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Isolation and Characterization of Lactic Acid Bacteria (LAB) from Bovine Milk and Study of their Antibiotic Susceptibility Patterns

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Abstract:
The present study focuses on the isolation, characterization, and antimicrobial susceptibility testing (AST) of lactic acid bacteria (LAB) from bovine milk samples obtained from Gir, Mehsana, and Holstein Friesian breeds in the Thane district of India. The LAB isolates were characterized morphologically and biochemically and identified using MALDI-TOF MS.Seven Gram-positive, catalase-negative isolates were used in this study. Antibiogram tests were performed to record the sensitivity or resistance of LAB towards antibiotics and results were interpreted according to the CLSI (2015) guidelines. *Leuconostocpseudomesenteroide* from the HF cow milk was resistant to all 18 tested antibiotics. *Lactobacillus plantarum* and *Lactobacillus casei*showed resistance to 16 and 15 out of 18 tested antibiotics respectively. *Lactococcus lactis* showed sensitivity to 6 tested antibiotics. All isolated LAB from bovine milk were resistant to oxacillin, vancomycin, ofloxacin, teicoplanin, ceftazidime, gentamycin, co-trimoxazole, and cloxacillin antibiotics.This study contributes to the ongoing efforts to understand the prevalence and implications of antibiotic resistance in probiotic bacteria, which is crucial for ensuring the safety and efficacy of probiotic products.

Keywords: Antibiotic susceptibility, Probiotic, Lactic acid bacteria, Bovine milk

Introduction

Probiotics are consumed globally in various forms, including food, dietary supplements, and as active components of registered medications. However, there is a need to reassess their safety, particularly regarding the potential spread of antibiotic resistance (ABR) (Sukmarini*et al.*, 2014). While antibiotic resistance is a significant concern, it has not received sufficient attention in the context of lactic acid bacteria (LAB), the most commonly consumed bacterial group.

The ability of LAB to help maintain or replenish beneficial gut microbiota has garnered significant attention for probiotics as health promoters. LABs have a long-standing 'Generally Recognized as Safe (GRAS)' status by the U.S. Food and Drug Administration (FDA) and are also candidates for Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA). Numerous research studies and reports have provided safety assurances for LAB, contributing to the growing interest in probiotics. The probiotic concept has evolved from traditional dairy products to a profitable market, where probiotic

bacteria are incorporated into various dairy products, health supplements, and functional foods (Arioli *et al*., 2013).

The probiotic market has witnessed substantial growth in recent decades. The global market for probiotic supplements was valued at nearly $7 billion in 2021 and projected to grow at a compound annual growth rate (CAGR) of 9.3% from 2022 to 2023 (Zavišić G. et al., 2023). This growth is driven by increasing consumer demand for healthy foods and nutritional supplements and a lifestyle focused on personal health and wellness.

There is a high possibility of horizontal gene transfer among bacteria in nature, leading to the further spread of these resistant strains between populations (Sukmarini*et al*., 2014). In the last decade, there has been an increase in reports documenting antibiotic resistance in LAB strains. Although LABs are generally considered safe, concerns exist regarding the possible mobility of resistance determinants to human and animal pathogenic and opportunistic bacteria. Some researchers acknowledge the presence of antibiotic resistance in LAB and suggest the possibility of co-administering them with antibiotic therapy to aid in replenishing the healthy gut flora, which is otherwise at high risk (Dixit *et al.*, 2013). However, this statement is controversial and a matter of debate. Resistance-coding genes and their transfer through plasmids and conjugative transposons have also been reported in *Lactobacillus* species (Jose *et al*., 2015). Genes conferring resistance to several antimicrobials are located on transferable genetic elements in various LAB strains (Gfeller *et al.*, 2003).The horizontal gene transfer from probiotic bacteria to gut commensals and pathogens raises safety concerns about probiotics (N. Toomey *et al*., 2009). While probiotics co-administered with antibiotics have benefits, horizontal transfer of multidrug resistance to gut microbes threatens the normal microflora (P. Courvalin, 2006).

Antimicrobial resistance (AMR) is a major healthcare issue causing treatment failures, morbidity, and death (N. Lu *et al*., 2014). Bacteria develop multidrug resistance through intrinsic or acquired mechanisms. These mechanisms may differ depending on the nature of the antibiotic, the target site of the drug, the bacterial species, and whether it is linked to a plasmid or chromosomal mutation (P. Sharma *et al*., 2014). The present study aimed to assess the antibiotic susceptibility pattern of LAB isolated from bovine milk, with the results interpreted according to the CLSI (2015) guidelines. This study contributes to the ongoing efforts to understand the prevalence and implications of antibiotic resistance in probiotic bacteria in bovine milk isolated LAB, which is crucial for ensuring the safety and efficacy of probiotic products.

