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Research Article

Characterization and Antibiotic Resistance of Lactic Acid Bacteria Isolated from Raw Milk

Anshika Sharma*

Department of Microbiology, Lovely Professional University, Phagwara, India

ABSTRACT

Breast milk is an important source of nutrition for infants which is required for the growth and development. It helps in regulating gastrointestinal function, improving immune system and prevent from acute illnesses. Breast milk contains a beneficial bacteria including *Lactobacillus* species, which acts as a natural probiotic for the infant's gut. *Lactobacillus* is a gram positive, rod-shaped, facultative anaerobes. They are non-spore forming, acid tolerance bacteria. They play an important role in fermentation process contributing to the flavour, texture and prevention of the foods. *Lactobacillus* is associated with various health benefits like improved digestion, Irritable Bowl Syndrome (IBS).

Probiotics are live microorganisms that provide health benefits when consumed in adequate quantity. There are various probiotic bacteria such as *Lactobacillus, Bifidobacterium* and certain strains of *Streptococcus*. These microorganisms are naturally present in the foods and supplements. These probiotics helps in maintaining health balance of gut bacteria, stimulate immune system, reduce the risk of illness.

Due to the excessive use of lactic acid bacteria in food manufacturing or in some other perspective it naturally achieved the antimicrobial resistance. The potential of LAB species to transmit genes for antibiotic resistance to disease renders them a significant threat, surpassing the innocent. Thus, caution is important to emphasize that the lactic acid bacteria utilized in the food sector do not carry genes for resistance and unconsciously are required.

This review explores the antibiotic susceptibility of Lactic Acid Bacteria (LAB) sourced from diverse milk types, including cow, goat, donkey, buffalo, sheep, camel and human milk. LAB serve essential functions in regulating gastrointestinal health, enhancing immunity and preventing acute illnesses. The study compares antibiotic resistance among various LAB species isolated from milk samples. Notably, two Lactobacilli species, *Lactobacillus johnsonii* and *Lactobacillus zeae*, were investigated for their detection and Antimicrobial Resistance (AMR) in raw milk (from cows, sheep, and goats). While Lactobacilli are commonly found in fermented dairy products and contribute to gut health, the emergence of AMR in these bacteria raises concerns about potential resistance transmission through the food chain.

Keywords: Antimicrobial resistance; *Lactobacillus johnsonii*; *Lactobacillus zeae*; Human colostrum; Cow milk; Sheep milk; Goat milk

INTRODUCTION

Most microorganisms possess distinct metabolic requirements, milk from mammals provides them with a rich medium that allows them to grow and thrive. Milk, composed of carbohydrates, fat, casein, protein, vitamins and minerals, is a nutrient-rich growing medium for a variety of bacteria. After being first isolated from milk, Lactic Acid Bacteria (LAB) were later found to be a naturally occurring microflora in raw milk. Further, milk-based products naturally contain it. Lactic acid is a primary by-product of sugar fermentation and LAB are gram-

Correspondence to: Anshika Sharma, Department of Microbiology, Lovely Professional University, Phagwara, India; E-mail: anshika252001@gmail.com

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positive, non-spore-forming bacteria used as starting cultures. Lactic acid bacteria that are facultative or anaerobic aerobic rods or cocci are found in raw milk and are a vital component of many food and feed fermentations. These bacteria are also found in other natural environments [1]. Fermented dairy products often contain LAB species, such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus* and *Streptococcus*.

Numerous vital nutrients, such as hormones, lipids, oligosaccharides, microRNAs, immune-mediated factors and many other therapeutically active substances, are found in human breast milk. For infants, it is regarded as the optimal source of nutrition. Especially during nursing, breast milk possesses a critical role in regulating gastrointestinal function, enhancing the immune system and preventing acute infections like acute otitis media. Cow's milk comprises up 90% of the milk on the market, while the remaining 10% composed of various forms of milk.

