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RESEARCH ARTICLE
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PREVALENCE OF PATHOGENIC BACTERIA AND ISOLATION OF LACTIC ACID BACTERIA (LAB) FROM PRE-PROCESSED CATTLE MILK ACROSS VARIOUS REGIONS OF PUNJAB, PAKISTAN

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ABSTRACT
Cattle milk is an equitable and nutritious food crucial for human health, but substantial nutritious components of milk cause prompt microbial invasion and spoilage. Lactose is a crucial part in the newborn mammal's development, being an important energy origin, crucial for heart, liver and kidneys. The current study focuses on milk analysis from four different milk sample collections. It involves a physiochemical milk survey (acidity, pH, temperature, fat contents, and lactometer reading). The microbial load (total plate count, *E.coli*, coliforms, and *Staphylococcus aureus*) and organoleptic properties of all milk samples were also measured. Further, the *lactobacillus* isolated from milk samples were evaluated and identified. Our results showed variations in different physiochemical properties from different sampling centers. The presence of microorganisms was confirmed on plate count agar, and the detection of metallic green sheen confirmed the presence of *Escherichia coli* on EMB agar, coliforms on VRB agar, and yellow colonies on Mannitol salt agar confirmed the presence of *Staphylococcus aureus.* AST results showed that the strain TQ1 was sensitive against azithromycin, cefoperazone, and chloramphenicol, whereas resistant against ciprofloxacin and gentamicin were found resistant. While strain TQ2 was sensitive against cefoperazone and chloramphenicol and resistant against azithromycin, ciprofloxacin, and gentamicin. The TQ3 was sensitive against all except azithromycin. This study surveys the bacterial prevalence and isolation of lactic acid bacteria from cattle milk from different milk sample collection centers.

INTRODUCTION
Milk plays an important role in maintaining balance of life and acts as a natural product, it is defined as secretion of mammary glands of animals, highly supplemented product that contains vitamins,

carbohydrates, minerals, fat, and lactose that provides us with the energy of 9.3 Cal per gram fat and 4.1 Cal per gram of protein and lactose. At milk collection centers, alcohol precipitation tests and specific gravity tests are performed to evaluate the quality and inventiveness of milk, while the Dye reduction test and pH and turbidity test are performed to analyze the milk's freshness in the dairy industry. Milk fat, milk SNF, total protein, whey protein, total mineral contents, and fat tests are performed for evaluating the composition of milk, and it is based on lactation stage, time of milking, breed and feed. Raw milk provides perfect nutritional conditions for many microorganisms (Cremonesi *et al.,* 2020). Raw milk contaminated with common foodborne pathogens like *Staphylococcus aureus, Salmonella spp,* and *Listeria monocytogenes* can lead to various foodborne diseases, posing a potential threat to human health (Berhanu *et al*., 2021).

Lipids, proteins, carbohydrates, minerals, and vitamins present in milk are an excellent source of nutrients readily absorbable by the human body. Lactose-milk sugar is the most important carbohydrate found in mammalian milk. In cow's milk, the primary component of milk solids is also lactose, apart from fat and protein (Guetouache *et al.,* 2014). Lactose is categorized as reducing disaccharides and hydrolyzed in solutions with a high acid concentration or in the digestive tract of mammals when treated with the ß-galactosidase enzyme, as well as in plants, bacteria, molds, and fungi cells. Lactose is broken down to glucose and galactose molecules by the hydrolysis process, and further in the presence of oxygen, oxidized to CO_2 and H_2O in the presence of O_2, while under anaerobic conditions, it converts to lactic acid or alcohol by fermentation (Gambelli *et al.,* 2017). Within the healthy udder cells of animals, when it leaves the udder, milk is supposed to be sterile. It usually contains a small number of lactic acid bacteria in small number, but possible exposure to external contaminants, a complex microbiota originating from different sources becomes part of milk (Berhanu *et al.,* 2021), the most common point of contaminants being the udder and teat surface of the animals (Parente *et al*., 2020). Microorganisms' type and count in raw milk are influenced by many factors, such as milking equipment cleanliness, season, water, feed, and animal health (Machado *et al.,* 2004).

Hygienic production of milk is major challenge and is important to understand the factors that positively or negatively affect the microbiota of raw milk, as milk food safety and quality is dependent on contaminants (Wang *et al.,* 2018). The sensory and quality characteristics of dairy products are dependent on milk microbial content and with increasing dairy products demand, bacterial contamination has become a global concern (Sharma *et al.,* 2023). Common microbes found in milk include *Streptococcus, Lactobacillus, Enterococcus,* and a subset of important psychrophilic bacteria, usually *Pseudomonas* and *Acinetobacter* (Yuan *et al., 2022).* Psychrophilic bacteria can grow and multiply at low temperatures and spoil milk by generating extracellular proteases and lipases (Quigley *et al*., 2013).

MATERIALS AND METHODS

Sample Collection

Twenty milk samples were collected from each cattle breeding centers of Arifwala, Chishtian, Jhang and Ellahbad. Samples were collected thrice a day for every third day based on storage conditions and farm practices. A sterile vessel was used for the collection of the sample and transferred to laboratory for further evaluation.

Physiochemical survey of milk

To study the chemical configuration of milk samples, the milk samples were analyzed for acidity, pH, temperature, fat contents, and Lactometer reading (Aydogdu *et al.,* 2023).

Microbiological testing

Microbiological testing of milk samples of cattle was performed for TPC, *Escherichia coli*, *Coliform, Staphylococcus aureus, Listeria, and Salmonella*. Microbiological testing for TPC, *E. coli*, Coliforms, and *S. aureus* was performed on Plate count agar, Eosin methylene blue agar, Violet red bile agar,

and Mannitol salt agar, respectively. A test for *Listeria* was performed on *Listeria* agar, and a *Salmonella* test was performed on *Salmonella-Shigella* agar.

Isolation of Lactic Acid Bacteria (LAB)

The Lactobacillus species were isolated as per protocol (Reuben *et al*., 2020). Milk sample (10ml) was added in 50ml MRS broth and supplemented with Tween 20 in 50ml falcon tube and incubated at 37ºC in the incubator. Sterile normal saline was used to synthesize a fresh culture with turbidity using the vortex method. After ten-fold dilutions had been made and 100ul of each sample were extracted and streaked on agar supplemented MRS medium and incubated at 37˚C for 1-3 days under anaerobic conditions. To collect a further pure culture, culturing was done.

Evaluation and identification of bacterial strains

The identification of isolated bacterial strains with probiotic properties was performed by two-stage screening. In first-stage screening, the preference was on physiological and morphological estimation, including catalase test and Gram staining, putting the strains on abiotic pressure mechanism to assess their temperature and salinity liberality. In the second stage their molecular identification was done. Three strains were isolated and named TQ1, TQ2, and TQ3.

DNA Extraction and Polymerase chain reaction (PCR)

The genomic DNA of TQ1, TQ2, and TQ3 was extracted using a GenomeJet DNA Purification Kit (Thermo Fischer Scientific) followed by electrophoresis on 1% agarose gel (Iqbal et al. 2014). The amplification of the 16S ribosomal RNA gene was performed using 25µl reaction of 12µl Master mix (Thermo Fischer Scientific), 1µl each of forward (27F) and reverse primers (1492R), 4µl of DNA template, and 7µl of sterile nuclease free water (Frank *et al.*, 2008). The denaturation was done at 94 ºC for 2 minutes, followed by annealing at 57.4 ºC for 20 seconds and extension at 70 ºC for 90 seconds. Thirty cycles were performed with a final extension at 70 ºC for 5 minutes (Barq *et al.*, 2021).

Safety evaluation of Lactic acid bacteria (LAB)

Acid tolerance

3mg/ml of pepsin enzyme was added to MRS broth to test the acid resistance of targeted bacterial strains. 1.0N HCl was utilized to diverse pH of broth at different values (3.0, 4.0, and 5.0) (Pereira *et al.*, 2002). The overnight bacterial cultures in the broth were used for the test, and their optical density was measured at 620nm. To assess the ability of bacteria to modify to acidic environments, a surplus number was counted.