Materials and Methods

Sampling: Milk samples (thirty each) from different bovine breeds (Gir, Mehsana and Holstein Friesian) were collected from nearby dairy farms in the surrounding areas of Thane district, India. Samples were collected using clean and sterile bottles and brought to the laboratory in an icebox for microbiological investigation. Samples were kept in a refrigerator (around 4°C) till the analysis began. Bovine milk samples were collected between January 2022 and March 2022. Milk samples were coded as 'G' for Gir cow milk; 'M'for Mehsana buffalo and 'HF' for Holstein Friesian.

Enrichment and isolation of LAB: Thirty samples of each bovine milk were pooled separately and homogenizedbefore isolation. From this 1ml of milk was added to 24ml of Rogosa broth and then incubated under anaerobic conditions for 24 hours. A loopful of mixture was then spread-plated on Rogosa agar plates and incubated for 48 hours to obtain isolated colonies.For further studies, the isolates were stored in 50% (w/v) glycerol.

Preliminary screening, morphological,and biochemical test: The preliminary screening of isolated LAB was performed using Gram staining to determine their Gram characteristics. Further screening of the isolates was conducted using catalase and oxidase tests. The catalase and oxidase-negative LAB were subsequently analyzed through biochemical and biophysical methods, including MALDI-TOF.

The biochemical tests included carbohydrate assimilation (arabinose, lactose, mannitol, sucrose, xylose, and starch),nitrate reduction, and the urease test.

MALDI-TOF Mass Spectrometry Analysis: The isolates were sent for MALDI-TOF MS analysis, which was performed by SRL Diagnostic Laboratory, Mumbai to identify the bacterial isolates at both the genus and species levels.

Test antibiotics used for AST testing: Eighteen antibiotics used in this study were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Antibiotic groups and their modes of action are shown in Table 1.

Table 1: List of antibiotics used in the study.

Sr no.	Antibiotic	Antibiotic group	Mode of action
1.	Amoxyclav	A combination of β lactam amoxicillin and β lactamase inhibitor clavulanic acid	Amoxicillin inhibits the synthesis of cell walls and clavulanic acid helps to overcome β lactamase-mediated resistance.
2.	Ofloxacin	Fluoroquinolone	Inhibit DNA gyrase
3.	Gentamycin	Aminoglycoside antibiotic	Inhibit protein synthesis by binding to the 30s subunit of a bacterial ribosome.
4.	Tetracycline	Tetracyclines	
5.	Clindamycin	Lincosamide antibiotic	Inhibit protein synthesis by binding to the 50s subunit of a bacterial ribosome.
6.	Lincomycin	Lincosamide antibiotic	
7.	Erythromycin	Macrolide	
8.	Penicillin G	β lactam	Inhibitors of the cell wall synthesis
9.	Cephalothin	1st generation cephalosporine	
10	Cefoxitin	2nd generation cephalosporin	
11	Cefuroxime	2nd generation cephalosporin	
12	Ceftazidime	3rd generation cephalosporin	
13	Cefotaxime	3rd generation cephalosporin	
14	Cloxacillin	Semisynthetic penicillin	
15	Oxacillin	Semisynthetic penicillin	
16	Vancomycin	Glycopeptide antibiotic	
17	Teicoplanin	Glycopeptide antibiotic	
18	Co-Trimoxazole	Sulphonamides antibiotic(combination of Trimethoprim and Sulfamethoxazole)	Trimethoprim inhibits the enzyme dihydrofolate reductase and Sulfamethoxazole inhibits dihydrofolate synthase.

Antibiotic susceptibility assay: An antibiotic susceptibility assaywas performed to determine the sensitivity or resistance of LAB to conventional antibiotics using a modified Kirby-Bauer method. This method is based on a standard disc diffusion assay (Bauer *et al*., 1966). In this assay, culture (100µL, 0.5 McFarland equivalent to 10^8cfu/ml) of all the tested isolates was spread plated onto the surface of freshly prepared Man Rogosa& Sharpe (MRS) plates. Antibiotic discs were aseptically placed using sterile forceps. Plates were incubated for 24 hours in anaerobic conditions. After overnight incubation, the diameter (mm) of the zone of inhibition (ZOI) was measured. Zone on de Man Rogosa& Sharpe (MRS) plates were depicted as sensitive/susceptible (S), intermediate (I), and resistant (R). The results were interpreted according to the recommended CLSI (2015) guidelines as follows: the isolates with a zone of inhibition less than or equal to 14 mm were considered as resistant (R) and those with more than 20 mm diameter as susceptible (S) and those having ZOI between 15 and 19 mm as intermediate (I).

Statistical evaluation: Three replicates of the disc diffusion method were carried out, and the recorded diameters are shown as resistant (R), sensitive (S), or intermediate (zone diameterin mm ± SD).

