



Research Article

Dairy-Associated Lactic Acid Bacteria: Isolation, Characterization, and Probiotic Evaluation

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Abstract

Probiotics are microorganisms that confer beneficial effects on their host. This study investigated the probiotic properties of lactic acid bacteria (LAB) isolated from cow milk and milk products (yogurt and sweets). Twelve isolates were screened based on their ability to form clear zones on Mann, Rogosa, and Sharpe (MRS) agar supplemented with 0.5% CaCO₃. These isolates were identified based on their cultural, morphological, and biochemical characteristics, all of which confirmed their classification under the genus *Lactobacillus*. The probiotic potential of these isolates was evaluated based on their resistance to acidic conditions (pH 3), tolerance to bile salts (0.3%), ability to grow at different temperatures, salt tolerance, antibiotic susceptibility, and antimicrobial activity. All isolates exhibited resistance to pH 3 and pH 5. Except for *L. delbrueckii* P18 and *L. delbrueckii* P20, all isolates also showed resistance to pH 10. Furthermore, all isolates tolerated bile salt concentrations ranging from 0.3% to 3%. They demonstrated the ability to grow at 10°C, 45°C, and 60°C. Optimal growth was observed at 4% NaCl, while growth was limited at 8% and 10% NaCl. Antibiotic susceptibility testing revealed that all isolates were resistant to metronidazole and vancomycin. Notably, *L. casei* P4 and *L. acidophilus* P9 exhibited resistance to all eight tested antibiotics. In addition, all isolates displayed antimicrobial activity against four pathogenic bacteria: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 35150, *Salmonella enteritidis* ATCC 13076, and *Listeria monocytogenes* ATCC 7644. The findings of this study suggest that these LAB strains with probiotic potential could contribute to maintaining and improving intestinal microbial flora. Their resistance to certain antibiotics and their antimicrobial activity highlight their potential for both preventive and therapeutic applications.

Keywords

Probiotics, Lactic Acid Bacteria, Antimicrobial Activity, Antibiotic Resistance, Milk and Dairy Products

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1. Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1]. Probiotics belong to a group of microbes that may directly enhance resistance against intestinal pathogens and aid in disease prevention. Probiotic bacteria can produce various compounds that inhibit pathogen growth, including organic acids (lactic and acetic acids), bacteriocins, and reuterin. Lactobacilli are known to produce many types of bacteriocins such as acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin, and lactobrevin [2]. In general, probiotic strains should exhibit desirable antibiotic resistance and sensitivity patterns. They may also act antagonistically toward potentially pathogenic microorganisms and have metabolic activities beneficial to the host's well-being. Intrinsically antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota has been disrupted or significantly reduced due to the administration of antimicrobial agents [3].

Probiotics are beneficial bacteria that positively influence intestinal microflora balance, inhibit harmful bacterial growth, promote digestion, boost immune function, and increase resistance to infection [4]. Lactic Acid Bacteria (LAB) are the most common type of microorganisms used as probiotics. Strains of the genera *Lactobacillus* and *Bifidobacterium* [5], as well as *Enterococcus* [6], are widely used and well-studied probiotic bacteria. These microorganisms have been considered probiotics based on several criteria, including their beneficial effects on the host, non-pathogenicity [7], and their ability to survive transit through the gastrointestinal (GI) tract [8-10].

Infectious diseases pose a significant global health concern, with gastrointestinal infections causing substantial morbidity and mortality each year [11]. There is growing evidence that probiotics are beneficial in treating gastrointestinal disturbances such as diarrhea, dysentery, and typhoid [12]. The rise in antibiotic-resistant bacteria has prompted scientists to explore the prophylactic and therapeutic potential of probiotics as alternatives to antibiotics [13]. Various commercial probiotic preparations are available in capsule, liquid, gel, and powdered forms, claiming to prevent infectious diseases. These preparations often include *Lactobacillus* alone or in combination with *Streptococcus* or *Saccharomyces*, demonstrating beneficial effects [14].