Antibiotic susceptibility testing (AST)

According to Baur and Kirby agar disc diffusion method was used to perform antibiotic susceptibility testing (Bauer et al. 1960).

Antimicrobial assay

Antimicrobial activity was performed on Muller Hinton agar by well diffusion method according to (Balouiri *et al.*, 2016).

Haemolytic activity

The haemolytic activity of bacterial isolates was processed by using cultures that were grown overnight. Then, it was streaked on blood agar plates and placed in an incubator at 37°C for 48 hours. (Mahmoudi *et al.*, 2019).

Organoleptic properties

The organoleptic properties of the milk sample were carried out by the international hedonic scale test method. It was tested in the form of taste, smell, texture, sight, and touch (Sharma *et al.,* 2023).

RESULTS

Physiochemical analysis of milk

The physiochemical analysis of milk samples (acidity, pH, temperature, fat and, lactometer reading) is shown in table 4.1 and figures 4.1, 4.2, 4.3, 4.4 and 4.5.

Table 4.1: Physiochemical analysis of cattle milk sampled from Arifwala (MSA), Chishtian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10). Different lowercase letters represent differences at $p < 0.05$.

Tests	MSA	MSC	MSJ	MSE
Acidity (%)	0.130a	0.131b	0.129a	0.128c
pH	6.75a	6.77b	6.77b	6.76b
Temperature (ºC)	6.5a	5.95a	6.04b	6.2b
Fat (%)	4.02a	4.30a	4.20b	4.15b
LR	26.43a	25.69b	25.78a	26.95c

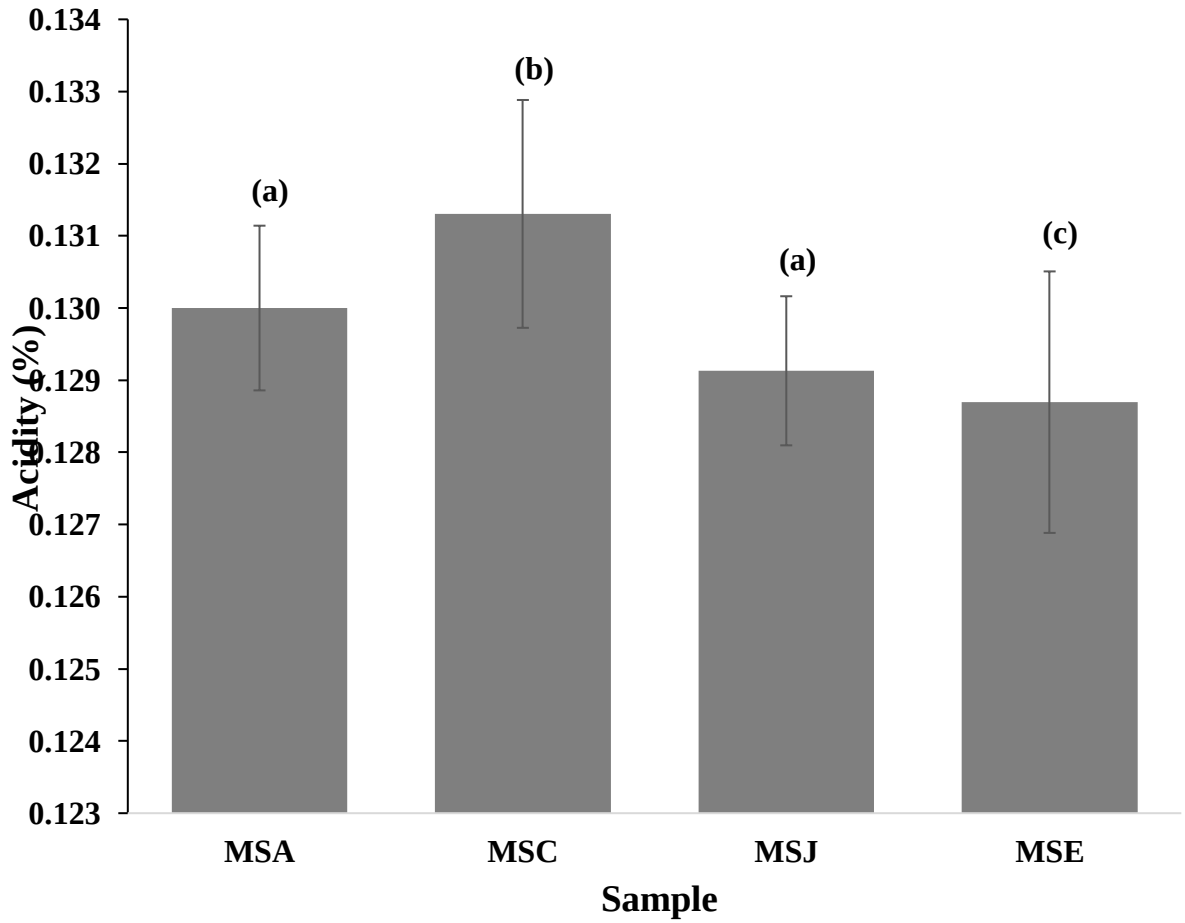

Figure 4.1: **Acidity variation in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).**

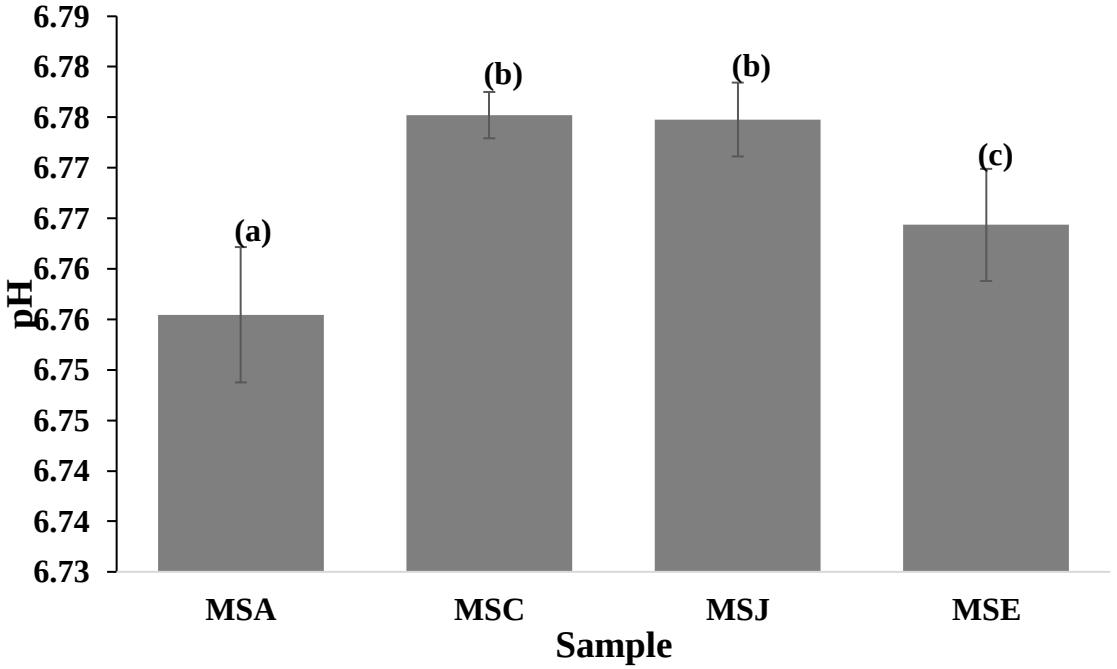

Figure 4.2: **pH variation in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC Test).**

