevičius M, Bimbraitė-Survilienė K, Naujokaitytė G, Šernienė L, Mulkytė K, Malakauskas M, Maruška A (2017) Current state of purification, isolation and analysis of bacteriocins produced by lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 101: 1323-1335.
- Krausova G, Hyrslova I, Hynstova I (2019) *In vitro* evaluation of adhesion capacity, hydrophobicity, and auto-aggregation of newly isolated potential probiotic strains. *Fermentation* 5: 100.
- Kumar M, Dhaka P, Vijay D, Vergis J, Mohan V, Kumar A, Kurkure NV, Barbuddhe SB, Malik SVS, Rawool DB (2016) Antimicrobial effects of *Lactobacillus plantarum* and *Lactobacillus acidophilus* against multidrug-resistant enteroaggregative *Escherichia coli*. *Int. J. Antimicrob. Agents.* 48: 265-270.
- Lai H, Chiu C, Kong M, Chang C (2019) Probiotic *Lactobacillus casei*: Effective for managing childhood diarrhea by altering gut microbiota and attenuating fecal inflammatory markers. *Nutrients* 11: 1150.
- Le NTT, Bach LG, Nguyen DC, Le THX, Pham KH, Nguyen DH, Thi TTH (2019) Evaluation of factors affecting antimicrobial activity of bacteriocin from *Lactobacillus plantarum* microencapsulated in alginate-gelatin capsules and its application on pork meat as a bio-preservative. *Int. J. Environ. Res. Public Health* 16: 1017.
- Li D, Ni K, Pang H, Wang Y, Cai Y, Jin Q (2015) Identification and antimicrobial activity detection of lactic acid bacteria isolated from corn stover silage. *Asian-Australas. J. Anim. Sci.* 28: 620-631.
- Li M, Wang Y, Cui H, Li Y, Sun Y, Qiu HJ (2020) Characterization of lactic acid Bbacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. *Front. Vet. Sci.* 7: 1-10.
- Li Y, Xu H, Ye JZ, Wu WR, Shi D, Fang DQ, Liu Y, Li LJ (2019) Efficacy of *Lactobacillus rhamnosus* GG in treatment of acute pediatric diarrhea: A systematic review with meta-analysis. *World J. Gastroenterol.* 25: 4999-5016.
- Łubiech K, Twarużek M (2020) Lactobacillus bacteria in breast milk. *Nutrients* 12: 1-13.
- Maldonado Galdeano C, Cazorla SI, Lemme Dumit JM, Vélez E, Perdigón G (2019) Beneficial effects of probiotic consumption on the immune system. *Ann. Nutr. Metab.* 74: 115-124.
- Michel E, Monfort C, Deffrasnes M, Guezenc S, Lhomme E, Barret M, Sicard D, Dousset X, Onno B (2016) Characterization of relative abundance of lactic acid bacteria species in French organic sourdough by cultural, qPCR and MiSeq high-throughput sequencing methods. *Int. J. Food Microbiol.* 239: 35-43.
- Orlando A, Linsalata M, Russo F (2016) Antiproliferative effects on colon adenocarcinoma cells induced by co-administration of vitamin K1 and *Lactobacillus rhamnosus* GG. *Int. J. Oncol.* 48: 2629-2638.
- Ren D, Zhu J, Gong S, Liu H, Yu H (2018) Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. *BioMed Res. Int.* 2018: 5416725.
- Rocha-Ramírez LM, Pérez-Solano RA, Castañón-Alonso SL, Moreno Guerrero SS, Ramírez Pacheco A, García Garibay M, Eslava C (2017) Probiotic *Lactobacillus* strains stimulate the inflammatory response and activate human macrophages. *J. Immunol. Res.* 2017: 1-14.
- Schirmer M, Smeekens S., Vlamakis H, Jaeger M, Oosting M, Franzosa EA, Jansen T, Jacobs L, Bonder MJ, Kurilshikov A, Fu J, Joosten LAB, Zhernakova A, Huttenhower C, Wijmenga C, Netea MG, Xavier RJ (2016) Linking the human gut microbiome to inflammatory cytokine production capacity. *Cell* 167: 1125-1136.
- Septiani Dewi EN, Wijayanti I (2017) Antibacterial activities of seagrass extracts (*Cymodocea rotundata*) against *Staphylococcus aureus* and *Escherichia coli*. *Indones. J. Fish. Sci. Technol.* 13: 1-6.
- Shehata MG, El-Sohaimy SA, El-Sahn MA, Youssef MM (2016) Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Ann. Agric. Sci.* 61: 65-75.
- Sinkiewicz G, Ljunggren L (2008) Occurrence of *Lactobacillus reuteri* in human breast milk. *Microb. Ecol. Health Dis.* 20: 122-126.
- Tilahun B, Tesfaye A, Muleta D, Bahiru A, Terefework Z, Wessel G (2018) Isolation and molecular identification of lactic acid bacteria using 16s rRNA genes from fermented teff (*Eragrostis tef* (Zucc.)) dough. *Int. J. Food Sci.* 2018: 1-7.
- Wang D, Liu W, Ren Y, De L, Zhang D, Yang Y, Bao Q, Zhang H, Menghe B (2016) Isolation and identification of lactic acid bacteria from traditional dairy products in Baotou and Bayannur of Midwestern Inner Mongolia and q-PCR analysis of predominant species. *Korean J. Food Sci. Anim. Resour.* 36: 499-507.
- Woodman ME (2008) Direct PCR of intact bacteria (Colony PCR). *Curr. Protoc. Microbiol.* 9: A.3D.1-A.3D.6.
- Wullur S, Napitupulu H, Wantania LL, Ginting EL, Warouw V, Tilaar S, Tallei TE, Rumengan IFM (2020) Molecular identification of bacteria isolated from culture medium of rotifer fed on fishery waste diet. *J. Biol. Biodivers.* 21: 2735-2740.
- Yeoh YK, Zuo T, Lui GCY, Zhang F, Liu Q, Li AYL, Chung ACK, Cheung CP, Tso EYK, Fung KSC, Chan V, Ling L, Joynt G, Hui DSC, Chow KM, Ng SSS, Li TCM, Ng RWY, Yip TCF, Ng SC (2021) Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 70: 698-706.