Milk and milk products are commonly associated with probiotic bacteria, providing supplements that help maintain a beneficial intestinal balance [15]. There is significant interest in identifying potential starter organisms from naturally occurring LAB in raw milk and raw milk products [16]. Novel probiotic strains can be discovered by exploring biodiversity in various ecological niches or through genetic modification of known production strains. Some key characteristics of these microorganisms include their ability to produce acid at a high and predictable rate, proteolytic activity, extracellular

polymeric substance (EPS) synthesis, and antimicrobial compound production, which are essential for fermented milk starter strains [17]. The present study aims to isolate, screen, identify, and characterize LAB from cow milk and milk products.

2. Materials and Methods

2.1. Mann, Rogosa and Sharpe (MRS) Agar Media

MRS agar media was prepared by adding bacteriological agar and CaCO_3 at a concentration of 1.5% (w/v) and 0.5% (w/v) respectively with MRS broth. The media composition of MRS broth (Oxoid) is as follows (g/l): Pepton, 10.0 g/l; Lab-Lemco powder, 8.0 g/l; Yeast Extract, 4.0 g/l; Glucose, 20.0 g/l; Sorbitan mono-oleate, 1 ml; Di-potassium hydrogen phosphate, 2.0 g/l; Sodium acetate $3\text{H}_2\text{O}$, 5.0 g/l; Tri-ammonium citrate, 2.0 g/l; Magnesium sulphate $7\text{H}_2\text{O}$, 0.2 g/l; Manganese sulphate $4\text{H}_2\text{O}$, 0.05 g/l; Distilled water, 1000 ml.

2.2. Sampling Area and Sample Collection

Cow milk and milk products (yogurt and sweet) samples were collected from different locations of Pabna district.

2.3. Milk and Milk Product Sampling

Cow milk samples were collected in pre-sterilized plastic bottles. Yogurt and Sweet (Chaana) samples were collected in gamma radiation sterilized polythene bag. All the samples were sealed and labeled and kept in ice box just after the collection. Samples were transported to the laboratory as early as possible for microbiological analysis.

2.4. Enumeration of Probiotic Lactic Acid Bacteria (LAB)

For enumeration of total lactic acid bacteria, 1 ml cow milk or 1 g milk products (Yogurt and Sweet) were mixed with 9 ml sterile distilled water. The samples were then diluted up to 10^{-7} fold. One ml of each dilution was inoculated on MRS agar medium supplemented with 0.5% CaCO_3 by spread plate technique. To obtain enriched cultures, milk (10 ml) and milk products (10 g) were inoculated in 90 ml MRS broth and incubated at 37°C for 48 h under static conditions. This provided suitable conditions for the growth of facultative anaerobic microorganisms and made it unnecessary to incubate the samples anaerobically. After 48 h of incubation, one ml of this culture was then inoculated in MRS agar plate supplemented with 0.5% CaCO_3 by spread plate technique.

Only the colonies which showed clear zones were enumerated for counting Total Lactic Acid Bacteria (TLAB).

2.5. Purification and Preservation of the Isolates

The isolated organisms were purified through repeated subculture method. MRS agar was used as media. For short term preservation of organism, single purified colony was inoculated on to MRS agar plate and incubated at 37°C for 48 h. Then the plate was tightly covered by parafilm and stored at 4°C. For long term preservation of organism, single purified colony was inoculated on to MRS broth slant containing 30% (v/v) glycerol and stored at -30°C.

2.6. Identification of the Bacterial Isolates

Bacterial cultural, morphological and biochemical procedures were studies for the bacterial identification.

2.7. Measurement of Cell Growth

The cell concentration was estimated by optical density (OD) at 600 nm using UV-Visible Spectrophotometer (Shimadzu 1601, Japan). In some special cases growth was monitored visually.

2.8. Probiotic Properties of the Isolated Species

2.8.1. Resistance to pH

Resistance to pH 3.0 is often used in vitro assays to determine the resistance to stomach pH [18]. 50 µl of overnight cultures were inoculated in 5 ml MRS broth with varying pH, i.e. pH 3, 5 and 10 at 37°C for 48 h.

2.8.2. Tolerance to Bile Salt

The mean intestinal bile concentration is believed to be 0.3% (w/v). To determine bile salt tolerance, 50 µl of overnight cultures were inoculated in 5 ml MRS broth with varying concentrations of bile salts, i.e. 0.3%, 1.0% and 3.0% at 37°C for 48 h.

2.8.3. Growth at Different Temperatures

To check the growth at different temperature, 50 µl of overnight cultures were inoculated in 5 ml MRS broth and incubated at 10°C, 45°C, and 60°C for 48 h.