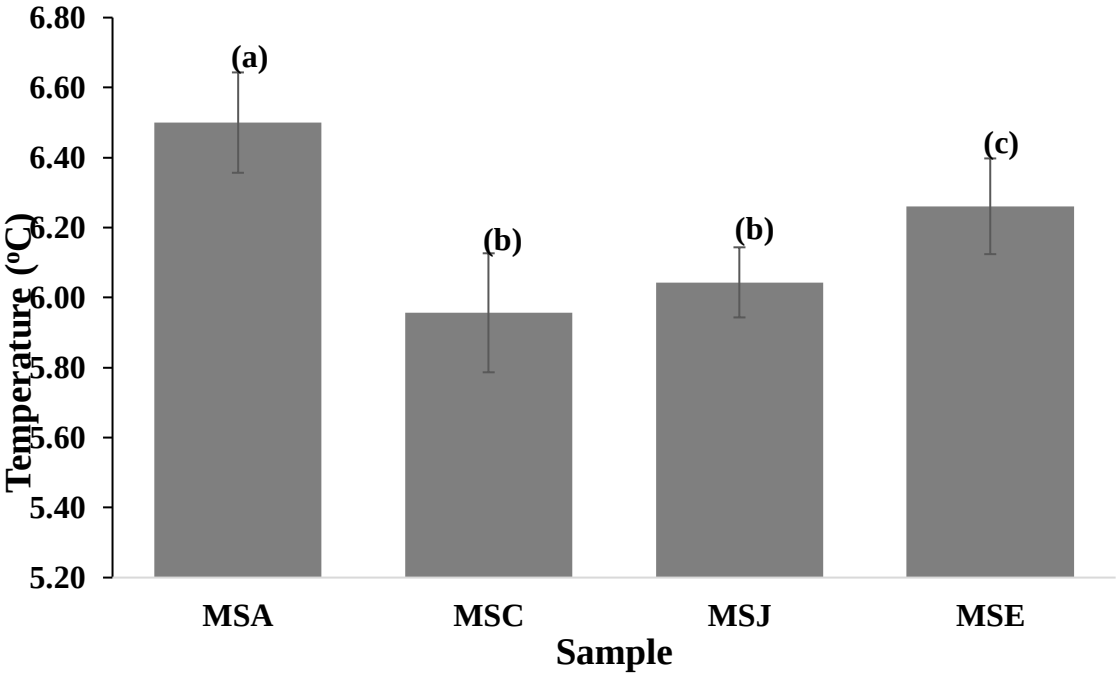

Figure 4.3: **Temperature variation in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).**

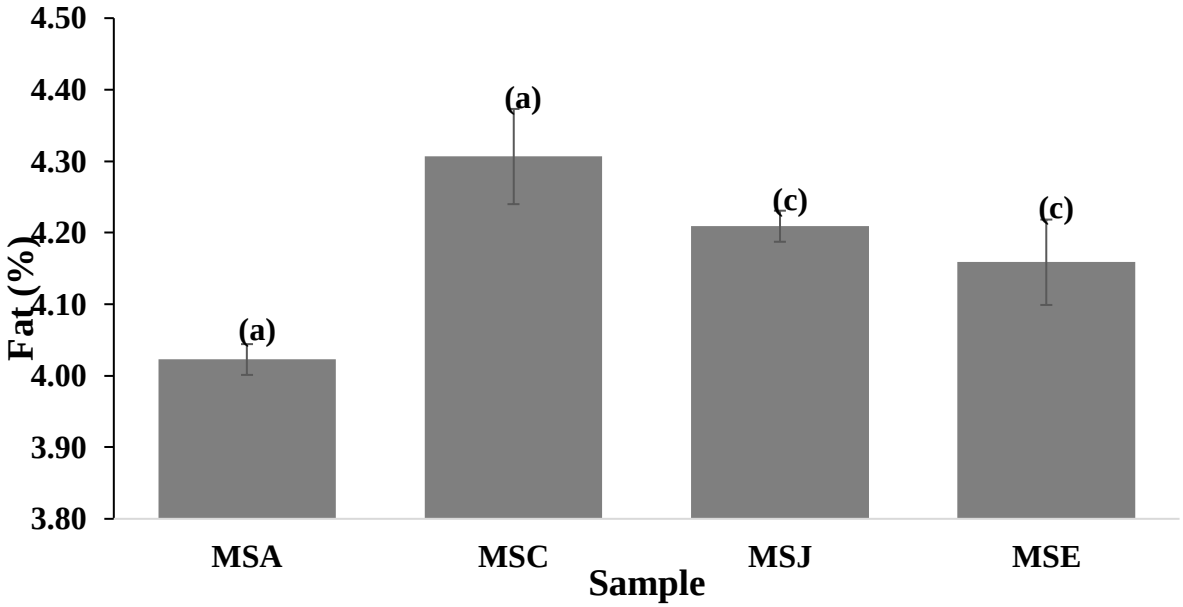

Figure 4.4: **Fat content variation in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).**

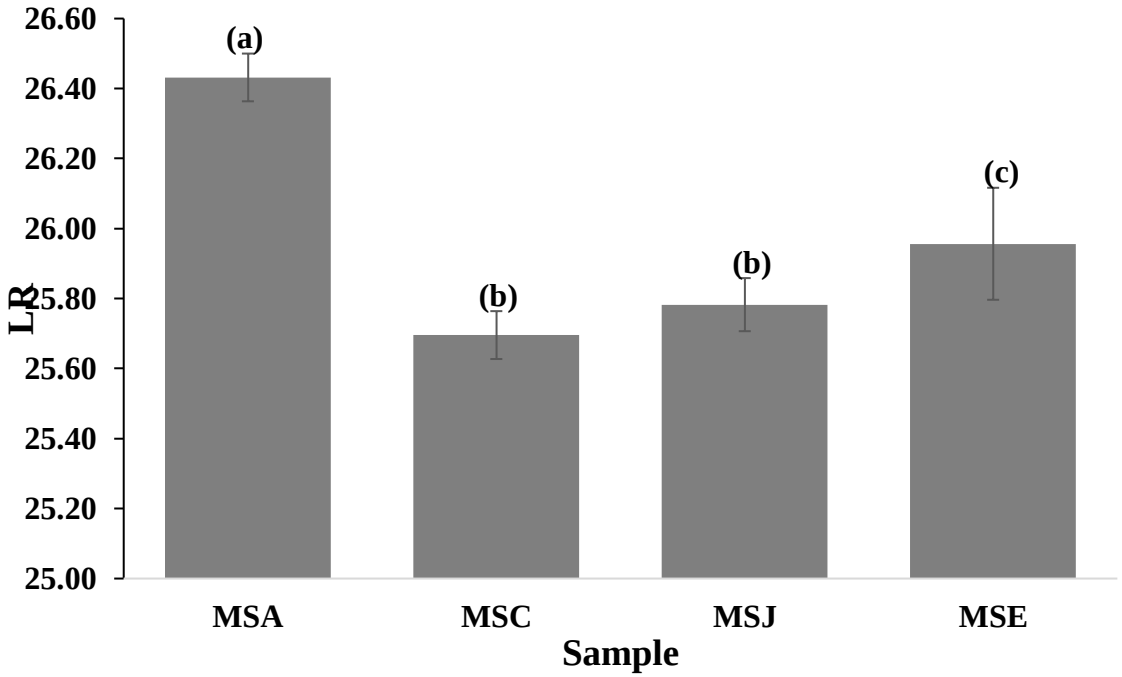

Figure 4.5: **Lactometer Reading variation in milk samples from collection centre in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).**

Microbiological testing

20 milk samples of cattle were processed to check contamination and the presence of harmful pathogens as Total Plate Count, *Escherichia coli,* Coliform test, *Staphylococcus aureus, Listeria monocytogene* test, and *Salmonella* test. Results showed that there is a presence of microorganism on

plate count agar, detection of metallic green sheen confirmed the presence of *Escherichia coli* on EMB agar, Coliforms showed the presence on VRB agar, and yellow colonies on Mannitol salt agar confirmed the presence of *Staphylococcus aureus* from all centers whereas *Listeria monocytogene* and *Salmonella* showed no growth as shown in table and graphs below. The proliferation of these pathogens may be attributed to sampling issues, poor hygiene conditions, contaminated soil and water, infected humans, contaminated animal udders, and various environmental factors, as shown in Figures 4.6, 4.7, 4.8, and 4.9.