Probiotics are defined as a kind of “live microorganisms which when administered in adequate amounts confer a health benefit on the consumer”. Probiotic strains' ability to proliferate through a stomach passageway, settle in the Gastrointestinal Tract (GIT) and outcompete pathogens determines their advantageous effects [2]. Certain qualities, such the capacity to flourish and establish colonies in a variety of environmental settings, are essential for robust probiotic isolates. The characteristics of probiotic bacteria, such as their resistance to bile salts and stomach acidity, determine their capacity to survive in the gastrointestinal tract.

LABs have a long history of being regarded as one of the most significant probiotics due to their wide range of positive effects on humans as well as animals. They also have a strong tolerance for low pH. LABs have been reported to have immunostimulatory, anti-cancer, anti-cholesterol, anti-depression, anti-anxiety, anti-obesity and anti-diabetic properties. LABs are thought to have an intriguing role in the respiratory system. Even though a variety of functional LABs have been used in probiotic fermented foods supplied commercially all over the world, there is still a significant need for bio- functional products derived from LABs.

Antimicrobial resistance can be naturally achieved and propagated through the use of lactic acid bacteria, which have become popular as probiotics in food manufacturing in recent years. The primary concern is the transmission of antibiotic resistance genes from commensal bacteria to pathogenic bacteria through lactic acid bacteria. Therefore, current research is examining the potential reservoir functions of lactic acid bacteria [3].

LAB species pose a serious threat in addition to being harmless because of their capacity to spread antibiotic resistance genes to other diseases. Thus, precautions must be made to ensure that lactic acid bacteria employed in food manufacturing lack resistance genes and policies that restrict the use of antibiotics that are undesirable and unnecessary are required. The creation of novel antibiotics or combination therapies to fight infections is another issue. In chemotherapy, Multi-Drug Resistance (MDR) is a significant issue. Identifying an isolate's multidrug resistance pattern is essential to overcoming this issue.

This review focuses on the antibiotic susceptibility of LAB from various milk sources and the emergence of antimicrobial resistance in specific Lactobacilli species.

MATERIALS AND METHODS

Antibiotic susceptibility of Lactic Acid Bacteria (LAB)

Milk samples were taken from a variety of animals, including humans, camels, goats, cows, buffalo, sheep and donkeys. These samples were sent to the lab using cold-chain protocols. The raw milk samples were homogenized in a 10 ml sample/90 ml Ringer's solution and dilutions between 10^{-3} and 10^{-6} were then made. Lactic Acid Bacteria (LAB) members were isolated using the double-layer sandwich method with the MRS and M-17 medium [4].

On MRS and M-17 agar petri dishes, typical colonies with creamy and smooth morphology were picked at random. These colonies were classified as LAB species as they showed a pH range of 3.5-5.0 in MRS broth, were gram-positive and were catalase-negative. Nine distinct standard antibiotic discs were used to test 42 LAB species from milk samples for antibiotic resistance. Using the Kirby Bauer disc diffusion technique, the LAB members' reproductive densities were adjusted to 0.5 Mac mice after they were resurrected in stock cultures of M17 and MRS liquid diets. Next, fresh cultures were used to inoculate 0.1 ml of MRS agar onto MRS agar and M17 agar plates, ensuring to spread it evenly. On the surfaces of petri dishes cultivated from these cultures, commercial antibiotic paper discs containing 30 µg of chloramphenicol, 10 µg of gentamicin, 10 units of penicillin G, 5 µg of streptomycin, 30 µg of tetracycline, 30 µg of kanamycin, 30 µg of teicoplanin and 30 µg of rifampin were placed at the appropriate intervals. Following the instructions provided by the Clinical and Laboratory Standards Institute (CLSI) the results were assessed in terms of the areas in which the isolates' inhibition zones against antibiotic discs.

Antibiotic susceptibility testing

Although Lactic Acid Bacteria (LAB) are essential to the fermentation processes of food, concerns have been raised about their resistance to antibiotics. The majority of study focuses on pathogenic microbes, however new results show that commensal LAB is safe. Antibiotic resistance genes, such as tetracycline, erythromycin and vancomycin, have been detected in strains of *Lactobacillus*, *Enterococci* and *Lactococcus lactis* that originate from fermented foods. In order to stop horizontal transmission of infections and the human gut microbiota, safety considerations place a strong emphasis on monitoring resistance. Resistance-free LAB is advised by the world health organization for use in the food industry. The majority of the LAB isolates that were discussed were resistant to penicillin G but still susceptible to tetracycline, penicillin and kanamycin. Preserving food safety and stopping the spread of resistant genes continue to be top concerns [5].