2.8.4. Growth Under Different NaCl Concentrations

To check the growth under different NaCl concentration, 50 µl overnight cultures were inoculated in 5 ml MRS broth containing 4%, 8% and 10% NaCl concentrations. Isolates were tested for their tolerance against different NaCl concentrations and incubated at 37°C for 48 h.

2.9. Antibiotic Susceptibility of the Isolates

Disk diffusion method proposed by Bauer et al. [19], was followed for antibiotic susceptibility test. Each isolate was inoculated into the MRS broth, which was incubated at 37°C for 24 h. Plates were made with Muller Hinton agar (Scharlau) and allowed to solidify. The cultures were inoculated in the plates using sterile swab. The antibiotic discs of penicillin (10 µg), ampicillin (10 µg), amoxicillin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), vancomycin (30 µg), bacitracin (10 µg), metronidazol (50 µg) (Oxoid) were placed in the plates. Agar plates with antibiotic disks were then incubated at 37°C for 24 h. The diameters of the inhibition zones were measured. The results were expressed as sensitive (S), intermediate (I) and resistant (R) as described by Oh et al. [20].

2.10. Antimicrobial Activity of the Isolates

The agar spot test was used for determining the antibacterial activity of the isolates and performed as described by Schillinger et al. [21] with some modifications. For this purpose, the isolates were grown in MRS broth at 37°C for 24 h. 10 µl of the isolates were spotted onto the MRS agar plates and incubated at 37°C for 24 h. On the other hand, cultures of the pathogen microorganisms were prepared in nutrient broth and incubated at 37°C for 24 h. Pathogens (100 µl) were transferred to the Muller Hinton agar tubes (8 ml) and overlaid to the MRS agar plates. This was allowed to solidify, before being incubated at 37°C for 24 h. Inhibition zone diameters (surrounding the spotted isolates) were measured. Isolates, which gave an inhibition zone bigger than spots size, were determined to the antimicrobial activity. Pathogenic bacteria used in this study are: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 35150, *Salmonella enteritidis* ATCC 13076, *Listeria monocytogenes* ATCC 7644.

2.11. Data Analysis

Data from the study was analyzed using Sigmaplot (2001). Statistical analysis was also performed by using Sigmaplot.

3. Results and Discussions

3.1. Enumeration of Total Lactic Acid Bacteria (TLAB)

The results of total lactic acid bacteria (TLAB) enumeration from the collected samples on MRS agar supplemented with 0.5% CaCO₃ are shown in Table 1. Cow milk and yogurt samples contained TLAB at levels of 10³-10⁴ CFU/mL and 10⁶-10⁷ CFU/g, respectively. However, no countable TLAB were detected in sweet samples. Both yogurt and sweet undergo heat treatment during preparation; however, unlike yogurt, sweet is not inoculated with a starter culture, which likely explains the negligible LAB presence

in sweet.

3.2. Isolation and Screening of Probiotic Lactic Acid Bacteria (LAB)

For the isolation of probiotic lactic acid bacteria, diluted milk and milk product samples were inoculated onto MRS agar supplemented with 0.5% CaCO₃ using the spread plate technique and incubated anaerobically at 37°C for 48 h. Based on visual observation, 12 morphologically distinct colonies with larger clear zones were selected for further study. This method was previously described by De Man et al. [22] for the isolation of *Lactobacillus*.

3.3. Identification of Probiotic Lactic Acid Bacteria (LAB)

Colony characteristics, morphological characteristics and various biochemical tests were performed for Identification of probiotic lactic acid bacteria (LAB). The colony characteristics of these 12 isolates were observed on MRS agar plate after 24 h incubation at 37°C.

3.4. Colony Morphology, Microscopic View and Gram Reaction of the Isolates

Colony morphology, microscopic view, gram reaction, biochemical test and carbohydrates fermentation tests of all isolates are shown in Table 2. By comparing the results from Table 2 with Bergey's Manual of Determinative Bacteriology [23] and Manual for the Identification of Medical Bacteria [24] it can be concluded that all the isolates were found to belong to the genus *Lactobacillus*. Among of the 12 isolates, 4 were identified as *L. delbrueckii*, 3 as *L. acidophilus*, 3 as *L. casei*, 3 as *L. plantarum* and 1 as *L. rhamnosus*. The designation of these isolates is shown in Table 3. The distribution of species among the isolates was as follows: *L. delbrueckii* (33%), *L. acidophilus* (25%), *L. plantarum* (17%), *L. casei* (17%), and *L. rhamnosus* (8%). These species are commonly isolated from raw milk and dairy products [25]. Mathara et al. [26] reported that *L. plantarum*, *L. acidophilus*, *L. rhamnosus*, and *L. fermentum* were frequently isolated from fermented dairy products, accounting for more than 60% of the identified *Lactobacillus* strains.

