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Determination of antimicrobial, pH, bile salt, and gastric juice tolerance properties of *Lactobacilli* isolated from human milk

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Abstract

Human milk is a complex biological fluid, and a source of nutrients and probiotics which plays a vital role in the growth and development of infants. Probiotics are helpful bacteria which are good for human health. The primary bacterial genera which are gaining status as probiotics are *Lactobacillus, Lactococcus*, and *Bifidobacterium*. The present work aimed to isolate, identify, and determine the probiotic potential of *Lactobacillus* bacteria from human milk. A total of 70 samples of human milk were collected from different lactating mothers. The milk samples were inoculated on the De Man, Rogosa, and Sharpe (MRS) agar plates to observe the growth of *Lactobacillus* bacteria. The bacteria were identified based on their morphology, culture characteristics, and biochemical properties. Isolated bacteria were evaluated for probiotic properties in which, tolerance to acidic pH, bile salts, and gastric juice as well as antibacterial activity and antibiotic susceptibility were determined. Out of the 70 milk samples, 57 were positive for *Lactobacillus*. Out of the positive sample, 10.5% of the samples tolerated acidic pH and high bile salt concentration, but a significant difference was obtained for gastric juice. In the antibacterial activity, *Pseudomonas* showed no action against *Lactobacillus*. In antibiotic susceptibility, the test isolates were resistant to penicillin. The present work proved the presence of beneficial bacteria in the human milk. Isolated *Lactobacillus* exhibited significant antibacterial activity against pathogenic bacteria, and tolerance to acidic pH, bile salt, and gastric juice. Therefore, human milk could be a good source of probiotics for infants.

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Introduction

Probiotics have been used for a long time as food constituents in foods and feeds without any side effects (Fernandez *et al.,* 2008). Probiotics are adequate as they are naturally found in the intestine of healthy individuals, and can withstand the chemical and physical hurdles existing in the gastrointestinal tract such as decreased pH, bile salts, and gastric juice (Piano *et al.,* 2006).

Lactobacillus and *Bifidobacterium* are two bacterial genera which are frequently used as probiotics, and are usually present in animal and human colon (Rajoka *et al.,* 2017). These bacteria are not pathogenic and are useful for the host's health (Gad El-Rab *et al.,* 2011). In humans and animals, they dominate the microflora of the upper gastrointestinal tract, as well as the oral cavity and lower intestine (Bourlieu *et al.,* 2020). On plants, *Lactobacillus* occur in a minimal number as plants are being dominated by *Leuconostoc*. *Leuconostoc* are naturally found in plants, milk, fermenting vegetables, wines, meats, and dairy products (Picard *et al.,* 2005; Rajoka *et al.,* 2018a).

In breastfed infants, *Lactobacillus* frequently prevail (Premkumar *et al.,* 2020). However, based on the feeding method, it has also been indicated that enterococci, coliforms, and *Bacteroides* predominately colonise the infants' intestinal tract (Brady *et al.,* 2000). Furthermore, preterm babies are mainly liable to strange colonies. The amalgamation of antibiotic usage delays the beginning of feedings and will expose babies to different microorganisms that inhabit the neonatal concentrated unit, and may additionally cause unusual colonisation (Soll, 2010).

Probiotic supplements have been suggested to improve continuous feeding and avoid sicknesses and nosocomial contaminations in preterm babies (Caglar *et al.,* 2005). There is a growing potential of

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L. acidophilus as probiotic species which is naturally present in human milk, together with other species such as *L. gasseri, L. rhamnosus, L. salivarius, L. fermentum*, and *L. plantarum* (Caplan and Jilling, 2000).

In recent years, approximately 200 diverse species of *Lactobacillus* have been recognised in human milk (Rajoka *et al.,* 2018b). There is a great interest in some probiotic species as previously mentioned. Previous study has also reported that many nutrients are present in human milk, which help in digestion (Beasley and Saris, 2004).