Results

A totalof 61 isolates were obtained, of which 25 were identified as lactic acid bacteria (LAB)(12 from Gir cow milk, 8 from Mehsana Buffalow milk, and 5 from Holstein Friesian cow milk) and 36 as non-LAB (18 from Gir milk, 10 from Mehsana milk, and 8 from Holstein Friesian cow milk). The non-LAB isolates were catalase-positive, oxidase-positive, and Gram-negative. For further investigation, 25 LAB isolates were used.These isolates were analyzed based on their morphological characteristics (Supplementary TableS1), and biochemical tests.Morphologically, some isolates appeared as cocci, while others displayed a rod shape under microscopic observation. The characteristic catalase and oxidase-negative twenty-five LAB were further screened using biochemical analysis and shown in Table 2 and represented in figure 1.

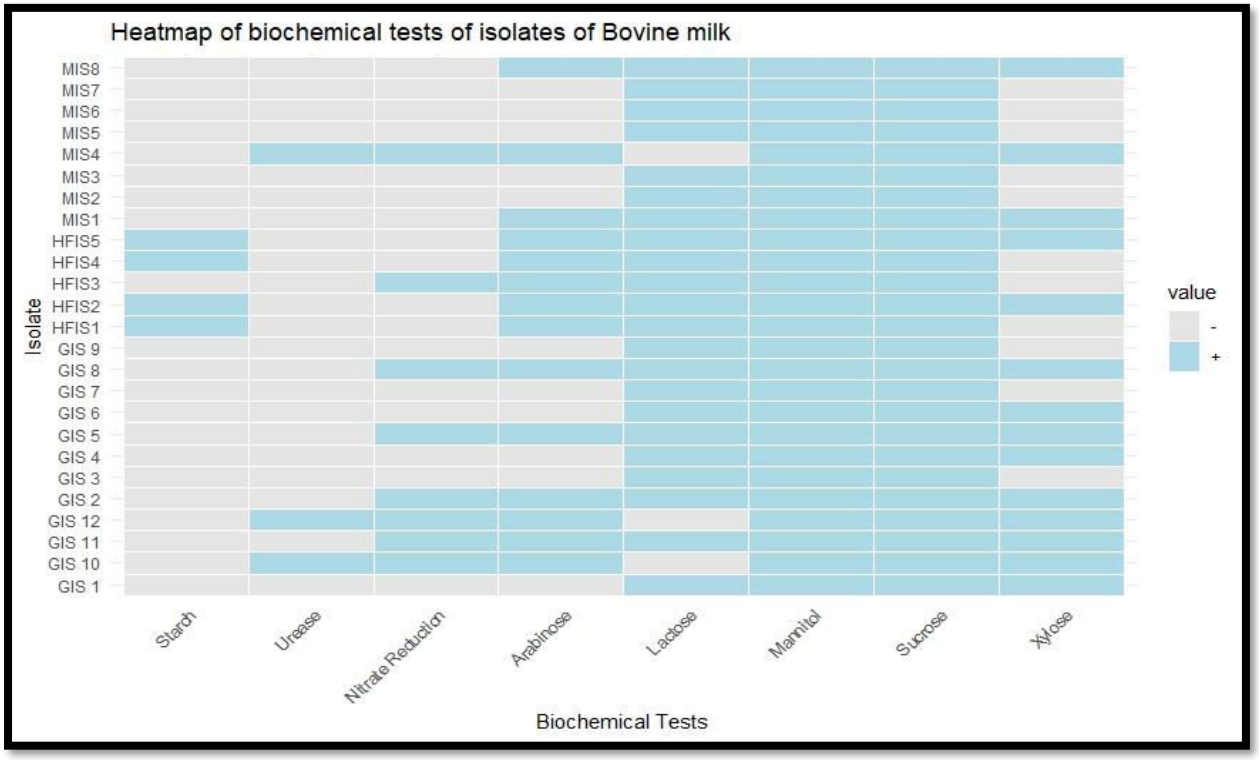
Table 2: Biochemical test of isolated LAB from bovine milk. '-' Negative; '+' Positive.

Source of isolate	Isolates	Starch	Arabinose	Lactose	Mannitol	Sucrose	Xylose	Nitrate Reduction	Urease
Gir cow Milk	GIS 1	-	-	+	+	+	+	-	-
	GIS 2	-	+	+	+	+	+	+	-
	GIS 3	-	-	+	+	+	-	-	-
	GIS 4	-	-	+	+	+	+	-	-
	GIS 5	-	+	+	+	+	+	+	-
	GIS 6	-	-	+	+	+	+	-	-
	GIS 7	-	-	+	+	+	-	-	-
	GIS 8	-	+	+	+	+	+	+	-
	GIS 9	-	-	+	+	+	-	-	-
	GIS 10	-	+	-	+	+	+	+	+
	GIS 11	-	+	+	+	+	+	+	-
	GIS 12	-	+	-	+	+	+	+	+
Mehsana Buffalo Milk	MIS1	-	+	+	+	+	+	-	-
	MIS2	-	-	+	+	+	-	-	-
	MIS3	-	-	+	+	+	-	-	-
	MIS4	-	+	-	+	+	+	+	+
	MIS5	-	-	+	+	+	-	-	-
	MIS6	-	-	+	+	+	-	-	-
	MIS7	-	-	+	+	+	-	-	-
	MIS8	-	+	+	+	+	+	-	-
Holstein Friesian cow milk	HFIS1	+	+	+	+	+	-	-	-
	HFIS2	+	+	+	+	+	+	-	-
	HFIS3	-	+	+	+	+	-	+	-
	HFIS4	+	+	+	+	+	-	-	-
	HFIS5	+	+	+	+	+	+	-	-