Table 1. Total lactic acid bacterial count in different milk and milk products samples collected from Pabna district.

Type of samples	Sample name	Total lactic acid bacteria (cfu/ml or cfu/g)
Cow milk	P-1	2.3×10 ³
	P-2	3.5×10 ⁴
	P-3	5.7×10 ³
	P-4	2.5×10 ³
	P-5	2.9×10 ⁴
	P-8	3.8×10 ⁴
Yogurt	P-9	2.2×10 ³
	P-12	4.6×10 ⁶
	P-14	4.3×10 ⁷
Sweet (Chaana)	P-16	TFTC
	P-18	TFTC
	P-20	TFTC

TFTC: Too few to count

3.5. Probiotic Properties of the Isolated Bacteria

3.5.1. Resistance to pH

Resistance to low pH is a crucial selection criterion for probiotic strains [27, 28], as they must survive the harsh acidic environment of the stomach to reach the small intestine [28, 29]. pH is a critical factor influencing bacterial growth. The resistance of the isolates to different pH levels is shown in Table 4. All isolates exhibited resistance to pH 3, which corresponds to the pH of human gastric juice [18]. However, *L. delbrueckii* P18 and *L. delbrueckii* P20 did not survive at pH 10 (Table 4). Previous studies by Gilliland and Walker [30] and Gotcheva et al. [31] have shown that probiotic microorganisms such as *L. acidophilus*, *L. casei*, and *L. plantarum* can withstand pH 3, allowing them to reach the small intestine and colon, where they contribute to the maintenance of intestinal microflora balance.

Table 2. Colony morphology, microscopic view, gram reaction, biochemical test and carbohydrates fermentation tests of the selected isolates.

Isolates	Microscopic Examinations		
	Gram Reaction	Microscopic view	Colony Morphology
P-1	G+	Rod Shaped	Round, smooth, medium size, white
P-2	G+	Rod Shaped	Round, smooth texture, slightly elevated, non lustrous, small size, white

Isolates	Microscopic Examinations		
	Gram Reaction	Microscopic view	Colony Morphology
P-3	G+	Rod Shaped	Round, creamy, irregular growth, medium size.
P-4	G+	Rod Shaped	Round, smooth texture, slightly elevated, non lustrous, small size, white
P-5	G+	Rod Shaped	Round, smooth, medium size, white
P-8	G+	Rod Shaped	Cream colored, little sticky, medium size.
P-9	G+	Rod Shaped	Round, smooth, medium size, white
P-12	G+	Rod Shaped	Whitish, round, small size.
P-14	G+	Rod Shaped	Whitish, round, small size.
P-16	G+	Rod Shaped	Cream colored, little sticky, medium size.
P-18	G+	Rod Shaped	Whitish, round, small size.
P-20	G+	Rod Shaped	Whitish, round, small size.

Table 2. Continued.

Isolates	Biochemical tests					
	Motility	Nitrate reduction	Urease	Gas production	Oxidase	Catalase
P-1	-	-	-	-	-	-
P-2	-	-	-	-	-	-
P-3	-	-	-	-	-	-
P-4	-	-	-	-	-	-
P-5	-	-	-	-	-	-
P-8	-	-	-	-	-	-
P-9	-	-	-	-	-	-
P-12	-	-	-	-	-	-
P-14	-	-	-	-	-	-
P-16	-	-	-	-	-	-
P-18	-	-	-	-	-	-
P-20	-	-	-	-	-	-

Table 2. Continued.

