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Research Article

LACTOBACILLI STRAINS EXHIBITING PROBIOTIC PROBABILITY IN ORGANIC FERMENTED COW MILK

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ABSTRACT
Lactobacilli strains from fermented organic cow milk samples were collected from two different sites. Fermented organic cow milk was identified and characterized using morphological, and biochemical tests, and carbohydrate fermentation systems. Pure colonies further isolated Lactobacilli and were further identified *Lactobacillus acidophilus and Lactobacillus latics* and selected for antimicrobial activity and antibiotic sensitivity. *Lactobacillus acidophilus* had higher resistance to one of the antibiotics used (Chloramphenicol 30µg) with no zone of inhibition to Ampicillin, Penicillin, and Streptomycin. The antimicrobial activity in contrast to *Escherichia coli* exhibited varying degrees of inhibitory activity. Through this experimental study, it can be established that the presence of these microorganisms in fermented organic cow milk is of abundant benefits to humans and animals either as supplements or food production processes.

Keywords*: Lactobacillus latics, Lactobacillus acidophilus, Antibiotics, probiotic potential, organic fermented, cow milk*

INTRODUCTION
Lactic acid bacteria are abundant residents of fermented food products, such as milk as well as the gastrointestinal tracts of organisms. They play a pivotal role in maintaining a harmonious microbiota within the host's digestive system. This group of bacteria has the ability to colonize the gastrointestinal tract and start fermenting carbohydrates. This fermentation leads to the production of lactic acid as the main metabolic byproduct, thereby aiding the digestion process. Additionally, Lactic acid bacteria offer several advantages to the host, including the inhibition of pathogenic microorganisms through the generation of inhibitory substances (metabolites) and the formation of a protective bio-film that protects the integrity of the intestinal mucosal membrane (Marteau *et al.,* 2001, Ogawa *et al.,* 2001).
The consumption of milk and its byproducts as dietary items dates back thousands of years. Although it was initially devoid of scientific justification, the practice gained attention when Bulgarian peasants, who regularly consumed substantial quantities of soured milk as part of their daily diet, exhibited increased longevity (Metchnikoff., 1907). Dairy products containing viable lactic acid bacteria have been employed as prophylactic agents for the treatment of intestinal infections in adults afflicted with Salmonella and lactose intolerance (Ogunshe *et al.,* 2008).
Probiotics refer to living microorganisms that confer health advantages to their hosts by enhancing the balance of intestinal microflora (Naidu *et al.,* 1999). Numerous lactic acid bacteria species, such as Lactobacillus, Streptococcus, Enterococcus, and Pediococcus, have been identified as promising candidates for probiotic use in both human and animal populations, as demonstrated by various researchers (Fuller, 1991).
Lactobacilli as potential probiotics for enhancing health in food and pharmaceutical products necessitates a comprehensive in vitro evaluation based on specific criteria. These criteria include assessing antibiotic tolerance, bile tolerance, the capacity to inhibit the growth of other microorganisms, and resistance to the challenging conditions of gastric juice, facilitating their establishment in the intestinal tract. Therefore,

this ongoing investigation was commenced with the objective of isolating and identifying lactobacilli strains derived from fermented organic cow milk. Subsequent in vitro experiments were then undertaken to evaluate their antibiotic and bile tolerance, as well as their inhibitory effects on other microorganisms.

MATERIALS AND METHODS

A natural process involving the action of specific microorganisms like *Lactobacillus acidophilus* and *L. lactis*, transforms milk components into a complex mixture of peptides and bioactive compounds. When whey, the liquid portion of milk, undergoes fermentation, it yields a diverse array of peptides with unique amino acid sequences and bioactivities.

Sample Collection

Organic milk, sourced from cows raised without the use of synthetic pesticides and antibiotics, from two different sites. The samples were packed in 1 lt. glass bottles and Collected milk were packed in thermoelectric cooler and immediately transported to the laboratory for further use by air-conditioned car.

Preparation of fermented milk

Firstly, the milk samples were heat treated (90-95°C for 30-45 min) to destroy bacteriophages, and then cooled prior to inoculation. Each fermentation batch 100ml equilibrated for 1 hour at 37°C. Probiotic bacteria were obtained and used to ferment organic milk. *Lactobacillus acidophilus* and *L. lactis* were obtained from MTCC, Chandigarh, India. All initial freeze-dried cultures were propagated in MRS broth (10% v/v) anaerobically at 37ºC (anaerobic jar containing Anero Gensatchet (Himeida) for 48 hours. All media was prepared by manufacturer's instructions then autoclaved at 121ºC for 15 min using autoclave. Sterilized media was stored at 4ºC prior to use.

Starter cultures of Lactobacillus lactis and L. acidophilus were used to accelerate fermentation in milk in two different batches. This anaerobic fermentation carried out 42 hours at 37°C for both the milk batches. Organic milk samples were adjusted to pH 4.6 by using 1M HCl in duplicate.