Maximum MDR value for LAB isolate from human milk was calculated to be 0.7 (resistant to antibiotics such as TE, C, S, CN, RD and K) or 0.6 (for h1> resistant to antibiotics such as TE, C, CN, P and K). Donkey milk isolates like D1 and human milk isolates like H1, H4 and H5 were discovered to have MDR due to antibiotic resistance levels of four or six. At least two antibiotics were discovered to be resistant in the isolates, with an MDR value of 0.2. Of the six LAB species isolated from camel milk, four had an MDR value of >0.1. They were discovered to be especially resistant to the antibiotic penicillin.

Four of the isolates from sheep milk were particularly resistant to kanamycin and all of the isolates showed an MDR value more than 0.1. At least three of the LAB members that were isolated from cow and buffalo milk had single antibiotic resistance or an MDR value of 0.1. Furthermore, it was shown that two LAB isolates from buffalo milk were resistant to three antibiotics with an MDR value of 0.3. Additionally, three LAB isolates with an MDR value of 0.2 from cow's milk showed resistance to the Antibiotics Ciprofloxacin (CIP), Penicillin (P) and Sulfamethoxazole (S). Three LAB strains, specifically resistant to Tetracycline (TE), Rifampicin (RD) and chloramphenicol antibiotics, exhibited an MDR value of 0.3 in goat milk isolates. Two further isolates of goat milk, particularly resistant to Kanamycin (K), with an MDR value of 0.2 (Table 1).

Table 1: Antibiotics and their isolates resistant's.

Antibiotic	Isolates resistant
Penicillin	18
Kanamycin	16
Gentamicin	14

Lactobacillus johnsonii and *Lactobacillus zeae* antimicrobial resistance in raw milk

Identification and confirmation: Researchers in eastern Slovakia examined *Lactobacillus* strains in a study using milk samples from sheep, goats and cows. They used the STN EN ISO 6887-1 (2017) guidelines to isolate these strains. A total of sixty milk samples were examined after being freshly milked, collected and kept. Spreading the inoculum over de Man, Rogosa, and Sharpe agar (MRS) medium and then incubating it anaerobically at 37°C were the steps in the isolation process. Colonies under suspicion that fit certain criteria were chosen for additional examination. Two techniques were used in the study to identify the genus and species: PCR (Polymerase Chain Reaction) and MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). By employing genome-based comparisons, these methods enabled the accurate classification of *Lactobacillus* strains, including those belonging to unique speciation like *Lactobacillus chiayiensis* sp. nov. and "*Lactobacillus zeae*".

To prepare samples for MALDI-TOF-MS identification, ethanol and formic acid were used. For analysis, the flex analysis software (version 3.0) and the Ultraflex III apparatus were used. Mass spectra similarity was assessed using Biotyper software (version 1.1), which produced identification reliability scores. A score of more than 2.30 denoted dependability at the species level and scores ranging from 2.00 to 2.29 indicated identification at the genus level. Less than 1.70 was an unreliable score. Using Chelex 100, DNA was isolated from multiplied isolates for confirmation *via* PCR. Using a 250 bp amplified fragment, particular primers (LbLMA and R16) were used to identify the genera *Lactobacillus* spp. ZeaI (TGT TTA GTT TTG AGG GGA CG) and ZeaII (CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA) primers, which are specific for *Lb. zeae* strains with amplified fragment sizes of 185 bp, were utilized to identify the species. *Lb. johnsonii* was identified using JohSI (GAC CTT GCC TAG ATG ATT TTA) 16SII (ACT ACC AGG GTA TCT AAT CC). These primers delimited a specific sequence of 750 bp [6].