Isolates	Carbohydrate Utilization									
	Sucrose	Sorbitol	Salicin	Mannitol	Maltose	Lactose	Galactose	Glucose	Cellobiose	Arabinose
P-1	+	-	+	-	+	+	+	+	+	-
P-2	+	+	+	+	+	+	+	+	+	-
P-3	+	+	+	+	+	+	+	+	+	+

Isolates	Carbohydrate Utilization									
	Sucrose	Sorbitol	Salicin	Mannitol	Maltose	Lactose	Galactose	Glucose	Cellobiose	Arabinose
P-4	+	+	+	+	+	+	+	+	+	-
P-5	+	-	+	-	+	+	+	+	+	-
P-8	+	+	+	+	+	+	-	+	+	+
P-9	+	-	+	-	+	+	+	+	+	-
P-12	-	-	-	-	-	-	-	+	+	-
P-14	-	-	-	-	-	-	-	+	+	-
P-16	+	+	+	+	+	+	-	+	+	+
P-18	-	-	-	-	-	-	-	+	+	-
P-20	-	-	-	-	-	-	-	+	+	-

Table 3. Identification of the selected isolates.

Type of sample	Isolates	Identified as
Cow milk	P-1	<i>Lactobacillus acidophilus</i> P1
	P-2	<i>Lactobacillus casei</i> P2
	P-3	<i>Lactobacillus rhamnosus</i> P3
	P-4	<i>Lactobacillus casei</i> P4
	P-5	<i>Lactobacillus acidophilus</i> P5
	P-8	<i>Lactobacillus plantarum</i> P8
	P-9	<i>Lactobacillus acidophilus</i> P9
Yogurt	P-12	<i>Lactobacillus delbrueckii</i> P12
	P-14	<i>Lactobacillus delbrueckii</i> P14
	P-16	<i>Lactobacillus plantarum</i> P16
Sweet (Chaana)	P-18	<i>Lactobacillus delbrueckii</i> P18
	P-20	<i>Lactobacillus delbrueckii</i> P20

Table 4. Growth of the isolates at pH 3, 5 and 10.

Isolates	Growth at pH 3	Growth at pH 5	Growth at pH 10
<i>L. acidophilus</i> P1	+	+	+
<i>L. casei</i> P2	+	+	+
<i>L. rhamnosus</i> P3	+	+	+
<i>L. casei</i> P4	+	+	+
<i>L. acidophilus</i> P5	+	+	+
<i>L. plantarum</i> P8	+	+	+
<i>L. acidophilus</i> P9	+	+	+

Isolates	Growth at pH 3	Growth at pH 5	Growth at pH 10
<i>L. delbrueckii</i> P12	+	+	+
<i>L. delbrueckii</i> P14	+	+	+
<i>L. plantarum</i> P16	+	+	+
<i>L. delbrueckii</i> P18	+	+	-
<i>L. delbrueckii</i> P20	+	+	-

+: Growth; -: No growth

3.5.2. Bile Salt Tolerance

Bile salt tolerance is another important selection criterion for probiotics. Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Although the bile concentration of human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v. Tolerance to 0.3% bile salt is an indicator to the ability to survive in the intestine [18]. Resistance to bile salts varies a lot among the lactic acid bacteria species and even between strains of the same species [32]. Following exposure to acidic conditions, the 12 isolates were tested for bile salt tolerance. In this study, MRS broth containing 0.3%, 1% and 3% bile salt were used to assess the resistance of the selected isolates. The results demonstrated that all isolates were able to survive at bile salt concentrations of 0.3%, 1%, and 3% (Figure 1).

3.5.3. Growth at Different Temperatures

Tolerances to a wide range of temperatures were studied for selection of potential probiotics isolated from food items by Abdullah and Osman [33] and El Soda et al. [17]. In this study growth at different temperatures was monitored visually. All isolates exhibited growth at temperature of 10°C, 45°C, and 60°C. Some isolates showed strong growth at 10°C, while others exhibited enhanced growth at 45°C. However, most isolates demonstrated poor growth at 60°C.

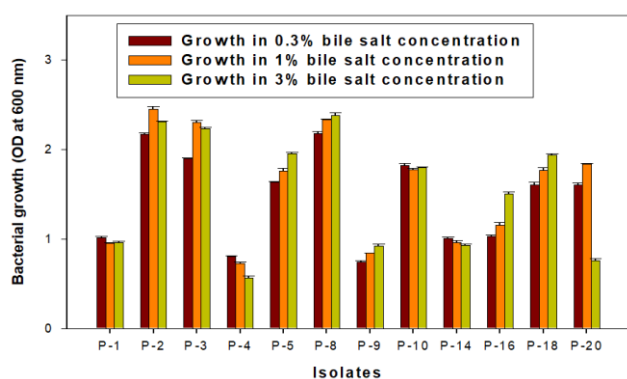


Figure 1. Growth of the isolates at 0.3, 1 and 3% bile salt.