Table 4.2: Presence of pathogens from milk samples

Centers	Arifwala	Chishtian	Jhang	Ellahabad
Listeria monocytogenes	Present	Absent	Absent	Present
Salmonella	Absent	Present	Absent	Present

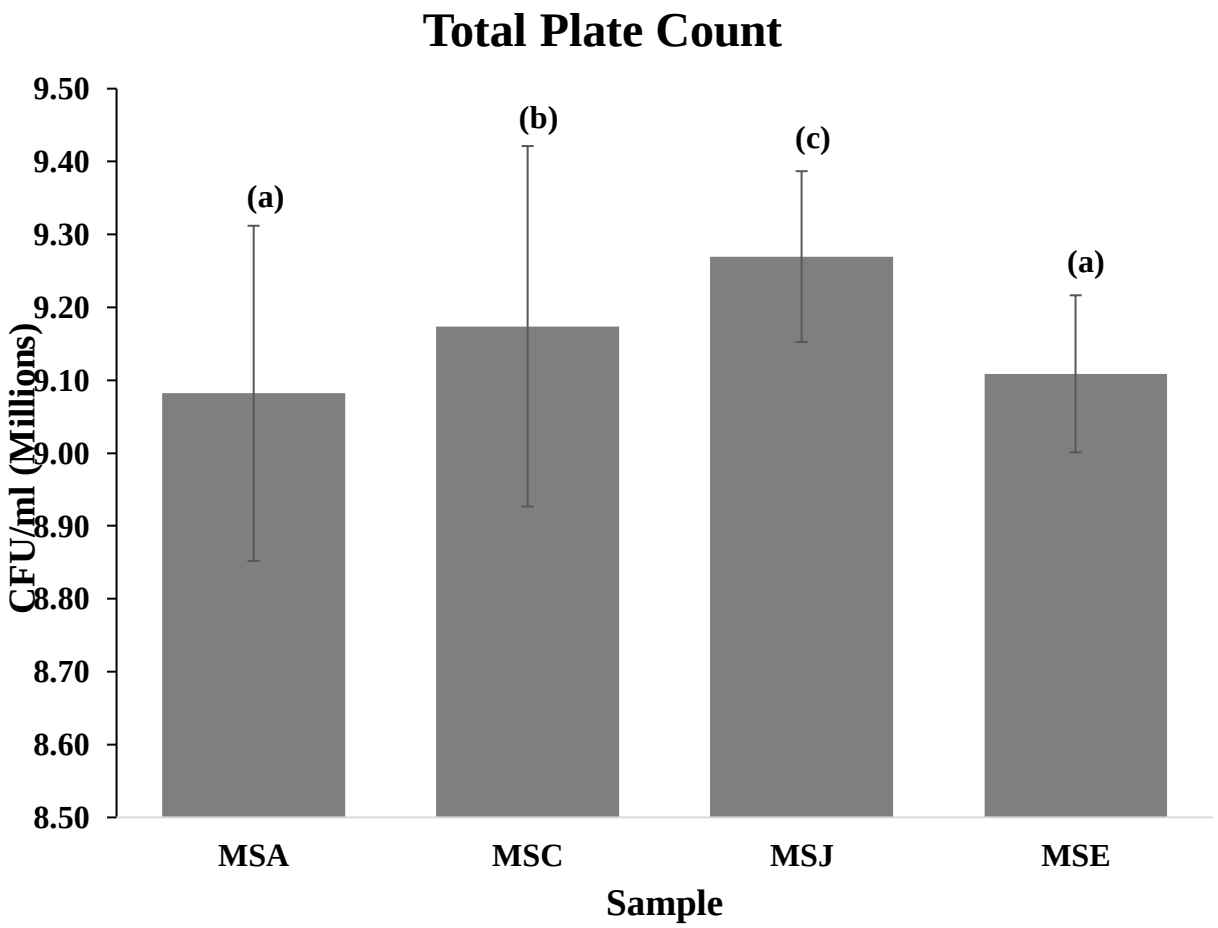

Figure 4.6: Total plate count variation in CFU per ml in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=3), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

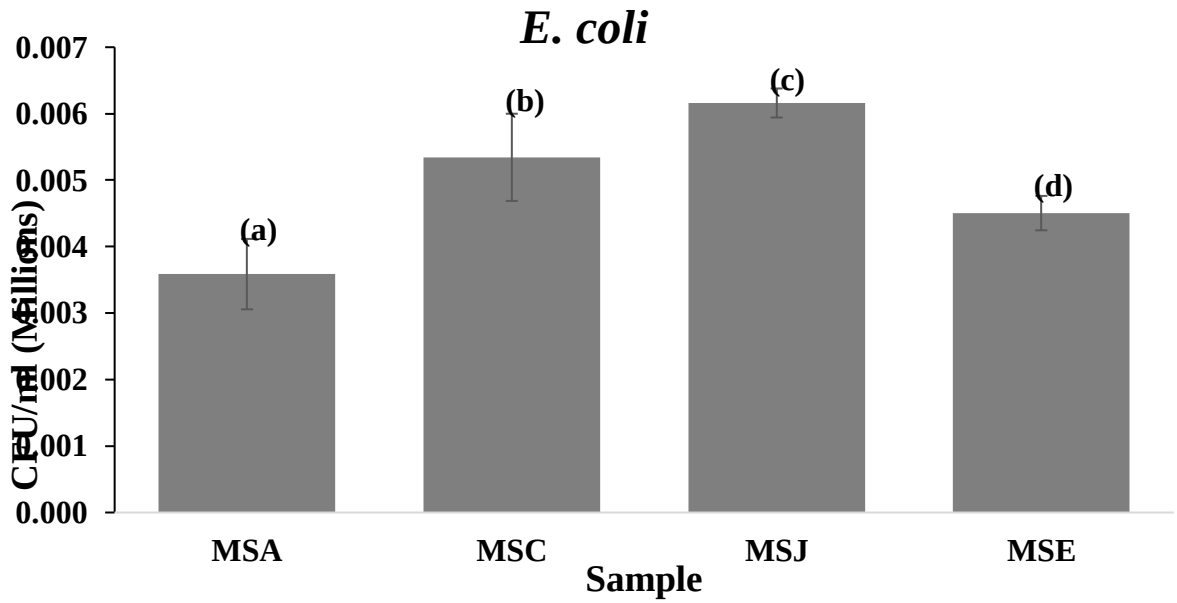

Figure 4.7: *E. coli* abundance CFU per ml in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=3), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

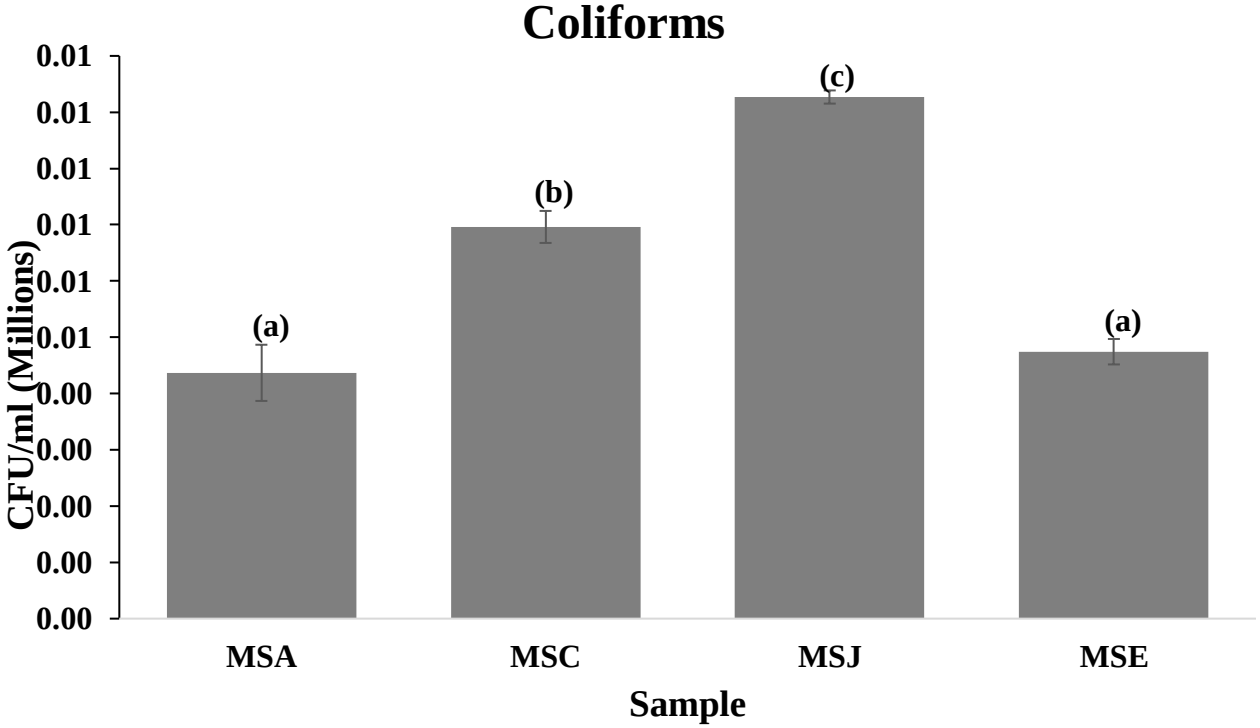

Figure 4.8: Total Coliforms in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=3), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

