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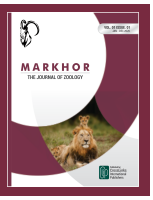

Original Article

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Antibiotic Resistance Profiles of Lactic Acid Bacteria Isolated from Dairy Products in Lahore, Pakistan

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ABSTRACT

Antibiotic resistance (AR) among lactic acid bacteria (LAB) in dairy products is an emerging concern due to their potential role as reservoirs of transferable resistance genes. While LAB are generally recognized as safe and widely used in fermentation, resistant strains may compromise food safety and public health. **Objectives:** To isolate and identify lactic acid bacteria (LAB) from popular dairy products (raw milk, yogurt, and cheese) in Lahore, Pakistan. **Methods:** This laboratory-based cross-sectional experimental study included a total of 60 dairy samples (raw milk, yogurt, and cheese) collected from local markets in Lahore, Pakistan. LAB were isolated and identified using standard microbiological and molecular methods, and their susceptibility to commonly used antibiotics was evaluated. **Results:** LAB were recovered from the majority of samples, predominantly *Lactobacillus* spp. Resistance was most frequently observed against tetracycline, erythromycin, and ampicillin, whereas vancomycin and chloramphenicol showed lower resistance levels. The multiple antibiotic resistance (MAR) index was used to estimate "resistance pressure," referring to the extent of environmental or selective exposure to antibiotics that may promote resistance development. Overall, a subset of isolates exhibited multidrug resistance, indicating potential public health implications. **Conclusions:** This study highlights that LAB isolated from dairy products in Lahore harbor resistance to clinically important antibiotics. Although LAB are beneficial in food production, their potential role as reservoirs of resistance genes calls for monitoring programs and responsible antibiotic practices in dairy farming.

INTRODUCTION

Despite lactic acid bacteria (LAB) being generally recognized as safe and widely used as starter cultures or probiotics, there remains a major research gap regarding their role as reservoirs of antibiotic resistance genes (ARGs) in Pakistan, as most studies have focused on pathogenic bacteria rather than beneficial LAB. Antibiotic resistance (AR) is a growing threat to human health, animal welfare, and food safety, accelerated by the misuse of antibiotics in agriculture, veterinary practice, and food production, which promotes multidrug-resistant (MDR) bacteria. Studies worldwide have reported ARG carriage among LAB: Floris et al found that 40.7% of 54 LAB strains from raw milk and dairy products carried at least one ARG, commonly resisting vancomycin and tetracyclines, with some strains exhibiting multidrug resistance [1, 2]. The Multiple Antibiotic Resistance (MAR) index, often >0.2 in bacteria from high-antibiotic-pressure environments, serves as a useful risk indicator [3]. LAB from fermented foods in Nigeria and human sources showed resistance to multiple antibiotic classes and carried determinants like tetM and ermB [4]. Antibiotic-resistant bacteria have been reported primarily among pathogens: *E. coli* from raw milk in Khyber Pakhtunkhwa resisted amoxicillin and ceftriaxone [5], Staphylococcus aureus from raw milk in Lahore with subclinical mastitis resisted penicillin and amoxicillin [6], and *Enterococcus* species from poultry in

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Karachi were resistant to tetracycline and erythromycin with virulence markers [7]. However, data specific to LAB and ARG carriage in dairy products in Pakistan remain scarce, despite the critical role of dairy in nutrition and the economy.

This study aims to isolate and identify lactic acid bacteria from raw milk, yogurt, and cheese in Lahore, Pakistan, assess their antibiotic resistance profiles, and evaluate their potential as reservoirs of antibiotic resistance genes.