Probiotic bacteria may induce special effect in the immune system of their hosts. Previous study has documented the outcomes of probiotics on immunity stimulation (Rajoka *et al.,* 2019). *In vitro* and *in vivo* researches were done on mice, and a few trials were run on humans (Camilia et al., 2016). Probiotics affect immunity in extraordinary means, for example, the generation of cytokines, the increase in secretory IgA, and the stimulation of macrophage concentrations (Fernandez *et al.,* 2013). The aim of the present work was therefore to isolate and identify *Lactobacillus* from human milk, and to evaluate their probiotic potential.

Materials and methods

A total of 70 healthy human milk samples were taken in groups; 35 samples were taken from 25 - 30 years old lactating mothers, and 35 samples from 30 - 35 years old lactating mothers. Based on antibiotic history, 35 samples were taken from lactating mothers with no antibiotic history, and 35 samples were taken from lactating mothers who received antibiotics two months prior to the milk collection. The milk samples were collected in sterilised 15 mL Falcon tubes using the hand-expression method, placed in an icebox during transportation to the laboratory, and then stored at 4°C upon arrival. Next, 1 mL of milk was 10-fold serially diluted, and 0.1 mL aliquots of appropriate dilution was poured onto the De Man, Rogosa, and Sharpe (MRS) agar plates. Plates were incubated at 37°C for 24 - 48 h (Carr *et al.,* 2002). The Gram-staining and biochemical tests (catalase test, Kliger's iron test, sugar fermentation test, and casein digestion test) were performed for further identification (Smibert and Krieg, 1994; Neamtu *et al.,* 2014).

Catalase test

A fresh culture was smeared onto a sterile glass slide, and 3% hydrogen peroxide was dropped and gently mixed (Mannan *et al.,* 2017). Immediate production of froth / bubble indicates positive result, and vice versa. *E. coli* and *S. aureus* served as negative and positive control, respectively.

Kliger's iron agar

KIA test was used for the determination of lactose and glucose utilisation. A fresh culture was streaked on the slant surface, and stabbed through the agar (Mannan *et al.,* 2017). Results were recorded after incubation at 37°C for 24 h. For glucose fermentation, the results showed the production of gas, H_2S, and colour change (acid in butt and alkaline in slant). Meanwhile, for lactose fermentation, acid was in slant, and alkaline was in butt. For both glucose and lactose, the acid was in both slant and butt. The H_2S production caused a blackening of the medium, and bubbles appeared due to the production of gas. *S. aureus* served as a positive control.

Sugar fermentation test

One percent (w/v) sugar in MRS broth was used along with glucose, fructose, sucrose, xylose, and lactose (Mannan *et al.,* 2017). Phenol red served as the indicator. To begin, 10 mL MRS broth was poured into the tube. In each tube, Durham's tube was invertedly inserted. Fresh culture was then added, and the results were recorded after incubation at 37°C for 24 h. Non-inoculated medium served as negative control. The change in the colour and formation of gas showed a positive test.

Casein digestion test

MRS agar plate with 1% skimmed milk was used to perform this test (Smibert and Krieg, 1994). Fresh bacterial cultures were streaked, and results were recorded after incubation at 37°C for 24 h. Clear zones of inhibition showed protease activity. *E. coli* and *Pseudomonas* spp. served as negative and positive control, respectively.

Tolerance to low pH

The isolates were incubated overnight at 37°C in MRS broth. Next, 0.1 mL aliquots of each strain were maintained at pH 7 to 2 by adding a few drops of HCl or NaCl, and then incubated for 3 h at 37°C. Cultures were diluted by 10-fold serial dilution in 0.1% distilled water, and then enumerated by pour plate method to check the viability of bacteria after every hour for 3 h. Bacterial isolates were monitored three times by measuring the absorbance using a spectrophotometer (Nova Spec II, Pharmacia) at 600 nm (Erkkila and Petaja, 2000).