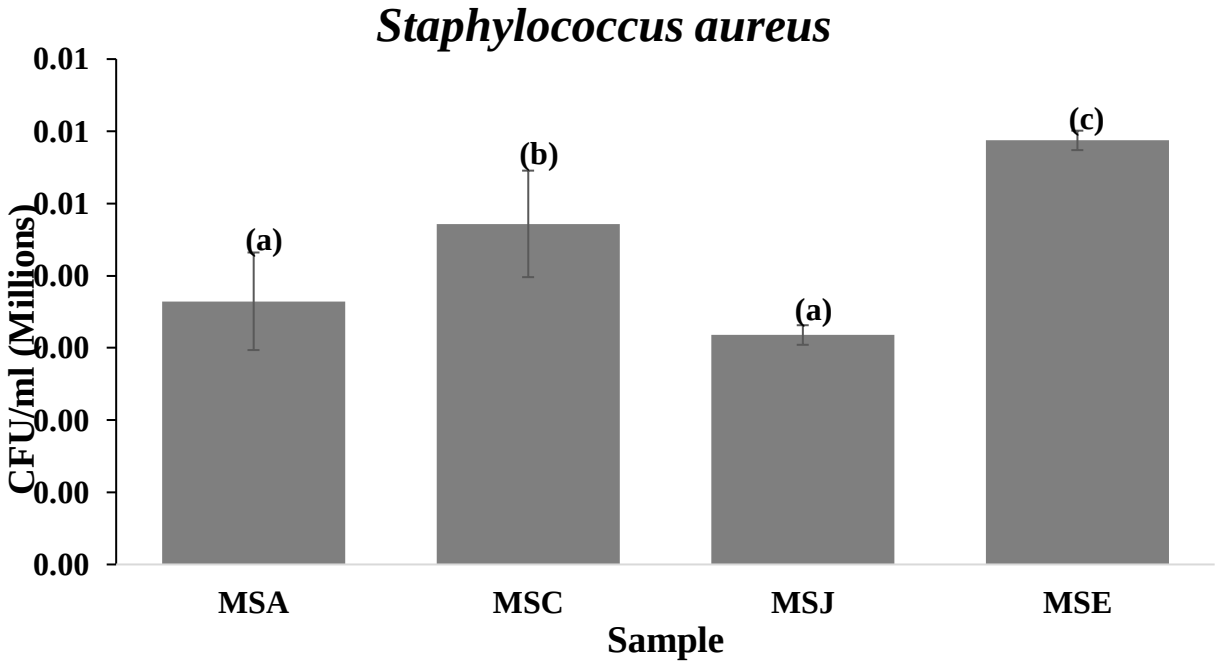

Figure 4.9: *Staphylococcus aureus* abundance in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=3), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

Isolation of Lactic Acid Bacteria

A sample taken of cattle was then isolated on MRS agar for further evaluation and identification of *Lactobacillus* species.

Figure 4.10: Isolation of Lactic acid bacteria on MRS agar.

Evaluation and identification of bacterial strains

Evaluation and Identification of lactic acid bacteria was done by two methods: Catalase test and Gram staining.

Catalase test

A catalase test was performed on a clean glass slide. This test is used to differentiate Gram- positive and Gram-negative category of bacteria. Test results shows there was no bubble formation by putting the drops of Hydrogen peroxide as shows that results were negative indicating the identification of

Lactic acid bacteria as shown in figure 4.11.

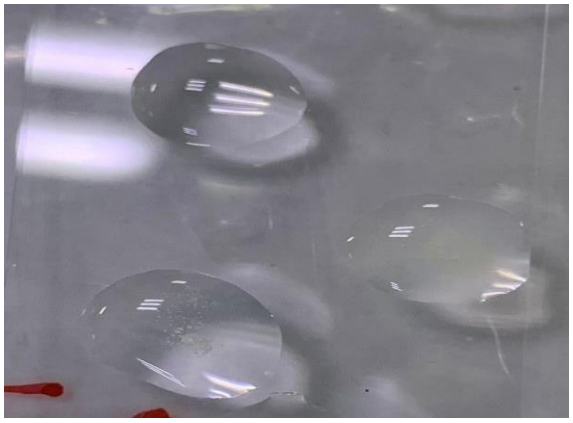

Figure 4.11: Catalase Test for isolated strains.

Gram staining

Gram staining was performed by the addition of crystal violet, iodine solution, ethanol and safranin into a colony present on a clean glass slide. Results showed the purple color appears indicating the identification of lactic acid bacteria as shown in figure below. The Gram- positive bacteria showed different array of staining, demonstrating the composition of cell wall and identification of bacterial species as shown in the figure 4.12.

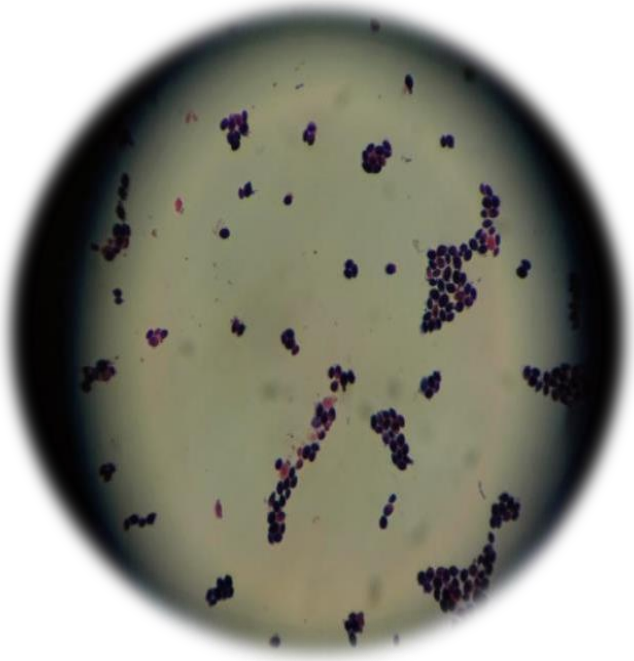

Figure 4.12: Gram Staining of Lactic Acid Bacteria.

DNA extraction, PCR, and sequencing

The genomic DNA was assessed on 1% agarose gel for the authentication of DNA extraction. The product was processed in 1% agarose gel agar and DNA gel was put into middle wall for sample TQ1, TQ2 and TQ3. On UV-transilluminator, at 1400~ bp, agarose gel was seen as shown in figure 4.13 and 4.14.

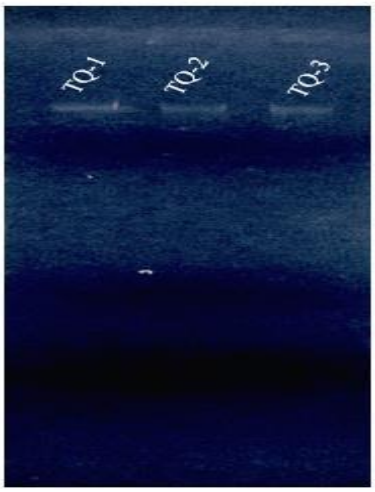

Figure 4.13 Agarose Gel % electrophoresis of extracted bacterial genomic DNA from three different Isolates. Lane 1= TQ1, Lane 2= TQ2, Lane 3= TQ3.