Genes associated with resistance: A semi-quantitative E-test approach was utilized in this investigation to evaluate the phenotypic expression of antimicrobial resistance in *Lactobacillus* strains against Ampicillin (AMP), Erythromycin (ERY) and Clindamycin (CLI). The procedure was adding 10% MRS agar-enriched Müller-Hinton agar medium to which an adjusted inoculum was added. After applying test strips with a predetermined amount of antibiotics to the agar surface, an inhibition zone in the shape of a teardrop developed during incubation. The manufacturer's instructions served as the basis for determining the Minimum Inhibitory Concentration (MIC) values. Furthermore, the Broth Microdilution Method (BMM) was used to assess antimicrobial resistance using different antibiotic concentrations. The E-test was read at the moment of total inhibition of all growth and the results were evaluated in accordance with CLSI recommendations. A semi-quantitative E-test approach was used to evaluate the phenotypic expression of antimicrobial resistance in *Lactobacillus* strains against Ampicillin (AMP), Erythromycin (ERY) and Clindamycin (CLI). The procedure was adding 10% MRS agar-enriched Müller-Hinton agar medium to which an adjusted inoculum was added. After being sterilely applied to the agar surface, test strips containing a predetermined amount of antibiotics created a teardrop-shaped zone of inhibition. The manufacturer's instructions served as the basis for determining the Minimum Inhibitory Concentration (MIC) values. Furthermore, the Broth Microdilution Method (BMM) was used to assess antimicrobial resistance using different antibiotic concentrations. The E-test was read at the moment of total inhibition of all growth and the results were evaluated in accordance with CLSI recommendations. Additionally, the ermB gene, which is the genetic determinant of erythromycin resistance, was found in molecular PCR method was used for accurate identification at the genus and species level (Tables 2 and 3).

Table 2: Identified strains of *Lactobacillus* sp. and *Lb. johnsonii* and *Lb. zeae* species in milk samples.

		Lactobacillus sp.	*Lb. zeae*	*Lb. johnsonii*
Cows' milk	MALDI-TOF-MS	131	8	10
	PCR	128	8	10
Sheep's milk	MALDI-TOF-MS	80	5	10
	PCR	77	5	7
Goats' milk	MALDI-TOF-MS	41	6	6
	PCR	40	5	6
Σ	MALDI-TOF-MS	252	19	26
	PCR	245	18	23

Table 3: Antibiotic resistance.

Milk source	Penicillin resistant (%)	Kanamycin resistant (%)	Gentamicin resistant (%)
Cow	10.7	14.3	7.1
Goat	14.3	21.4	14.3
Donkey	21.4	14.3	14.3
Buffalo	7.1	14.3	7.1
Sheep	14.3	14.3	7.1
Camel	14.3	14.3	14.3
Human	14.3	14.3	14

RESULTS AND DISCUSSION

Strains of Lactic Acid Bacteria (LAB), which are frequently classified as Generally Recognized as Safe (GRAS), are essential components of probiotics and are being studied for potential health advantages when consumed by humans and animals. Nevertheless, certain strains of LAB have grown resistant to drugs used in veterinary and human medicine. Antibiotic resistance may spread as a result of the possible transmission of these resistance genes to harmful bacteria. Although LAB species are generally thought to be innocuous, there have been isolated reports of serious diseases such as meningitis, pleuropneumonia, endocarditis are caused by LAB [7]. To determine their level of antibiotic resistance and guarantee safety, LAB strains indicated for use as feed additives must be closely observed. Regulatory agencies like as the European Food Safety Authority (EFSA) closely assess LAB strains in order to address antimicrobial resistance concerns. Numerous studies have documented the antibiotic resistance of *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus reuteri* and *Lactobacillus acidophilus* in food. The aph-IIIa and ant genes, which are linked to pathogenicity, were discovered to be present in starter cultures that were recovered from yogurt in China. This finding underscores the significance of this field of study for public health. Moreover, Lactobacilli, Streptococci, Lactococci and *Leuconostoc* sp. have all been shown to exhibit intrinsic streptomycin resistance, indicating a minimal probability of transmission without raising any particular safety issues. This suggests that the level of streptomycin resistance in these isolates might be an inherent characteristic, akin to what is seen in members of LAB, meaning there is no risk [8].