Tolerance to bile salts

Selected bacteria were cultured in MRS

broth overnight at 37°C. Bile extract powder (Oxoid) was dissolved in peptone water to make bile solution. A bile solution was passed through a sterilised filter size of 4 µm. Then, the bile solution was mixed with two of the cultures to attain 0.3% concentration. While the other culture of bacteria served as a control with 0% bile concentration, *Lactobacillus* bacteria were diluted by 10-fold serial dilution prepared in 0.1% distilled water, and then enumerated by pour plate method to check the viability of bacteria after every hour for 3 h. Bacterial isolates were monitored three times by measuring the absorbance using a spectrophotometer (Nova Spec II, Pharmacia) at 600 nm (Hyronimus *et al.,* 2000).

Tolerance to gastric juice

Gastric juice tolerance was evaluated as described by Dunne *et al.* (2001). Freshly prepared gastric juice included 3% pepsin, 0.5% NaCl, and pH 0.2. Acidic pH was attained with 1 M HCl/0.5 M NaOH. Freshly prepared 3 mL gastric juice and 1 mL phosphate buffer saline was mixed with the culture of bacteria, and all the test tubes were mixed with gastric juice. All the tubes were anaerobically incubated at 37°C. The bacterial count was determined on nutrient agar by plate count technique after 30, 60, 90, 120, 150, and 180 min of incubation (Papamanol *et al.,* 2003). Results were calculated using Eq. 1, and expressed in CFU/mL:

Bacterial count = Average number of total colonies × dilution factor (Eq. 1)

Antibacterial activity

Antibacterial activity of isolated *Lactobacillus* against selected bacterial pathogens was evaluated by the well diffusion method. The bacterial pathogens were *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. For this purpose, 24 h old culture of bacterial pathogens was inoculated separately on Mueller Hinton (MH) agar plates at 37°C. Wells (8 mm diameter) were cut off the agar plates, and 100 µL of an overnight culture of *Lactobacillus* was filled into each well. The plates were incubated at 37°C for 24 h, and the diameter of inhibition zones was measured in mm after incubation. The inoculated plate with only *Lactobacillus* culture served as negative control (Poonam *et al.,* 2016).

Antibiotic susceptibility test

For antibiotic susceptibility test, *Lactobacillus* were assessed by the disc diffusion method against frequently used antibiotics, as described by Bao *et al.* (2010). A loopful bacterial culture in MRS broth was incubated for 3 h at 37°C until the turbidity was visible. Bacteria were then streaked onto the MH agar plate surface. The streaked plates were left to dry for 5 min to remove excess humidity. The antibiotic discs were placed on the agar plate and placed in an anaerobic incubator at 37°C for 24 h in upside position. The diameter of the zones of inhibition was determined. The experiment was conducted in replicates, and the results were reported in accordance with the CLSI manual (Ammor *et al.,* 2007).

Statistical analysis

Microsoft Office Excel was used to analyse the data, and the data were further confirmed through Minitab 15 software. All values were stated as the mean ± SD. Significance of means was determined at p value < 0.05.

Results

All isolates were confirmed by their colony characteristics on MRS agar; the observed isolated colonies of *Lactobacillus* were whitish and creamy in colour, as shown in Figure 1. Out of 70 samples, 57

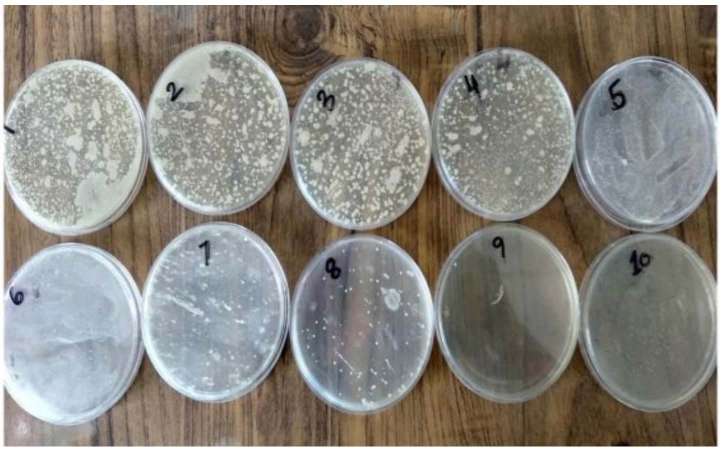

Figure 1. Colonies of isolated *Lactobacillus* after incubation on plate count agar.

lactic acid bacteria were isolated from human milk. Biochemical characteristics showed that out of 57 positive samples, 42 were catalase, indole, VP, citrate negative, and methyl red positive.