The biochemical analysis of lactic acid bacteria (LAB) isolates from Gir cow, Mehsana buffalo, and Holstein Friesian cow milk provides insights into their potential genus identification based on fermentation profiles and metabolic characteristics. In Gir cow milk, the isolates (GIS 1-12) predominantly exhibited negative results for starch and urease, with varying fermentation capabilities primarily for lactose and mannitol, suggesting the presence of genera such as *Lactococcus* and *Leuconostoc*. The Mehsana buffalo isolates (MIS 1-8) displayed

similar trends but showed a higher incidence of lactose fermentation, further supporting the likelihood of *Lactobacillus* species. Conversely, Holstein Friesian isolates (HFIS 1-5) revealed a more diverse profile, with some testing positive for starch and notable lactose fermentation, indicating the presence of *Lactobacillus* species. Overall, the combination of fermentation abilities, particularly with lactose and mannitol, along with the negative urease results, suggests that the isolates belong to various LAB genera.

Figure 1: Biochemical analysis of LAB isolated from bovine milk. Light blue indicates positive results and grey indicates negative results.



Further identification of LAB colonies isolated from bovine milk was carried out according to their protein and peptide registered information, and analysis using MALDI-TOF MS. All isolates were identified to species level with the help of MALDI TOF which is done by either comparing the Protein mass fingerprints (PMF) of unknown organisms with the PMFs contained in the database or by matching the masses of biomarkers of unknown organisms with the proteome database.This analysis utilized the bioMérieux-developed automated MALDI-TOF system with the VITEK® MS PRIME database. Table 3 shows the MALDI TOF MS identified LAB.

Table 3: Identification of LAB using MALDI-TOF MS.

Source of isolate	Isolates	Organism
Gir cow Milk	GIS 1	*Pediococcuspentosaceus*
	GIS 2	*Lactobacillus plantarum*
	GIS 3	*Lactococcus lactis*
	GIS 4	*Pediococcuspentosaceus*
	GIS 5	*Lactobacillus plantarum*
	GIS 6	*Pediococcuspentosaceus*
	GIS 7	*Lactobacillus delbrueckii*
	GIS 8	*Lactobacillus plantarum*
	GIS 9	*Lactobacillus delbrueckii*
	GIS 10	*Lactobacillus fermentum*
	GIS 11	*Lactobacillus plantarum*
	GIS 12	*Lactobacillus fermentum*
Mehsana Buffalo Milk	MIS 1	*Weisellaconfuse*
	MIS 2	*Lactobacillus casei*
	MIS 3	*Lactobacillus delbrueckii*
	MIS 4	*Lactobacillus fermentum*
	MIS 5	*Lactobacillus casei*
	MIS 6	*Lactobacillus casei*
	MIS 7	*Lactobacillus fermentum*
	MIS 8	*Weisellaconfuse*
Holstein Friesian cow milk	HFIS 1	*Leuconostocmesenteroide*
	HFIS 2	*Leuconostocpseudomesenteroide*
	HFIS 3	*Lactobacillus plantarum*
	HFIS 4	*Leuconostocmesenteroide*
	HFIS 5	*Leuconostocpseudomesenteroide*

Due to repeated sub culturing, some of the isolates were unable to grow. Whereas during MALDI-TOF identification, some of the isolates were identified as similar LAB species. Those isolates were not used in further studies.
Lactococcus lactis, *Lactobacillus plantarum*, and *Pediococcuspentosaceus*isolates from Gir cow milk samples; *Lactobacillus casei* and *Weisellaconfusa* isolate from buffalo Mehsana milk samples, and *Leuconostocpseudomesenteroide* & *Leuconostocmesenteroide* isolate from hybrid Holstein Friesian cow milk samplewere used for the further study.

Antibiotic disk susceptibility assays were performed according to the modified Kirby-Bauer method (Bauer et al 1966) and results were interpreted according to CLSI(2015) guidelines. The growth of all tested LAB isolates from the bovine milk

sample was homogenous over MRS agar, and the zone of inhibition was noticeably seen. The results of isolated LAB have been presented in (Table 4, Data on actual diameter of zone is shown in supplementary Table S2).