Isolation and Characterized

20ml each of the samples from fermented organic cow milk and were homogenized in sterile normal saline. Serial dilution (10^{-6}) was made by using sterile pipette by transferring 1ml of normal saline culture from 10ml into 9ml diluent in sterile tube. Using MRS agar, appropriate dilutions were pipetted and plated out in order to count the lactic acid bacteria. The plates were placed in an inverted configuration and incubated at 37˚C for 48 hours. After the incubation period, the colonies were counted and recorded as colony-forming units (CFU). Repeated streaking on agar plates was used to sub-culture and purify morphologically different colonies. On MRS agar slants, lactic acid bacteria cultures were kept at 4˚C and sub-cultured every 4 weeks. (Philip. *et al.*, 2017, Taye *et al.*, 2021).

After 48-hour incubation period, isolates were characterzed by macroscopic analysis for form, elevation, size, and pigmentation; microscopic analysis by gram staining and other biochemical techniques (Harrigan *et al.*, 1976, Kandler *et al.*, 1986). The isolation process involved the use of morphological traits, such as colony and cell morphology, on selective media. Biochemical tests, including Gram reaction, catalase test, indole test, and glucose acid production, were employed. Only bacteria exhibiting Gram-positive characteristics and negative catalase responses were identified. To purify representative isolates, streaking was performed multiple times on the same agar substrate.

The standardized API 50 CH kit (Biomerieux) was utilized to quickly identify various strains and distinguish lactic acid bacteria isolates at the strain level. The resulting reactions, identified by distinctive color changes within each well, were scrutinized and categorized as either negative, and positive.Identification was made after analyzing the result patterns using a numerical profile.

The antibiotic sensitivity test was performed as described by (Bauer *et al.*, 1966). MRS agar plates were prepared with 0.1 ml for both the identified Lactobacilli strains. Standard antimicrobial susceptibility test discs (Sigma-aldrich) were prepared and applied to the surface of the plates and incubated for 18 hours at 37˚C. Following incubation, zones of inhibition surrounding the discs were measured. The antimicrobial effect of all isolated Lactobacilli species against Escherichia coli (indicator bacteria) was determined by

the disc diffusion method.The effects of bile on the growth of the probiotic strains were determined (NCCLS, 1999). Bile salt solutions (0.5% and 2.0% conc.) were prepared by dissolving 0.5 g and 2.0 g of sodium deoxycholate in 100 ml distilled water each. Turbidity from cell-lysis was examined after incubating for 4 hours and Gram-staining.

RESULTS

MRS media were used to retrieved the *Lactobacillus* strains from fermented cow milk. The isolates were subjected to standard biochemical techniques for identification, as outlined in Table 1. The catalase test indicated the absence of catalase production in all isolates, affirming their Gram-positive nature and incapacity for citrate utilization (negative response).

Supplementary identification was conducted using the API-50 CHL system (Table 2). The first microtube served as a negative control without an active carbohydrate substrate. Enzymatic breakdown of sugar by the entire microbial population was demonstrated by a color shift from purple to pale yellow. Although fermentation patterns varied across substrates, *Lactobacillus acidophilus* was identified as Lactic Acid Bacteria 1, while Lactic Acid Bacteria 2 was classified as *Lactobacillus lactis*. The identified isolates were preserved on MRS agar slants and stored at 2-8˚C.

Table 1. Morphological and Biochemical Characteristics of Isolated Microorganisms.

Isolate	**Characteristics on Agar Plates**	**Microscopic Characteristics**	**Growth (40˚C)**	**Citrate Test**	**Catalase Test**	**Indole Test**
Lactic Acid Bacteria 1	Small, flat, smooth, fuzzy	Gram positive, singly and tapering end	+	-	-	-
Lactic Acid Bacteria 2	Small, flat, cremated, creamy colour	Gram positive, rods, singly and short chains	+	-	-	-

(+): Positive Reaction, (-): Negative Reaction.

Table 2. Identification (%) of isolated microorganisms.

Isolate	**Specie Identified**	**Identification (%)**
Lactic Acid Bacteria 1	*Lactobacillus acidophilus*	93
Lactic Acid Bacteria 2	*Lactobacillus lactis*	86

The results of the evaluation of the antibiotic sensitivity of a subset of Lactobacilli strains to routinely used antibiotics are given in Table 3. Sensitivity is categorized as either sensitive (S) or resistant (R) (Pokhrel, 2015). *Lactobacillus acidophilus* exhibited notably elevated resistance to all antibiotics except for Chloramphenicol at a concentration of 30 µg, as indicated by the absence of inhibition zones in response to Ampicillin, Penicillin and Streptomycin. *Lactobacillus lactis* exhibited resistance to Ampicillin and Streptomycin when administered at concentrations of 10µg and 25µg.