Tolerance to acidic pH

Out of 57 isolates, six isolates survived in pH 2, 3, and 3.5. At different pH values, the tolerance level was significantly variable. The test was performed repeatedly for six times. The tolerance towards bile salts assays was observed, and the growth was about 2.37×10^8 CFU/mL with the average of 2.51×10^8, as shown in Table 1.

Table 1. Colony-forming unit (CFU)/mL of *Lactobacillus* after incubation at different pH conditions.

Group	CFU/mL
Group 1	$2.3700 \times 10^8 \pm 0.0420^A$
Group 2	$2.5183 \times 10^8 \pm 0.0757^B$

Values are mean ± SD. Means with different superscripts are statically significantly different. Group 1 had pH = 7, and Group 2 had pH = 2.

Tolerance to bile salts

All of the six isolates could survive the presence of 0.3% bile salts concentration. The plate count technique was used to test the tolerance to bile salts from 24 h isolated culture of *Lactobacillus*. This experiment was performed repeatedly for six times. The mean and average value was 1.01×10^9 unit evaluated and matched with the control. The tolerance to bile salt concentrations was 1.10×10^9, as shown in Table 2. This confirms that *Lactobacillus* expressed tolerance to bile salts.

Table 2. Colony-forming unit (CFU)/mL of *Lactobacillus* after incubation at different bile salt conditions.

Group	CFU/mL
Group 1	$1.018 \times 10^9 \pm 0.0309^A$
Group 2	$1.1012 \times 10^9 \pm 0.0757^B$

Values are mean ± SD. Means with different superscripts are statically significantly different. Group 1 had no bile salts, and Group 2 had bile salts.

Tolerance to gastric juice

Gastric juice tolerance of six isolated *Lactobacillus* was performed after 30, 60, 90, 120, 150, and 180 min of incubation, and the mean average value was evaluated and matched with the control value. The mean value of the CFU/mL used for control was 2.03×10^{10}, and for gastric juice was 2.43×10^{10}, as shown in Table 3. This confirms that *Lactobacillus* expressed tolerance to gastric juice.

Table 3. Colony-forming unit (CFU)/mL of *Lactobacillus* after incubation at different gastric juice conditions.

Group	CFU/mL
Group 1	$2.0377 \times 108 \pm 0.0422^A$
Group 2	$2.4383 \times 108 \pm 0.0257^B$

Values are mean ± SD. Means with different superscripts are statically significantly different. Group 1 had no gastric juice, and Group 2 had gastric juice.

Antibacterial activity

Lactobacillus exhibited inhibitory activity of varying degrees against *P. aeruginosa, K. pneumoniae, E. coli,* and *S. aureus* as shown in Figure 2. It was observed that *Lactobacillus* presented a very strong inhibitory zone against *E. coli.* Moderate and strong inhibitory responses were determined against *K. pneumoniae* and *S. aureus*, respectively, while there was no response against *P. aeruginosa*.