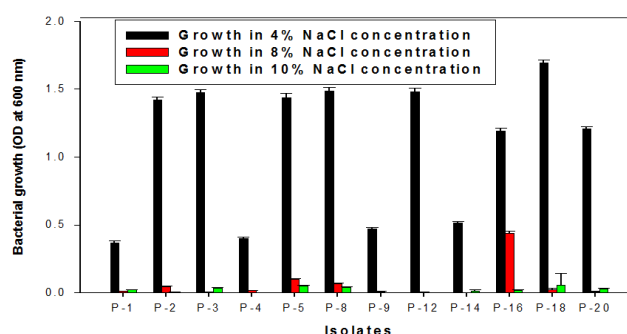


Figure 2. Growth of the isolates at 4, 8 and 10% NaCl.

3.5.4. Growth at Different NaCl Concentrations

Growth at different NaCl concentrations was monitored spectrophotometrically at 600 nm. All isolates showed the ability to grow well at 4% NaCl concentration, but growth was significantly reduced at 8% and 10% NaCl concentrations (Figure 2). These findings were in agreement with the findings of Hoque et al. [34] and Elezete and Carlos [35]. Figure 2 shows that *L. plantarum* P8 exhibited the highest growth, whereas *L. acidophilus* P1 displayed the lowest growth at 4% NaCl concentration. Furthermore, *L. plantarum* P16 and *L. delbrueckii* P18 exhibited maximum growth at 8% and 10% NaCl concentrations, respectively. Most isolates exhibited significantly reduced growth at 8% and 10% NaCl concentrations.

3.5.5. Antibiotic Susceptibility of the Isolates

The susceptibility of probiotic bacteria to antibiotics is generally recognized as one of the basic characteristics of these organisms. The antibiotic susceptibility of the isolated LAB strains was assessed using the disc diffusion method. Susceptibility to antibiotics is graded as R (Resistant) or S (Susceptible) or I (Intermediate) [20]. The results of the study are shown in Table 5. The results indicated that all the isolates were resistant to metronidazol and vancomycin. *L. casei* P4 and *L. acidophilus* P9 were resistant to all eight tested antibiotics. *L. acidophilus* P1, *L. plantarum* P8, *L. delbrueckii* P14 and *L. plantarum* P16 were resistant to 5 antibiotics. *L. delbrueckii* P18 was resistant to 4 antibiotics. *L. casei* P2, *L. rhamnosus* P3, *L. delbrueckii* P12 and *L. delbrueckii* P20 were resistant to 3 antibi-

otics. *L. acidophilus* P5 was resistant to 2 antibiotics.

The resistance's attributes of many LAB to antibiotics are often intrinsic and non-transmissible [36]. Intrinsically antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of various antimicrobial agents [37]. The resistance to antibiotics also indicates the isolates can potentially minimize the negative effects of antibiotic therapy on the host bacterial ecosystem [38]. *Lactobacillus plantarum* P8 and *Lactobacillus plantarum* P16 were resistant to the penicillin and sensitive to the erythromycin. This result confirms the finding of Nguyen et al. [39] who reported that *Lactobacillus plantarum* is resistant to the penicillin and sensitive to the erythromycin. It was observed that 75% isolates were resistant to penicillin. This result confirms the finding of Shitandi and Sternesjo [40]. Resistance to several antibiotics has been noted in strains isolated from craft-made Spanish cheeses [41]. According to Condon [42], the impermeability of the *Lactobacillus* cell wall is the main reason for their resistance, but several non-specific mechanisms could cause differences among strains within the same species [43]. Most *Lactobacillus* are resistant

to glycopeptides (vancomycin), while susceptibility to bacitracin is variable [44, 45]. *Lactobacillus* are usually sensitive to inhibitors of protein synthesis such as chloramphenicol, erythromycin [45-47]. This is consistent with the results of the present study, where most of the isolates were sensitive to chloramphenicol and erythromycin. Hoque et al. [34] found that *Lactobacillus* spp. isolated from yogurt were resistant to metronidazol.