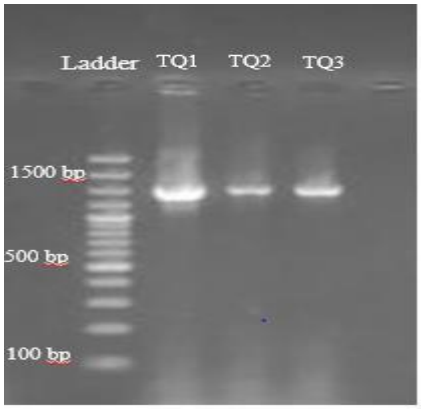

Figure 4.14: PCR amplification of the 16S rRNA for TQ1, TQ2, and TQ3.

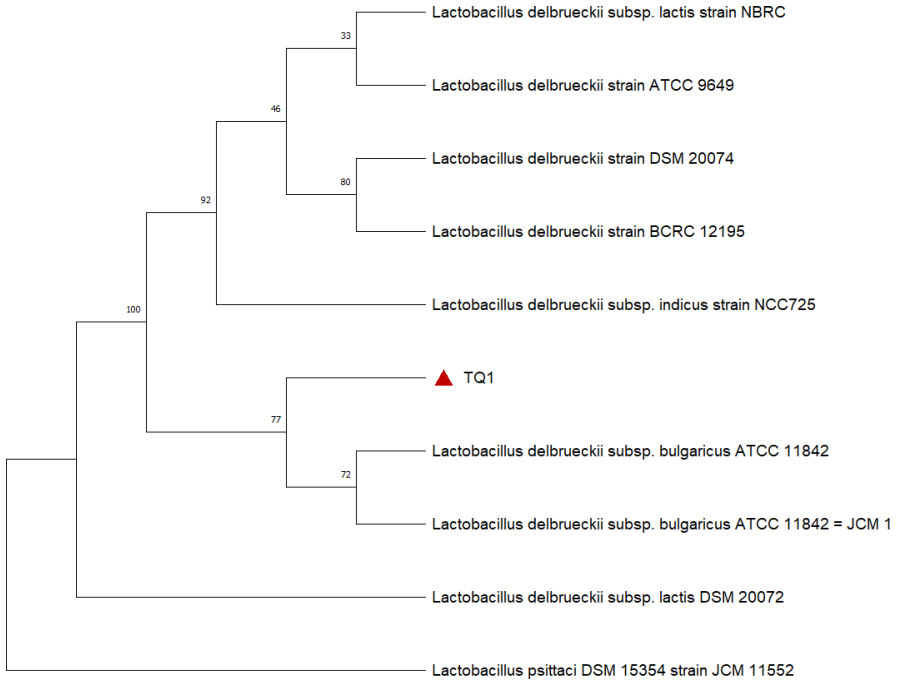

Figure 4.15: **Neighbor-Joining Phylogenetic Tree based on 16S rRNA shows the Position of *Lactobacillus delbrueckii* (TQ1).**

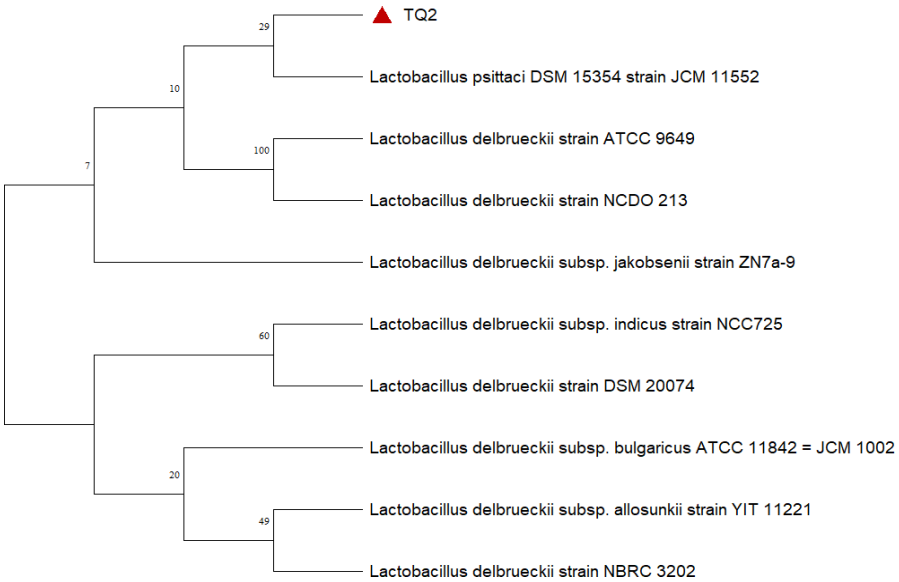

Figure 4.16 **Neighbour-Joining Phylogenetic Tree Based on 16S rRNA Shows the Position of *Lactobacillus delbrueckii* (TQ2).**

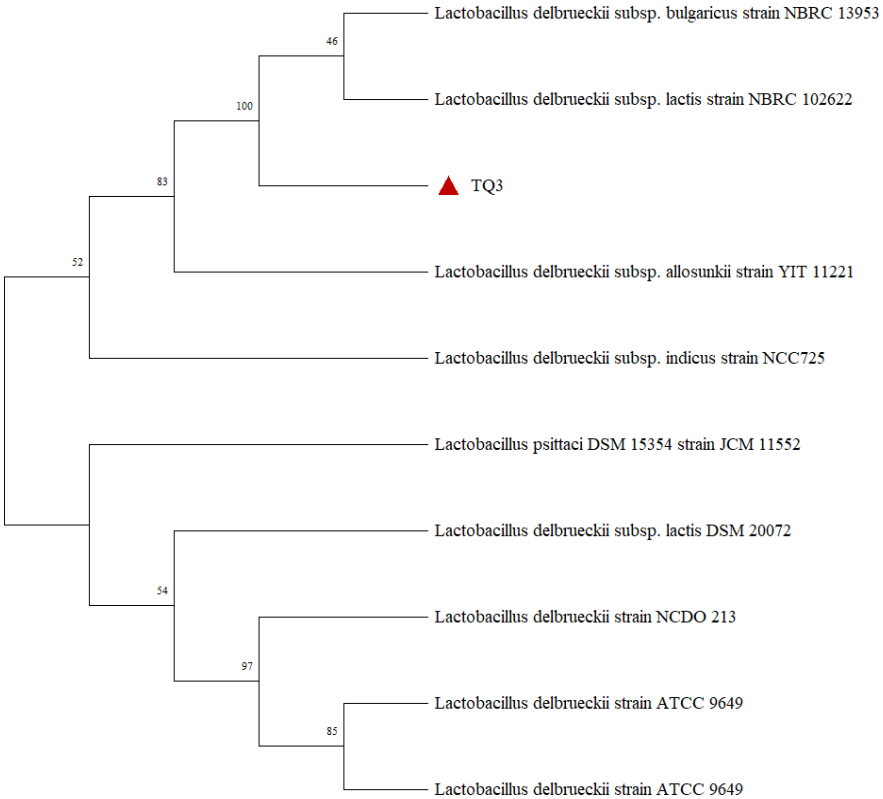

Figure 4.17 Neighbour-Joining Phylogenetic Tree Based on 16S rRNA Shows the Position of *Lactobacillus delbrueckii* (TQ3).

Antimicrobial assay

Antimicrobial activity was performed by well diffusion method against *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes and Salmonella Species.* Well has been made on Muller Hinton agar plates and 100ml of tested culture was added. The results showed a Zone of Inhibition to confirm the antimicrobial assay. The zone of inhibition was measured in (mm) with the help of a scale. The results showed promising steps in the inhibition of *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, and Salmonella,* as shown in table 4.3 and figure 4.18.

Table 4.3: Antimicrobial activity of TQ1, TQ2, and TQ3 against *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*.