Researchers used the Broth Microdilution Method (BMM) and the E-test to look into antibiotic resistance in *Lactobacillus* strains. Three antibiotics were tested: Erythromycin, clindamycin and ampicillin. The resistance rates of *Lb. johnsonii* and *Lb. zeae* were 33.3% and 34.8%, respectively. Erythromycin showed the greatest degree of agreement between the approaches, but ampicillin and clindamycin showed lower levels of agreement. Similar differences between the two methods have been documented in other investigations, highlighting the necessity of taking method-specific variances into account. Overall, the BMM approach verified antibiotic resistance in Lactobacilli, while the E-test tended to provide lower MICs for specific antibiotics. A major concern with Lactobacilli is antimicrobial resistance. Sixty-four percent of the Lactobacilli isolates showed resistance to three or more antimicrobial classes. Furthermore, 43.5% of isolates exhibited erythromycin and lincomycin cross-resistance. In 6% of cases, simultaneous resistance to gentamicin and streptomycin was found. It was discovered that erythromycin, lincomycin and tetracycline were acquired resistant. Lactobacilli strains were also reported to exhibit poly and multi resistance [9]. Notably, *erm (A)*, *erm©*, *erm(T)* and *erm(B)* are the main genes for erythromycin resistance. Lactobacilli have been shown to be more resistant to erythromycin and the phenotypic presentation of antimicrobial resistance has been found to be associated with the presence of the *ermB* gene. Over one-third of the forty Lactobacillus isolates that Drago et al., proved to be resistant to erythromycin were largely linked to the *ermB* gene.

These studies highlight the importance of monitoring antibiotic resistance in LAB, especially those with probiotic properties. The presence of resistant strains in raw milk and their potential spread through the food chain is a concern. Further research is needed to address the impact of antibiotic-resistant LAB on food safety and human health [10].

CONCLUSION

The review article emphasizes the possible dangers of Antimicrobial Resistance (AMR) in Lactic Acid Bacteria (LAB) that have been isolated from different sources of milk. Live Cultures (LAB) are found in raw milk by nature and are essential to the fermentation process of dairy products. But because of their overuse in food production, Antibiotic-Resistant Bacteria (AMR) have emerged, which has raised concerns about the spread of resistant strains throughout the food chain.

The study discovered that LAB isolates from a variety of milk sources, including human, cow, goat, sheep, buffalo and donkey milk, had differing levels of antibiotic resistance. Interestingly, strains of *Lactobacillus johnsonii* and *L. zeae* that were isolated from raw milk samples from sheep, goats and cows showed notable resistance to antibiotics such as ampicillin, clindamycin and erythromycin. The phenotypic expression of AMR in these *Lactobacillus* strains was linked to the presence of resistance genes, including *ermB*.

The findings underscore the potential risk of AMR transmission from commensal LAB to pathogenic bacteria through the food chain, posing a threat to food safety and human health. Continuous monitoring and surveillance of AMR in LAB, especially those used as probiotics or in food production, are crucial to mitigate these risks and ensure food safety.

FUTURE PERSPECTIVES

Further research is needed to understand the mechanisms and genetics of AMR in LAB, as well as the potential for horizontal gene transfer of resistance determinants to pathogenic bacteria. Developing strategies to minimize the use of antibiotics in food production and identifying alternative antimicrobial compounds or combination therapies are essential to combat AMR in LAB and other microorganisms. Strict regulations and guidelines should be implemented to ensure that LAB strains used in the food industry are free from AMR genes and do not contribute to the dissemination of resistance. Consumer awareness and education about the potential risks associated with AMR in food products are also necessary to promote responsible use and consumption of probiotics and fermented foods. Overall, the review emphasizes the importance of addressing AMR in LAB to maintain food safety and prevent the potential public health consequences associated with the spread of resistant strains through the food chain. A multifaceted approach involving research, regulation and public awareness is crucial to tackle this emerging challenge effectively.

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