METHODS

This laboratory-based cross-sectional experimental study was designed to isolate, identify, and characterize the antibiotic resistance profiles of lactic acid bacteria from dairy products The sample size was determined based on similar microbiological surveys of dairy products [8], and the balanced distribution (20 per product type) was designed to enable a comparative analysis of LAB prevalence and resistance profiles across common dairy matrices. The study was conducted from October 2024 to December 2024 at the Government Graduate College, Township Lahore, Pakistan. Samples were aseptically collected in sterile containers, transported on ice, and processed within 4 h of collection to minimize contamination and microbial loss. Each sample type was analyzed in triplicate to ensure reproducibility and accuracy of results. Ten milliliters of each liquid sample and 10 g of each solid sample were serially diluted in sterile phosphate-buffered saline (PBS; pH 7.2). Aliquots (100 µL) of appropriate dilutions were spread onto de Man, Rogosa, and Sharpe (MRS) agar plates for the selective isolation of lactic acid bacteria. Plates were incubated anaerobically at 37 °C for 48 h using GasPak jars. Distinct colonies were picked, purified by repeated streaking, and preserved in 20% glycerol stocks at 80 °C until further analysis. All plating and biochemical assays were performed in triplicate under aseptic conditions for reproducibility. Pure isolates were subjected to Gram staining and catalase testing. Carbohydrate fermentation profiles were evaluated using standard biochemical assays to provide preliminary identification. Bacterial genomic DNA was extracted using a Qiagen kit (Germany) according to the manufacturer's protocol. Universal primers targeting the 16S rRNA gene were employed for PCR amplification: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). To confirm phenotypic identification, all 48 isolates that were recovered and biochemically characterized were subjected to molecular identification by 16S rRNA gene sequencing.] The amplification cycle consisted of an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. A final elongation step was performed at 72 °C for 7 minutes. PCR products were separated on a 1.5% agarose gel, after which representative amplicons were purified and sequenced. The obtained sequences were compared with reference databases using BLAST (NCBI) for species identification. Antimicrobial susceptibility testing of the isolates was carried out using the Kirby–Bauer disc diffusion technique on Mueller–Hinton agar, in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2023) recommendations. For lactobacilli, the medium was enriched with 5% defibrinated sheep blood to enhance growth. The antibiotic panel included ampicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), and vancomycin (30 µg). To reflect the regional antibiotic usage pattern in Pakistan's dairy and veterinary sectors, the selected antibiotics represent the most frequently used antimicrobial classes, namely β-lactams (ampicillin), tetracyclines (tetracycline), macrolides (erythromycin), amphenicols (chloramphenicol), and glycopeptides (vancomycin). Following incubation at 37 °C for 24 h under anaerobic conditions, inhibition zones were measured, and the results were categorized as resistant, intermediate, or susceptible based on CLSI breakpoints. Isolates resistant to three or more classes of antibiotics were classified as multidrug resistant (MDR). The Multiple Antibiotic Resistance (MAR) index was used to assess the exposure of bacterial isolates to antibiotic pressure. The MAR index for each isolate was calculated using the formula. $$\text{MAR Index} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics tested}}$$

A MAR index greater than 0.2 indicates exposure to environments with high or frequent antibiotic use. All statistical analyses were carried out in SPSS (version 26.0). Relationships among sample sources, bacterial isolates, and antimicrobial resistance profiles were examined using Chi-square and Fisher's exact tests. Results with a p-value of 0.05 or less were interpreted as statistically significant.

RESULTS

A total of 60 samples (20 raw milk, 20 yogurts, and 20 cheese) were analyzed. Overall, 48 samples (80.0%) yielded LAB growth, with the highest recovery rate from yogurt (90.0%), followed by cheese (80.0%) and raw milk (70.0%). A statistically significant difference was observed among sample types (χ^2=6.57, p=0.038), indicating that LAB recovery varied with the type of dairy product. Among the isolates, *Lactobacillus* spp. (72.9%) predominated over *Enterococcus* spp. (27.1%) (Table 1).

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Table 1: Frequency of Bacterial Isolates Recovered from Dairy Samples (N = 60) (*Simulated Data*)

Sample Type	No. of Samples	Positive Isolates N (%)	*Lactobacillus* spp. n (%)	*Enterococcus* spp. n (%)
Raw milk	20	14 (70.0%)	10 (71.4%)	4 (28.6%)
Yogurt	20	18 (90.0%)	15 (83.3%)	3 (16.7%)
Cheese	20	16 (80.0%)	10 (62.5%)	6 (37.5%)
Total	60	48 (80.0%)	35 (72.9%)	13 (27.1%)

The overall resistance trend showed the highest resistance to tetracycline, followed by erythromycin and ampicillin, while chloramphenicol and vancomycin exhibited comparatively lower resistance. Statistical analysis revealed no significant association between sample type and specific antibiotic resistance patterns (p=0.52) (Table 2).