Microorganism	TQ1	TQ2	TQ3
Escherichia coli	S	S	S
Staphylococcus aureus	R	S	S
Listeria monocytogenes	S	R	R

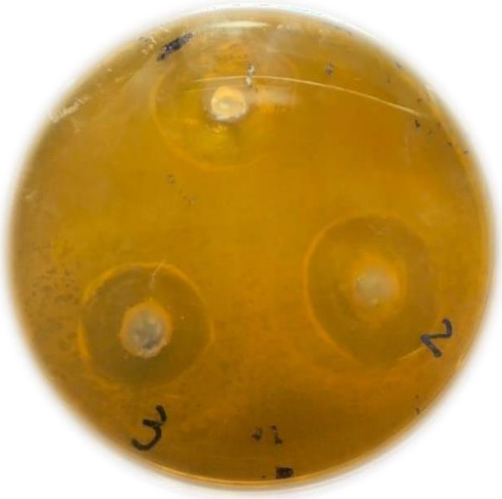

Figure 4.18: Antimicrobial activity of isolated lactic acid bacteria

Antibiotic susceptibility testing

AST was performed by the disk diffusion method to detect the sensitivity and resistant pattern of Lactic acid bacteria. Antibiotic disks of azithromycin, ciprofloxacin, gentamicin, cefoperazone, and chloramphenicol were added into MRS agar. The zone of inhibition around antibiotics was measured in mm with the help of a scale. The result showed that on TQ1 azithromycin, cefoperazone, and chloramphenicol were found sensitive, whereas ciprofloxacin and gentamicin were found resistant. In TQ2, only cefoperazone and chloramphenicol were found to be sensitive. Azithromycin, ciprofloxacin, and gentamicin were found to be resistant. In TQ3, all antibiotics were sensitive except azithromycin, as shown in table 4.4.

Table 4.4: AST Results of TQ1, TQ2, and TQ3 against Azithromycin, Ciprofloxacin, Gentamicin, Cefoperazone, and Chloramphenicol.

Antibiotics	**TQ1**	**TQ2**	**TQ3**
Azithromycin	S	R	R
Ciprofloxacin	R	R	S
Gentamicin	R	R	S
Cefoperazone	S	S	S
Chloramphenicol	S	S	S

Acid tolerance test

An acid tolerance test was performed in the presence of MRS medium before H_2SO_4 to check the ability of a strain to survive in low acidic conditions. Results demonstrate the resistance pattern as strains can be survived in a low pH condition showing their ability to thrive in an acidic environment. Such survival is important to be able to compete with other microorganisms. The acid tolerance of cattle milk was assessed by using different pH values (3, 4, and 5). Acid tolerance in the TQ1 sample at pH 3 was ranged as 5×10^3. At pH 4 and 5, it was detected 2×10^5. In sample TQ2 pH 3, results

showed the value of 1.4×10^5, pH 4 showed acid tolerance of 3×10^5, and pH 5 shows acid tolerance of 1×10^5. In sample TQ3 at pH 3, results showed the value of 6×10^5, pH 4 results showed the value of 1×10^5, and 5 acid tolerances was 2.5×10^5 as shown in graph and table 4.5.

Table 4.5: Acid Tolerance test of isolated strains, i.e., TQ1, TQ2, and TQ3 at pH 3.0, 4.0, and 5.0.

Sample	pH 3	pH 4	pH 5
TQ1	5×10^3	2×10^5	2×10^5
TQ2	1.4×10^5	3×10^5	1×10^5
TQ3	6×10^5	1×10^5	2.5×10^5

Haemolytic activity

The sample was cultured on blood agar plates and then incubated at 37°C for 24 hours. Results demonstrate that the *Lactobacillus* strain showed no color change, which indicates negative test results that mean the *Lactobacillus* strain was not able to diminish red blood cells in blood agar medium. This shows that *Lactobacillus* species are not declared pathogenic, as shown in table.
4.6 and Figure 4.19.

Table 4.6: Haemolytic Activity results of TQ1, TQ2, and TQ3.

Sample name	Haemolytic activity
TQ1	No colour change
TQ2	No colour change
TQ3	No colour change

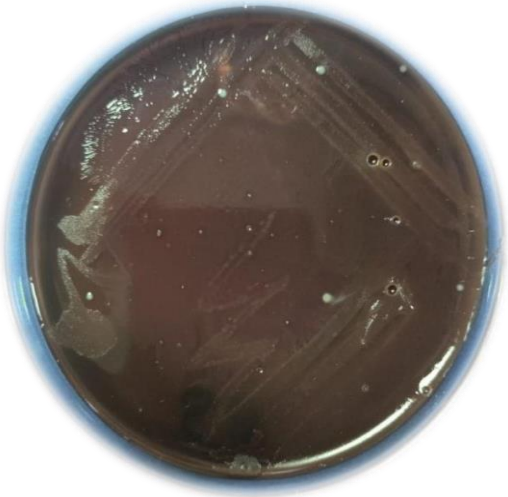

Figure 4.19 Hemolytic activity of isolated bacterial strain

Organoleptic properties

The taste, smell, and texture of milk taken from the Arifwala center was declared as 9 according to the standard set. Taste, smell, and texture of milk was also declared as 9 according to standard value whereas taste smell, and texture of milk was also declared as 9, in the end milk taken from Ellahabad showed value of 9 in taste and smell, and 8 in texture as shown in graph 4.20, 4.21 and 4.22.

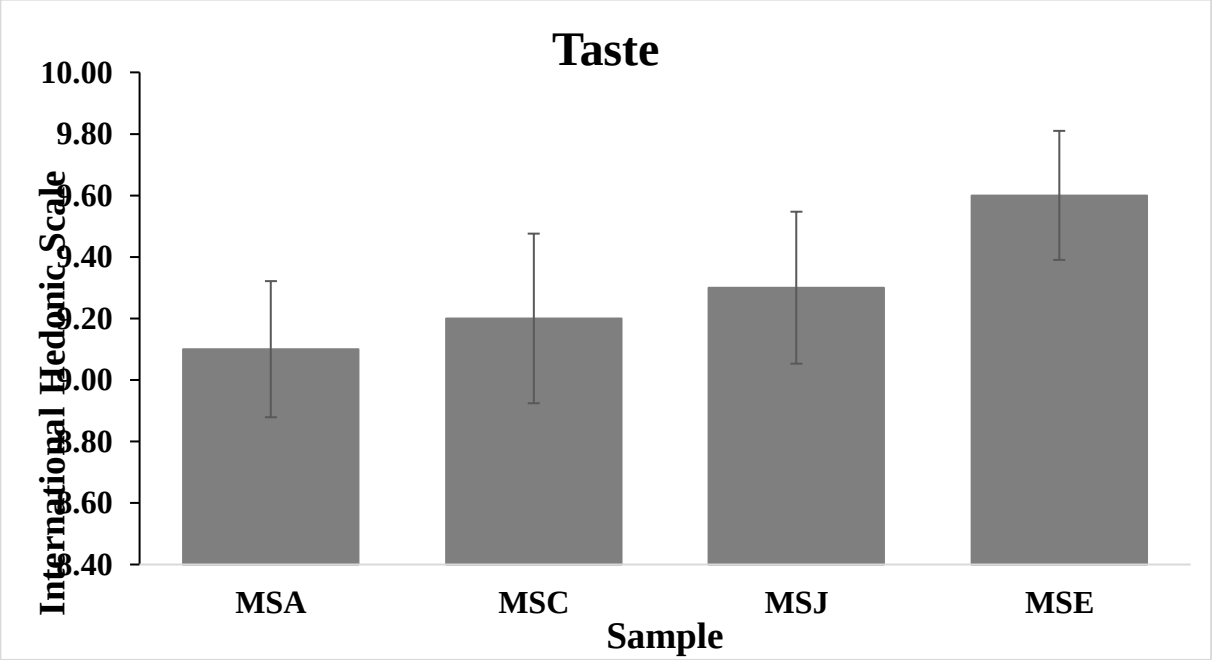

Figure 4.20: Taste variations (as per the international hedonic scale) in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