3.5.6. Antimicrobial Activity of the Isolates

The agar spot method was used to determine the antibacterial activity of the isolates. The selected isolates were evaluated based on their antimicrobial activity. For this purpose, isolates were tested against four pathogenic microorganisms: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 35150, *Salmonella enteritidis* ATCC 13076, *Listeria monocytogenes* ATCC 7644. The diameter of inhibition zones indicated that all the isolates exhibited an antimicrobial effect on the pathogenic microorganisms. The tests were done duplicate, and the averages of diameters of the inhibition zones are provided in Table 6.

Table 5. Antibiotic susceptibility of isolates using the disk diffusion method (Unit: mm).

Isolates	Diameter of inhibition zone in (mm) ^a							
	P ₁₀	Ap ₁₀	Am ₁₀	C ₃₀	E ₁₅	B ₁₀	Mt ₅₀	Va ₃₀
<i>L. acidophilus</i> P1	0 _(R)	14 _(I)	26 _(S)	0 _(R)	0 _(R)	16 _(S)	0 _(R)	0 _(R)
<i>L. casei</i> P2	15 _(I)	8 _(R)	21 _(S)	28 _(S)	28 _(S)	15 _(S)	0 _(R)	0 _(R)
<i>L. rhamnosus</i> P3	20 _(I)	10 _(R)	25 _(S)	24 _(S)	29 _(S)	16 _(S)	0 _(R)	0 _(R)
<i>L. casei</i> P4	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)
<i>L. acidophilus</i> P5	29 _(S)	20 _(S)	31 _(S)	24 _(S)	31 _(S)	17 _(S)	0 _(R)	0 _(R)
<i>L. plantarum</i> P8	0 _(R)	10 _(R)	0 _(R)	24 _(S)	28 _(S)	13 _(S)	0 _(R)	0 _(R)
<i>L. acidophilus</i> P9	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)	0 _(R)
<i>L. delbrueckii</i> P12	0 _(R)	18 _(S)	27 _(S)	35 _(S)	37 _(S)	30 _(S)	0 _(R)	0 _(R)
<i>L. delbrueckii</i> P14	0 _(R)	0 _(R)	0 _(R)	20 _(S)	25 _(S)	15 _(S)	0 _(R)	0 _(R)
<i>L. plantarum</i> P16	0 _(R)	0 _(R)	0 _(R)	30 _(S)	35 _(S)	23 _(S)	0 _(R)	0 _(R)
<i>L. delbrueckii</i> P18	0 _(R)	0 _(R)	16 _(I)	24 _(S)	28 _(S)	17 _(S)	0 _(R)	0 _(R)
<i>L. delbrueckii</i> P20	31 _(S)	0 _(R)	30 _(S)	27 _(S)	29 _(S)	30 _(S)	0 _(R)	0 _(R)

Antibiotics (Disk potency): P₁₀: Penicillin G (10 units), Ap₁₀: Ampicillin (10 µg), Am₁₀: Amoxicillin (10 µg), C₃₀: Chloramphenicol (30 µg), E₁₅: Erythromycin (15 µg), B₁₀: Bacitracin (10 µg), Mt₅₀: Metronidazol (50 µg), Va₃₀: vancomycin (30 µg), R: Resistant, I: Intermediate, S: Susceptible. a: diameters of the discs are inclusive.

Table 6. Diameter of inhibition zones (mm) among the isolates by agar spot method.

Isolates	Zone of inhibition against pathogens (mm)*			
	<i>Staphylococcus aureus</i> ATCC 6538	<i>Eschericia coli</i> ATCC 35150	<i>Salmonella enteritidis</i> ATCC 13076	<i>Listeria monocytogenes</i> ATCC 7644
<i>L. acidophilus</i> P1	20	24	30	24
<i>L. casei</i> P2	12	15	17	14
<i>L. rhamnosus</i> P3	13	16	15	17
<i>L. casei</i> P4	18	29	30	29
<i>L. acidophilus</i> P5	12	14	16	18
<i>L. plantarum</i> P8	14	16	23	20
<i>L. acidophilus</i> P9	20	22	29	20
<i>L. delbrucckii</i> P12	19	23	29	30
<i>L. delbrucckii</i> P14	25	30	30	29
<i>L. plantarum</i> P16	20	24	29	30
<i>L. delbrucckii</i> P18	29	30	29	30
<i>L. delbrucckii</i> P20	29	30	30	29

*Inhibition Zone Diameters of the isolates are inclusive.