Table 4: Antimicrobial susceptibility profile of LAB isolated from bovine milk to antibiotics. The results were interpreted according to the recommended CLSI (2015) guidelines

Antibiotics	*Gir cow milk*			*Mehsana buffalo milk*		*Holstein Friesian cow milk*	
	Lactobacillus plantarum	*Pediococcus Pentosaceus*	*Lactococcus lactis*	*Lactobacillus casei*	*Weisella confusa*	*Leuconostoc mesenteroide*	*Leuconost pseudome*
Penicillin G	I	S	S	I	I	I	I
Oxacillin	R	R	R	R	R	R	R
Cephalothin	R	S	S	R	R	I	I
Clindamycin	I	S	S	S	S	I	I
Erythromycin	S	S	I	R	S	S	I
Amoxyclav	R	R	R	R	R	I	R
Vancomycin	R	R	R	R	R	R	R
Ofloxacin	R	R	R	R	R	R	R
Teicoplanin	R	R	R	R	R	R	R
Ceftazidime	R	R	R	R	R	R	R
Gentamycin	R	R	R	R	R	R	R
Cefoxitin	R	I	S	R	R	S	R
Tetracycline	R	R	R	I	S	I	R
Co-Trimoxazole	R	R	R	R	R	R	R
Cloxacillin	R	R	R	R	R	R	R
Lincomycin	R	R	I	R	R	R	R
Cefuroxime	R	I	S	R	I	S	R
Cefotaxime	R	R	S	R	R	S	R

LAB isolated from bovine milk showed a mixed response to tested antibiotics. *Lactobacillus plantarum, Lactobacillus casei, and Leuconostocpseudomesenteroide* from Gir cow milk, Mehsana buffalo milk, and HF cow milk, respectively, showed the most resistant patterns towards tested antibiotics. All isolated LAB from bovine milk were resistant towards oxacillin(semisynthetic penicillin), vancomycin (glycopeptide), ofloxacin (fluoroquinolone), teicoplanin (glycopeptide), ceftazidime (3rd generation cephalosporine), gentamycin (aminoglycoside), co-trimoxazole (sulphonamide), and cloxacillin (semisynthetic penicillin) antibiotics. Clindamycin is a lincosamide-class antibiotic that is effective against most of the Lactic Acid Bacteria (LAB) that were taken from cow's milk. About 4 out of 7 isolates that were tested were sensitive to the antibiotic. In contrast, efficacy of tetracycline was found against only one isolate, i.e., *Weisellaconfusa*.

Lactococcus lactis isolated from Gir cow milk showed sensitivity toward 6 antibiotics viz. penicillin G, cephalothin, clindamycin, cefoxitin, cefuroxime, and Cefotaxime (3rd generation cephalosporine); intermediate resistance to erythromycin (macrolide) and lincomycin (lincosamide) (Table 4, 5).*Lactobacillus plantarum*(Gir cow milk isolate) and *Lactobacillus casei*(Mehsana buffalo milk isolate) showed resistance to 15 antibiotics and intermediate resistance to 2 antibiotics each out of total 18 antibiotics tested.*Lactobacillus plantarum* showed sensitivity only to Erythromycin (macrolide) whereas*Lactobacillus casei* was sensitive to only Clindamycin.*Leuconostocpseudomesenteroide*from the HF milk sample showed resistance to 14 antibiotics and intermediate resistance to 4 antibiotics out of a total 18 antibiotics tested.*Leuconostocmesenteroide*(HF milk isolate) showed resistance to 9 antibiotics and intermediate resistance to 5 antibiotics(Penicillin G, cephalothin, clindamycin, tetracycline, and amoxiclav)out of a total of 18 tested antibiotics. It was sensitive to four antibiotics viz. erythromycin, cefoxitin, cefuroxime, and cefotaxime.

Table 5: Antibiotic sensitivity pattern of the LAB isolates

Isolate	No. of antibiotics		
	Resistant	Intermediate resistant	Sensitive
Lactobacillus plantarum	15	2	1
Pediococcuspentosaceus	12	2	4
Lactococcus lactis	10	2	6
Lactobacillus casei	15	2	1
Weisellaconfuse	13	2	3
Leuconostocmesenteroide	9	5	4
Leuconostocpseudomesenteroide	14	4	0

Pediococcuspentosaceus showed sensitivity to4antibioticsviz. penicillin G(β-lactam), clindamycin(lincosamide), oxacillin(semisynthetic penicillin), and cephalothin(1st generation cephalosporine);intermediate resistance to 2 antibiotics (2nd generation cephalosporin antibiotic viz. cefoxitin and cefuroxime) and resistance to12antibiotics.

Weisellaconfusa was sensitive to 3 tested antibiotics viz. clindamycin, erythromycin, and tetracycline; It showed intermediate resistance to 2antibioticsviz.penicillin G, and cefuroxime; and was resistant to remaining13 antibiotics tested.

Interestingly all isolated LAB showed mixed antibiotic susceptibility patterns as shown in Table 6.*Leuconostocpseudomesenteroide*is the only isolatethat did not show sensitivity toward the 18 tested antibiotics.