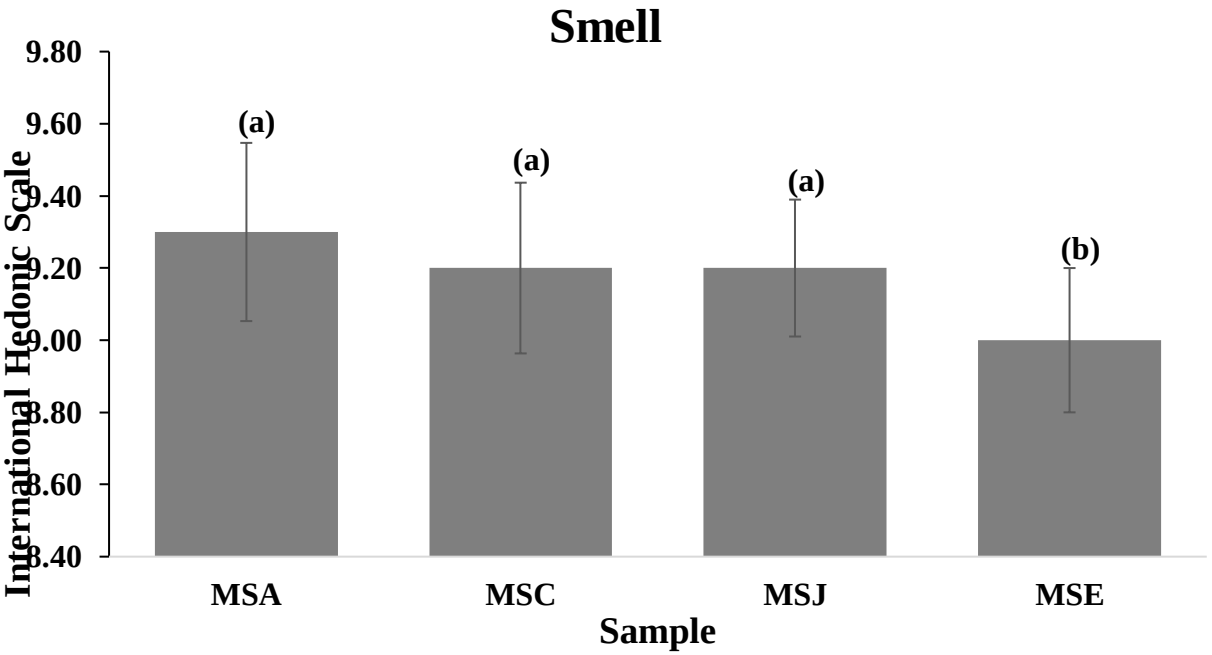

Figure 4.21: Smell variations (as per the international hedonic scale) in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

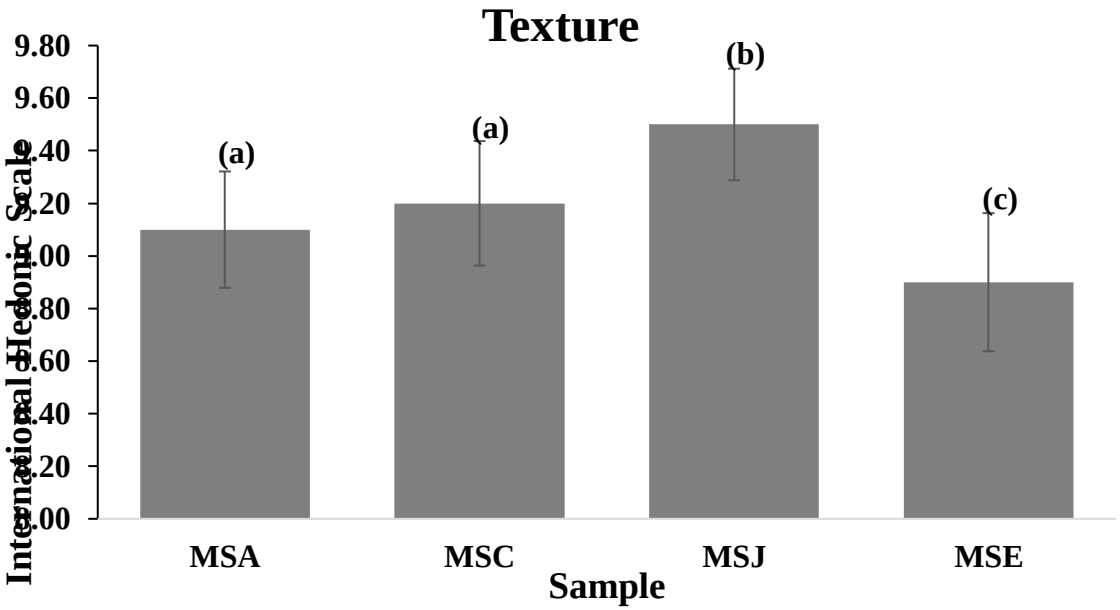

Figure 4.22: Texture variations (as per the international hedonic scale) in milk samples from collection centers in Arifwala (MSA), Chistian (MSC), Jhang (MSJ), and Ellahbad (MSE). Values represent means (n=10), and error bars signify standard error. Different lower-case letters show significant differences at P < 0.05 (Tukey's Post HOC test).

DISCUSSION

It is said that more than 2000 species produce milk to satisfy our basic and daily nutritional requirements. The dairy animal consumption for human need has been set by different factors including animal availability, facility of milking, and organoleptic characteristics of milk (Leblanc *et al*., 2010). This study is about to evaluate probiotic characteristics of LAB isolates including their acidity, fat %, lactometer reading, bile salt, acid tolerance, and antibacterial activity against other pathogens. This study shows how lactic acid bacteria affect pathogens present in milk. Four different samples of milk were collected from Arifwala, Chishtian Jhang, and Ellahbad and processed for further testing. To test the physiochemical analysis of the milk sample, tests were performed to ensure its quality and safety as follows: acidity, pH level, temperature, fat %, lactometer reading, and density, lactose. The physiochemical properties of cow's milk were evaluated, as shown by Preka *et al*. (2016). In this study, pH, fat %, protein %, lactose, freezing point, and density of cow's milk were assessed. The presence of pathogens in milk samples was tested. Results showed the presence of *Escherichia coli*, Coliform pathogens, and *Staphylococcus aureus*. The proliferation of these pathogens may be attributed to sampling issues, poor hygiene conditions, contaminated soil and water, infected humans, contaminated animal udders, and various environmental factors. A study by Holzhauer *et al*. (2023) showed the detection of microorganisms in cattle are *Bacillus, Brucella, Campylobacter, Chlamydia, Clostridium, Escherichia, Leptospira, Listeria, Salmonella,* and *Mycobacterium.* Results showed that ampicillin, imipenem, meropenem, cefotaxime, chloramphenicol, erythromycin, and tigecycline were found sensitive in all isolates of *Lactobacillus* tested, whereas streptomycin, tobramycin, vancomycin, ciprofloxacin, ofloxacin, teicoplanin, cefoxitin, oxacillin, and methicillin were found resistant. In our study, Haemolytic activity showed no colour change when *Lactobacilli* streaked on blood agar. In comparison to that, a study by Yasmin *et al.* (2020) showed that there was no haemolytic activity determined; it was gamma-haemolysis. In our study, the antimicrobial activity of *Lactobacillus* was performed on *Escherichia coli, Salmonella, Staphylococcus aureus,* and *Listeria monocytogene.* It showed a zone of inhibition of 4.0-4.25 mm against *Escherichia coli,* and others showed a 10mm zone of inhibition against *Staph*ylococcus *aureus*, *Listeria monocytogene*, and *Salmonella typhimurium.* Results showed that all strains of *Lactobacillus* were found to be sensitive against all pathogenic organisms. In our study, Organoleptic properties showed that taste, smell, and

texture gave the value of 9 according to the standard value set by the international hedonic scale method.

CONCLUSION

This study represents the bacterial prevalence and isolation of lactic acid bacteria from cattle milk. The pre-processed cattle milk samples from various regions in Punjab showed significant bacterial contamination, indicating the potential health risks associated with milk before pasteurization. Three lactic acid bacteria isolates were successfully isolated from the milk samples, showing promising antimicrobial activity. These LAB isolates could potentially be used as bio-preservatives to enhance the safety of dairy products. The acid tolerance, antibiotic resistance, and haemolytic tests demonstrated that the LAB isolates are not only effective against specific pathogens but also safe for use in food products. This supports their potential application in improving the microbial quality and safety of dairy products in the region.

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