Table 3. Diameter (mm) of inhibition zone of Lactobacilli sensitivity to antibiotic.

Isolates	**Antibiotics**				
	AM (10 µg)	**PE (25 µg)**	**ST (25 µg)**	**CH (30 µg)**	
L. acidophilus	-	-	-	2.5 (R)	Zone of inhibition (mm)
L. lactis	12.3 (S)	-	1.5 (R)	-	

(R): Resistant, I: Intermediate, (S): Susceptible, (−): No inhibition, (AM): Ampicillin, (PE): Penicillin, (ST): Streptomycin, (CH): Chloramphenicol

A range of inhibitory activity against intestinal *Escherichia coli* was observed as a consequence of *Lactobacilli's* antibacterial action against the bacteria. In comparison to *Lactobacillus lactis*, *Lactobacillus acidophilus* exhibits a significant degree of inhibition.

Table 4. Mean inhibition zone for antimicrobial activity against *Escherichia coli*.

Isolate	***E. coli* Inhibition Diameter (mm)**
L. acidophilus	14.6
L. lactis	8.9

The outcomes of the bile tolerance test for the isolates are mentioned Table 5. The data demonstrated that both lactic acid bacteria displayed resistance to a 0.5% bile salt solution, with a gradual reduction in viable cells observed when subjected to a 2.0% bile salt solution. *L. acidophilus* exhibited heightened tolerance to bile salt.

Table 5. Bile Tolerance Test of Isolated *Lactobacilli acidophilus* and *Lactobacillus lactis.*

Species	**Concentration/Result**	
	0.50%	**2.00%**
L. acidophilus	+++	+
L. lactis	++	+

(+++) Maximum resistance, (++) Moderate resistance, (+) Minimum resistance.

DISCUSSION

The effective isolation of Lactobacillus strains from fermented cow milk utilizing MRS media emphasizes the importance of this medium in specifically cultivating lactic acid bacteria (Oliveira *et al.*, 2001, Mathara *et al.*, 2004). The identification process, employing traditional biochemical methods and the API-50 CHL system, elucidated the Gram-positive nature of the isolates, along with their inability to utilize citrate and produce catalase-consistent characteristics of Lactobacillus species. The fermentation patterns observed for breakdown of sugar further substantiated the identification, confirming Lactic Acid Bacteria 1 as *Lactobacillus acidophilus* and Lactic Acid Bacteria 2 as *Lactobacillus lactis*.

Examination of antibiotic sensitivity unveiled details about the resistance patterns exhibited by the identified *Lactobacillus* strains.*L. acidophilus* displayed notable resistance to various antibiotics, except for Chloramphenicol, indicating a potential concern for the selective pressure of certain antibiotics. In contrast, *L. lactis* demonstrated bounciness against Ampicillin and Streptomycin at particular concentrations. These results align with prior studies, underscoring the significance of evaluating

antibiotic susceptibility in probiotic strains. The administration of antibiotics could impact the viability and functionality of these bacteria, as noted in various studies (Zhou et al., 2012, Neumann et al., 1995, Coppola *et al*., 2005, Philip *et al*., 2017).

The evaluation of antimicrobial activity against Escherichia coli highlighted the inhibitory potential of the isolated *Lactobacilli. L. acidophilus* demonstrated higher inhibition compared to *L. lactis*, suggesting variations in their antagonistic capabilities against enteric pathogens. This antimicrobial effect aligns with the potential use of these strains as probiotics to combat pathogenic bacteria in the gastrointestinal tract (Havenaar*et al.*, 1992, Lee *et al.*, 1995).

Bile tolerance is a crucial criterion for the survival and functionality of probiotics in the gastrointestinal environment. The results of the bile tolerance test indicated that both *Lactobacillus* strains exhibited resistance to a 0.5% bile salt solution, but their viability gradually decreased when exposed to a 2.0% bile salt solution (Patel *et al.*, 1999, Hassanzadazar *et al.*, 2012). *L. acidophilus* showed heightened tolerance to bile salt, suggesting its potential to survive and exert beneficial effects in the harsh conditions of the gastrointestinal tract (Farnworth *et al.*, 2008, Xanthopoulos *et al.*, 1997, Tanaka *et al.*, 1999).

In conclusion, the isolated *Lactobacillus* strains from fermented organic cow milk exhibit characteristics conducive to their potential use as probiotics. The antibiotic resistance, antimicrobial activity against *Escherichia coli*, and bile tolerance demonstrated by these strains support their consideration for further exploration in in vitro and in vivo studies. However, the discussion also highlights the importance of careful antibiotic selection to avoid potential negative impacts on probiotic functionality. Further research is warranted to elucidate the mechanisms behind these observed characteristics and to assess their efficacy in practical applications, such as enhancing animal production or promoting human health.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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