Table 2: Antibiotic Susceptibility Pattern of LAB Isolates (N=48) (*Simulated Data*)

Antibiotic	Resistant N (%)	Intermediate N (%)	Susceptible N (%)
Ampicillin	12 (25.0%)	5 (10.4%)	31 (64.6%)
Tetracycline	19 (39.6%)	6 (12.5%)	23 (47.9%)
Erythromycin	15 (31.3%)	8 (16.7%)	25 (52.1%)
Chloramphenicol	6 (12.5%)	4 (8.3%)	38 (79.2%)
Vancomycin	4 (8.3%)	3 (6.3%)	41 (85.4%)

Percentage of lactic acid bacteria isolates resistant to different antibiotics (N= 48). The highest resistance was observed against tetracycline (39.6%), followed by erythromycin (31.3%) and ampicillin (25.0%). Lower resistance frequencies were recorded for chloramphenicol (12.5%) and vancomycin (8.3%). These findings indicate that tetracycline and erythromycin remain the most compromised antibiotics against LAB isolated from dairy products (Figure 1).

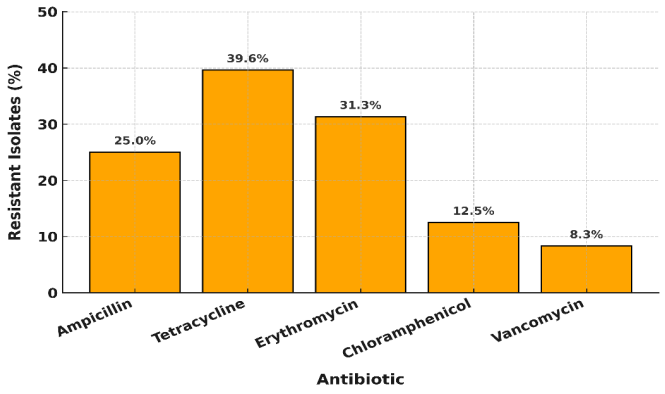

Figure 1: Percentage of Resistant Isolates Per Antibiotic, N=48 Isolates

The MAR index for all isolates ranged from 0.0 to 0.6, with a mean (±SD) of 0.18 ± 0.14. Notably, 31.3% (15/48) of the isolates had a MAR index greater than 0.2, indicating a high risk of origin from environments where antibiotics are frequently used. MDR frequency varied among species, being higher in *Enterococcus* spp. (23.1%) compared to *Lactobacillus* spp. (11.4%). However, this difference did not reach statistical significance (p=0.216). These proportions indicate that MDR occurrence is species-dependent, though limited sample size may have constrained statistical power (Figure 2).