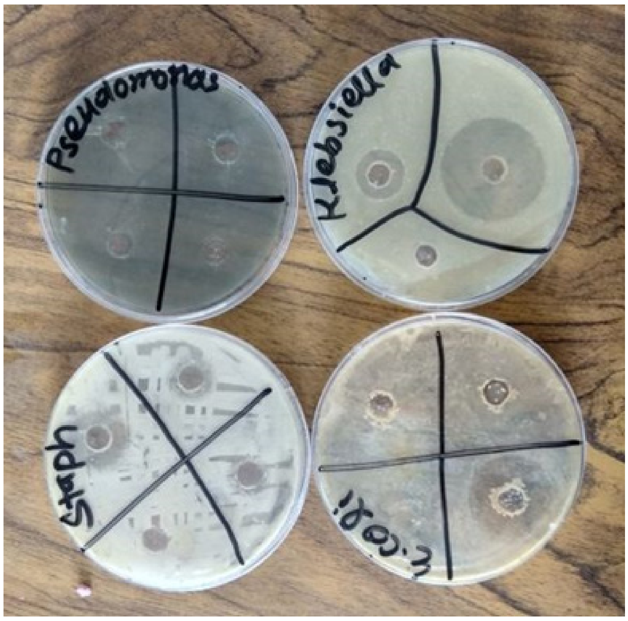

Figure 2. Antibacterial activity exhibited by the isolated *Lactobacillus* against pathogenic bacteria using the well diffusion method.

Antibiotic susceptibility

The antibiotic profiling of *Lactobacillus* was evaluated against generally used antibiotics by the disc diffusion method. The interpretation was abbreviated as resistant (R), intermediate (I), or sensitive (S). Inhibition zones were evaluated and measured by CLSI manual standards. *Lactobacillus* presented resistance against penicillin, intermediate against amoxicillin and kanamycin, and sensitive against gentamycin, oxytetracycline, ceftriaxone, pipemidic acid, tobramycin, and trimethoprim-sulphamethoxazole (Figure 3).

Figure 3. Antibiotic susceptibility exhibited by isolated *Lactobacillus* against pathogenic bacterium using the disc diffusion method. (a) penicillin, (b) gentamycin, (c) oxytetracycline, and (d) ceftriaxone.

Discussion

The probiotic bacteria isolated in the present work was from human milk, and included *Lactobacillus, Lactococcus*, and *Bifidobacterium*. Many of the LAB strains from this source has been observed, and have previously been proven to retain the properties of probiotic together with a broad-spectrum inhibition of newborn pathogenic bacteria by the production of antimicrobial compounds such as natural acids, bacteriocins, or hydrogen peroxide (Luo *et al.,* 2012).

The microbiota of the gastrointestinal tract could survive the pancreatic enzymes, gastric acid, peristalsis, pH, and bile salts. This is why it is important to screen the tolerance of the probiotic strains of interest. Martin *et al.* (2003) concluded that *Lactobacillus* could survive at a pH ranged between 2 and 3. They also determined that *Lactobacillus* could tolerate bile salt at a concentration of 0.3%. Although not all of the isolated *Lactobacillus* in the present work exhibited these characteristics, the results were within the range.

The antibacterial test verified that *Lactobacillus* was capable of inhibiting pathogenic bacterial growth. This agrees with the results of previous works. Klinberg and Gottschalk (1987) reported that *Lactobacillus* inhibited *E. coli* and *S. aureus*. Nieto-Lozano *et al.* (2002) and Papamanol *et al.* (2003) also reported the inhibitory effect of *Lactobacillus* against *S. aureus*.

In terms of antibiotic susceptibility, the isolated *Lactobacillus* showed resistance to penicillin, intermediate to amoxicillin and kanamycin, and susceptible to gentamycin, oxytetracycline, ceftriaxone, tobramycin, pipemidic acid, and trimethoprim-sulfamethoxazole. D'Aimmo *et al.* (2007) reported that *L. acidophilus, L. casei,* and *L. delbrueckii* were resistance to kanamycin and spectinomycin, and susceptible to penicillin and clindamycin. Klare *et al.* (2007) reported that *L. plantarum, L. paracasei*, and *L. rhamnosus* were susceptible to penicillin, chloramphenicol, and ampicillin. Huys *et al.* (2006) reported that *L. plantarum* was susceptible to clindamycin and ampicillin, and resistant to gentamycin than streptomycin.

Conclusion

The present work demonstrated the presence of beneficial bacteria in human milk. The possible source for these bacteria can be originated from the mother's gut. Studies suggested that immune cells use the lymphatic system to carry the bacteria over the body, and ends at the mammary gland, and into the milk. The isolated *Lactobacillus* exhibited significant antibacterial activity against the pathogenic bacteria, and tolerance to acidic pH, bile salt, and gastric juice. Therefore, human milk could be a good source of probiotics for infants.

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