Discussion

The study evaluat ed the antibiotic susceptibility profile of LAB isolated from different bovine milks in the regions of Thane district, India. All the isolates used in the study were initially screened using morphological characteristics, and biochemical tests. They were further identified using MALDI-TOF MS. This quick, cost-effective, and dependable approach to bacterial characterization provides an important alternative to traditional methods, particularly for applications in the food industry.(Nacef M et al 2016).

For cell wall synthesis inhibitors such as β-lactams (e.g., Penicillin G, Cephalothin, Oxacillin, Amoxiclav), the key mode of action is inhibiting the synthesis of the bacterial cell wall by binding to penicillin-binding proteins (PBPs) involved in peptidoglycan cross-linking. However, differences in susceptibility can occur due to variations in PBPs. Some bacteria have low-affinity PBPs, which bind poorly to β-lactams, leading to resistance, as seen in *Lactobacillus plantarum*. Additionally, some bacteria produce β-lactamase enzymes that hydrolyze the β-lactam ring of these antibiotics, rendering them ineffective. This explains why certain strains, like *Lactococcus lactis and Lactobacillus casei*, are resistant. Moreover, the intrinsic structure of the cell wall in certain Gram-positive bacteria, including many lactic acid bacteria (LAB), may also confer resistance by preventing antibiotics from accessing their target.

For protein synthesis inhibitors, such as macrolides (e.g., Erythromycin, Clindamycin) and aminoglycosides (e.g., Gentamicin), which act by targeting the bacterial ribosome, differences in susceptibility can arise due to alterations in the ribosomal binding sites. Some bacteria develop resistance by mutating these sites or producing modification enzymes (Golakar T *et al* 2018). For example, *Lactobacillus casei* is resistant to Lincomycin but susceptible to Clindamycin, likely due to differences in how these antibiotics interact with the ribosome. Additionally, efflux pumps in certain bacteria actively expel antibiotics, lowering their intracellular concentration and effectiveness, as seen in *Lactobacillus plantarum* (Rossi F *et al* 201). For aminoglycosides like Gentamicin, bacteria may produce modifying enzymes that inactivate the drug(Zárate S *et al* 2018), explaining the resistance observed in *Weissellaconfusa.*

For fluoroquinolones like Ofloxacin, which target bacterial DNA gyrase or topoisomerase IV to inhibit DNA replication, resistance can occur due to mutations in the genes encoding these enzymes (George A. Jacoby 2005). These mutations prevent the antibiotic from binding, leading to resistance, as observed in *Lactobacillus plantarum and Leuconostocmesenteroides*. Efflux pumps may also

expel fluoroquinolones, contributing to resistance in species such as *Pediococcuspentosaceus.*

For cell membrane disruptors like glycopeptides antibioticssuch as Vancomycin, resistance often stems from changes in the bacterial peptidoglycan structure. For example, many LABs, including *Weissellaconfusa and Leuconostoc*species, modify their peptidoglycan precursors from D-Ala-D-Ala to D-Ala-D-Lac, preventing glycopeptides from binding and thus conferring resistance (Yuan S *et al* 2021). Additionally, some bacteria develop thickened cell walls, making it harder for Vancomycin to penetrate and act effectively(Zhang S *et al* 2018).

In the case of folate synthesis inhibitors like Co-Trimoxazole, which targets dihydropteroate synthase and dihydrofolate reductase, resistance can develop due to mutations in the target enzymes (). These mutations reduce the drug's binding ability and inhibit the bacterial folate pathway. Bacteria may also produce excess amounts of folate precursors, overcoming the blockage caused by the antibiotic. This explains the resistance observed in *Lactobacillus plantarum and Leuconostocmesenteroides.*

Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit,as seen in *Weissellaconfusa (*Ian Chopra and Marilyn Roberts 2001). Some bacteria also produce ribosomal protection proteins that dislodge tetracycline from its binding site, leading to resistance in organisms like *Leuconostocpseudomesenteroides(*Connell, S. R *et al* 2003*)*

For lincosamides like Clindamycin and Lincomycin, resistance often arises from the methylation of the 23S rRNA binding site, preventing the antibiotic from binding effectively (Marilyn C. Roberts 2008). This mayexplain why *Lactococcus lactis* shows intermediate resistance to Lincomycin but is still susceptible to Clindamycin. Efflux pumps may also play a role in reducing the intracellular concentration of lincosamides, as seen in *Leuconostocpseudomesenteroides.*

In some studies, by Sharma *et al.* (2017), curd isolates against oxacillin were recorded as susceptible or mildly effective; however, the scenario was reversed for all the bovine milk-isolated LAB*.Earlier*, Sharma *et al*. (2015) reported the susceptibility of different LAB species from different isolation sources to penicillin. In our study, *Lactococcus lactis and Pediococcuspentosaceus*were the only two isolates that showed susceptibility to penicillin G. Genes coding for β-lactam, which have been shown to transmit conjugally within distinct groups, are responsible for resistance to β-lactam. All of the LAB isolatesthat were tested showed a general resistance to oxacillin, which is consistent with the pattern found in the isolates of cheese Lactobacillus by Erdourul and Erbulur (2006).