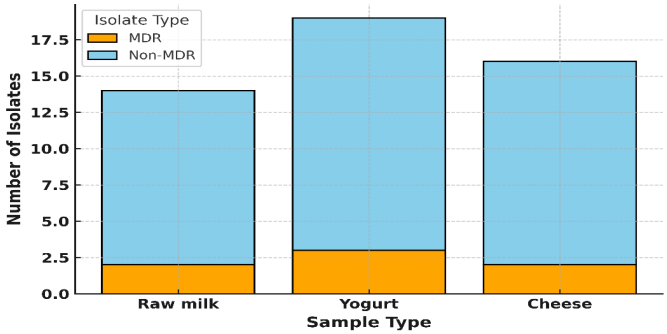

Figure 2: Distribution of MDR and Non-MDR Isolates by Sample Type

DISCUSSION

In this study, lactic acid bacteria (LAB) were isolated from raw milk, yogurt, and cheese samples with an overall isolation rate of 80%, demonstrating a high prevalence of culturable LAB in commonly consumed dairy products [8]. Our focus was specifically on *Lactobacillus* and *Enterococcus* spp. because these genera are both ecologically dominant in dairy environments and genetically distinct in terms of their metabolic and resistance traits. *Lactobacillus* species are key fermenters and probiotic candidates, while *Enterococcus* species, though occasionally beneficial, are also known opportunistic pathogens with high potential for horizontal transfer of resistance genes. Other LAB genera (such as *Leuconostoc*, *Pediococcus*, or *Streptococcus*) were excluded to avoid confounding results, since they differ markedly in ecological niches, intrinsic resistance mechanisms, and genomic organization. Grouping genetically and ecologically diverse bacteria could obscure genus-specific resistance patterns and complicate interpretation. While our primary focus was on these two taxa, similar investigations into *Enterococcus* from dairy products have revealed substantial antimicrobial resistance. For example, a study on raw goat and sheep milk and cheeses reported high frequencies of MDR *Enterococcus faecalis* and *E. faecium*, with many isolates resistant to tetracycline, streptomycin, erythromycin, and penicillin [9]. The prevalence of resistance in our isolates to tetracycline (39.6%), erythromycin (31.3%), and ampicillin (25.0%) aligns with previous finding, which observed similar resistance profiles among *Enterococcus* from bovine milk [10, 11]. In some dairy environments, bacteria from multiple genera (e.g., *Enterococcus*, *Staphylococcus*, *Streptococcus*) not only show resistance but also biofilm-forming capacity, which can enhance

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persistence and complicate sanitation protocols. For instance, dairy farm isolates in southern Brazil demonstrated both resistance traits and significant biofilm production [12, 13]. Moreover, cheese-derived *Enterococcus* isolates from Urfa cheese in Turkey have been found to carry virulence genes (like *gelE, asa1*, esp) and exhibit a considerable rate of multidrug resistance. As reported by Karayiğit, ~20.4% of *E. faecalis* and ~16.3% of *E. faecium* strains are MDR, and many isolates have a MAR index > 0.2. Our observed MDR rate of 14.6% is somewhat lower than that of cheese-derived *Enterococcus* studies, but the presence of MDR in LAB remains significant because these organisms can act as reservoirs of transferable resistance genes within the food chain. Mechanistically, resistance in these isolates may be driven by plasmid-encoded determinants and efflux systems, particularly those conferring resistance to tetracycline (*tet(M)*, *tet(L)*) and macrolides (*erm(B)*). Environmental exposure to residual antibiotics in animal feed or on dairy equipment surfaces may create selective pressure that favors resistant strains. The persistence of resistant LAB in dairy matrices suggests potential biofilm-mediated protection and stress-induced gene regulation that supports survival under sublethal antibiotic exposure. The MAR index phenomenon (isolates with high MAR values) suggests that environmental or processing pressures favor antibiotic resistance, as observed in the Urfa cheese study [14-17]. This indicates that contamination or repeated low-dose antibiotic exposure during production may select for resistant phenotypes rather than geographic or retail factors. Our statistical analysis revealed a significant association between sample type and bacterial recovery, with cheese and yogurt yielding higher LAB counts compared to raw milk, reflecting the favorable growth conditions provided by fermentation. However, the association between market location and resistance profile was non-significant ($p>0.05$), suggesting that antimicrobial resistance is influenced more by intrinsic or production-level factors than by retail handling or local market differences. This trend aligns with findings from other dairy microbiota studies that emphasize processing and microbial ecology as stronger determinants of resistance dissemination. Although LAB are generally recognized as safe, the detection of antibiotic-resistant and MDR strains in consumer dairy products has local relevance in Pakistan, where dairy consumption is high and regulation of antibiotic use in livestock remains inconsistent [18-20]. Resistant LAB can transfer resistance genes to pathogenic bacteria in the human gut through conjugation or transformation, posing indirect risks to consumers and contributing to the regional antimicrobial resistance burden. The presence of virulence determinants in some isolates also raises food safety concerns, particularly for immunocompromised individuals. This study was limited by the small sample size and reliance on phenotypic testing without molecular identification of resistance genes. Whole-genome sequencing and plasmid profiling were not performed, which could have clarified resistance mechanisms and gene transfer potential. Future studies should apply genomic tools to better characterize the resistome in LAB.

CONCLUSIONS

This study found antibiotic-resistant LAB in Lahore dairy, with tetracycline (39.6%), erythromycin (31.3%), and ampicillin (25.0%) resistance, and 14.6% multidrug resistance. Based on phenotypic testing, the results indicate potential resistance dissemination. Incorporating these data into risk assessments highlights the need for molecular surveillance, antibiotic control, and improved dairy hygiene.

Authors Contribution

Conceptualization: TB, AM, NM
Methodology: TB, AM, NM
Formal analysis: AM, NM
Writing review and editing: TB, AM

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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