All the isolates showed antimicrobial activity against all four tested pathogenic microorganisms. *L. delbrucckii* P18 and *L. delbrucckii* P20 showed maximum and *L. casei* P2 and *L. acidophilus* P5 showed minimum antimicrobial activity against *S. aureus* ATCC 6538. *L. delbrucckii* P14, *L. delbrucckii* P18 and *L. delbrucckii* P20 showed the highest, whereas *L. acidophilus* P5 showed the lowest antimicrobial activity against *Eschericia coli* ATCC 35150. *L. acidophilus* P1, *L. casei* P4, *L. delbrucckii* P14 and *L. delbrucckii* P20 showed the highest, while *L. rhamnosus* P3 showed the lowest antimicrobial activity against *Salmonella enteritidis* ATCC 13076. *L. delbrucckii* P12, *L. plantarum* P16, *L. delbrucckii* P18 demonstrated the highest, whereas *L. casei* P2 showed minimum antimicrobial activity against *Listeria monocytogenes* ATCC 7644.

In general, the antimicrobial activity of *Lactobacillus* may be due to organic acids, hydrogen peroxide, bacteriocins, or other inhibitory substances produced as [48, 49]. In this study, the observed growth inhibition on agar-spot plates indicates that *Lactobacillus* produced antimicrobial products such as organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins that were able to inhibit the growth of *S. aureus* ATCC 6538, *E. coli* ATCC 35150, *S. enteritidis* ATCC 13076, and *L. monocytogenes* ATCC 7644 all of which are food contaminants and pathogens. All of the isolates exhibited inhibition in the agar spot test, which suggests that they produce acetic acid and/or lactic acids that lower the pH of the medium. Inhibition may also be through competition for nutrients, production of bacteriocin or other antibacterial compounds [50]. Obadina et

al. [51] observed that the fermentation process involving *L. plantarum* caused a reduction in pathogen levels, such as *S. typhi*, *E. coli* and *S. aureus*. In the present study, it was observed that *L. plantarum* P16 showed good antimicrobial activity against all four tested pathogens. Olarte [52] noted that the presence of *L. plantarum* during the production of Cameros cheese from goat's milk decreased the number of enterobacteria and fecal coliforms in the final product.

4. Conclusions

Following conclusions can be drawn from results of this study:

Twelve LAB were isolated and identified from cow milk and milk products (yogurt and sweets). Identified bacterial isolates were: *L. acidophilus* P1, *L. casei* P2, *L. rhamnosus* P3, *L. casei* P4, *L. acidophilus* P5, *L. plantarum* P8, *L. acidophilus* P9, *L. delbrucckii* P12, *L. delbrucckii* P14, *L. plantarum* P16, *L. delbrucckii* P18 and *L. delbrucckii* P20.

All the isolates were resistant to pH 3 and pH 5. Except for *L. delbrucckii* P18 and *L. delbrucckii* P20, the remaining isolates were resistant to pH 10. All the isolates were resistant to bile salt concentrations ranging from 0.3% to 3%. These isolates also showed the ability to grow at temperatures of 10°C, 45°C and 60°C. The isolates grew well at 4% NaCl concentration, however, growth was not satisfactory at 8% and 10% NaCl concentrations.

All the isolates were resistant to metronidazol and vanco-

mycin. *L. casei* P4 and *L. acidophilus* P9 were resistant to all eight tested antibiotics.

All the isolates showed antimicrobial activity against the pathogenic microorganisms, including *S. aureus* ATCC 6538, *E. coli* ATCC 35150, *S. enteritidis* ATCC 13076 and *L. monocytogenes* ATCC 7644.

Abbreviations

LAB	Lactic Acid Bacteria
MRS	Mann, Rogosa, and Sharpe
TLAB	Total Lactic Acid Bacteria
OD	Optical Density

Author Contributions

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Sirajee Arif Mahmud: Data curation, unding acquisition, Validation, Writing – review & editing

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Abdullah-Al-Mahin: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing

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Conflicts of Interest

The authors declare no conflicts of interest.

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