The high resistance towards glycopeptides (teicoplanin, vancomycin) and quinolones (ofloxacin) exhibited by lactobacilli in this study can be substantiated by the reports supporting the presence of intrinsic resistance mechanisms towards both antibiotic families (Nawaz et al. 2011). Intrinsic resistance refers to the insensitivity of bacterial strains to the approved drug doses, regulated by permeability barriers and active efflux. Intrinsic resistance is usually non-transferable and poses no risk to LABs. Lactobacilli are known for their innately high resistance to several medicines, especially vancomycin. Resistance towards vancomycin is due to the presence of peptidoglycan precursors terminating in D-alanyl-D-lactate, preventing the binding of vancomycin (Gueimonde et al. 2013). Resistance to quinolones can be attributed to mutation in topoisomerase IV, which is the primary target for ofloxacin (Hummel et al. 2007). Our results are in co-occurrence with the reported results bySharma et al 2017), who also reported quinolone and glycopeptides resistance of maximum numbers of LAB isolates. Among cephalosporins; cephalothin, cefoxitin, cefuroxime, and cefotaxime are most effective against LAB isolated from bovine milk. The susceptibility of curd lactobacilli to cefotaxime was discovered by Halder and Mandel (2015). High cephalosporin resistance was studied by Ammor et al. 2007 in several investigations. Variants of broad-spectrum β-lactam and efflux pumps linked to cell wall impermeability (Delgado *et al.* 2005) are responsible for resistance to cephalosporins, a structural subtype of β-lactam antibiotics (Pfeifer *et al*. 2010).In our findings, some of the isolates such as*Pediococcuspentosaceus* and *Lactococcus lactis* from the gir cow milk sample and *Leuconostocmesenteroide*fromthe HF cow milk sample showedsusceptibility towards cephalosporins.The absence of cytochrome-mediated electron transport that prevents antibiotic uptake is another variable related to the resistance phenotype to aminoglycosides (Charteris *et al.* 2001). In our findings, all the isolates from bovine milk show resistance towards gentamycin.

All isolates except *Lactococcus lactis, lactobacillus casei, and Leuconostocpseudomesenteroide* showed sensitivity towards erythromycin. Resistance to erythromycin, which was a macrolide type of antibiotic inhibitsbacterial protein synthesis by binding to the 50s subunit of the bacterial ribosome. This disrupts the translation process and prevents the bacteria from producing essential proteins required for their growth and survival. All isolates except *Weisellaconfusa* showed resistance towards tetracycline. Klare *et al*. (2007) found that the vast majority of LAB isolates showed a significant amount of clindamycin resistance, which is by previous studies reporting resistant characteristics. Similar to this study except *Lactobacillus plantarum*, *Leuconostocmesenteroide,*and *Leuconostocpseudomesenteroide*, all other isolates showed resistance toward clindamycin. Karapetkov*et al* (2011) observed that Lactobacillus strains were susceptible to erythromycin, tetracycline, and clindamycin. According to Danielsen and Wind (2003), lactobacilli are resistant by

nature to sulphonamides (co-trimoxazole) and trimethoprim, both of which are inhibitors of nucleic acid synthesis.A combination of trimethoprim and co-trimoxazole has been extensively employed against different clinical scenarios in humans since the late 1960s. Owing to its low cost, low toxicity, availability through both oral and intravenous routes, and high bactericidal activity, it offers an attractive option, especially for developing countries (Goldberg and Bishara 2012). A marked resistance was seen in all the isolated LAB from bovine milk.

When comparing the AST results, reported earlier (Sharma *et al,*2017) shows a noticeable difference, possibly due to the environmental niche of the selected strains from the culture collection centre. These strains have had no direct exposure to antibiotics for decades, which may have allowed them to avoid developing drug resistance. So it is important to note that exposure to antibiotics plays a critical role in the development of resistance; effective utilization and maximizing compliance to specific medications may delay the development of antibiotic resistance. The inherent antimicrobial characteristics of LAB may have a synergistic effect when combined with antibiotic treatment, potentially enhancing the eradication of pathogenic bacterial strains.

Hence, we conclude that the current study will be the first step toward determining antibiotic resistance in isolated LAB from bovine milk, as the emergence of multidrug resistance in probiotics is a serious issue. LAB is an important component in dietary supplements and pharmaceuticals. Before considering a probiotic, its safety must be determined by evaluating its antibiotic resistance.

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Supplementary Table

Table S1: Morphological characteristics of bovine milk isolated LAB.

Source of isolate	**Isolates**	**Catalase**	**Oxidase**	**Gram nature**	**Cocci/ Rod**	**Motility**	**Colony characteristics**			
							Surface	**Shape**	**Margin**	**Colour**
Gir cow milk	GIS 1	-	-	+	Cocci	Nonmotile	Smooth, mucoid	Circular	Entire	Creamy white
	GIS 2	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	GIS 3	-	-	+	Cocci	Nonmotile	Smooth	Circular	Entire	Whitish
	GIS 4	-	-	+	Cocci	Nonmotile	Smooth, mucoid	Circular	Entire	Creamy white

	GIS 5	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	GIS 6	-	-	+	Cocci	Nonmotile	Smooth	Circular	Entire	Whitish
	GIS 7	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	GIS 8	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	GIS 9	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Off white
	GIS 10	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	GIS 11	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	GIS 12	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
Mehsana Buffalo milk	MIS1	-	-	+	Cocci	Nonmotile	Smooth, Mucoid	Circular	Entire	off-white
	MIS2	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	MIS3	-	-	+	Cocci	Nonmotile	Smooth, Mucoid	Circular	Entire	off-white
	MIS4	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	MIS5	-	-	+	Rod	Nonmotile	Smooth, Mucoid	Circular	Entire	off-white
	MIS6	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	MIS7	-	-	+	Rod	Nonmotile	Smooth, Mucoid	Circular	Entire	off-white
	MIS8	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
Friesian g	HFIS1	-	-	+	Cocci	Nonmotile	Slightly	Circular	Entire	off-white

							granular			
	HFIS 2	-	-	+	Cocci	Nonmotile	Smooth	Circular	Entire	off-white
	HFIS 3	-	-	+	Rod	Nonmotile	Smooth	Circular	Entire	Creamy white
	HFIS 4	-	-	+	Cocci	Nonmotile	Slightly granular	Circular	Entire	off-white
	HFIS 5	-	-	+	Cocci	Nonmotile	Smooth	Circular	Entire	off-white

Table S2: Antibiotic susceptibility assay of isolated LAB from bovine milk against antibiotics. The zone of inhibition was measured in mm, results represented Mean ± SD, n=3.

Sr. no.	Antibiotics	*Gir cow milk*			*Mehsana buffalo milk*		*Holstein Friesian cow milk*	
		Lactobacillus Plantarum	*Pediococcus pentosaceus*	*Lactococcus Lactis*	*Lactobacillus casei*	*Weisella confusa*	*Leuconostoc mesenteroide*	*Leuconostoc pseudomesenteroide*
1.	Penicillin G	17.3±1.15	26.7±1.15	29.0±1.73	17.3±1.53	19.0±1.73	17.0±1.73	17.7±1.53
2.	Oxacillin	2.3±0.28	1.7±0.67	4.7±1.15	1.7±1.15	1.3±0.58	11.3±1.53	3.7±1.15
3.	Cephalothin	7.3±1.15	19.7±0.58	20.7±1.15	7.3±1.15	10.0±1.00	19.0±1.73	18.3±0.58
4.	Clindamycin	15.3±0.58	28.3±1.15	24.0±1.73	21.7±0.58	20.0±1.73	18.7±0.58	19.0±1.73
5.	Erythromycin	22.3±0.58	22.7±1.53	17.3±1.53	13.7±0.58	23.7±1.15	20.0±1.73	17.3±1.15
6.	Amoxyclav	13.3±1.15	8.3±0.58	12.0±1.00	8.3±1.53	11.7±0.58	19.7±1.15	6.7±1.53
7.	Vancomycin	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition
8.	Ofloxacin	No zone of inhibitio	No zone of inhibitio	No zone of inhibition	5.0±1.73	4.0±1.0	12.0±1.00	2.7±0.58

		n	n					
9.	Teicoplanin	7.0±1.73	1.3±0.58	1.3±0.58	2.3±0.58	3.3±0.58	No zone of inhibition	No zone of inhibition
10.	Ceftazidime	4.7±0.58	1.0±0.0	10.3±1.15	3.3±0.58	8.7±1.15	12.7±1.15	5.7±1.15
11.	Gentamycin	6.7±1.15	13.7±1.53	10.3±1.15	6.3±0.58	3.7±1.15	13.3±0.58	10.7±1.15
12.	Cefoxitin	12.7±0.58	18.3±0.58	20.0±1.73	11.3±1.15	10.0±0.58	20.3±0.58	12.0±1.73
13.	Tetracycline	9.3±1.53	13.0±1.00	12.3±0.58	17.7±0.58	26.0±1.73	19.7±1.15	11.3±1.53
14.	Co-Trimoxazole	14.0±1.73	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition	No zone of inhibition
15.	Cloxacillin	No zone of inhibition	No zone of inhibition	3.3±0.58	3.7±1.15	3.7±1.15	7.0±1.73	13.±0.58
16.	Lincomycin	2.3±0.58	14.3±1.53	16.0±1.73	4.7±0.58	4.0±1.00	7.7±0.58	13.3±0.58
17.	Cefuroxime	14.0±1.73	18.7±1.15	27.0±1.73	8.7±0.67	18.3±1.53	20.3±0.58	14.3±1.15
18.	Cefotaxime	8.7±1.15	13.3±1.15	29.7±1.33	13.0±1.53	14.0±1.73	20.7±0.58	13.7±1.15