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Potential Probiotics in Donkey Milk: A New Frontier in Microbiota Research

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ABSTRACT:
Non-bovine milk, enriched with bioactive compounds, has been shown to modulate immune responses, support bone health, and influence gut microbiota composition. Donkey milk, historically valued for its therapeutic benefits, remains underexplored in terms of its microbial diversity and probiotic potential. In current research, isolate and characterize lactic acid bacteria (LAB) from donkey milk is to assess their suitability for functional applications. Twenty-five LAB isolates were obtained, of which eight were selected for detailed phenotypic evaluation. Two strains demonstrating robust acid and bile salt tolerance, alongside significant antibacterial activity, were further characterized at the molecular level. *Bacillus paramycoides strain RED9* [OQ746321] and *Brevibacillusborstelensis strain 3.1* [OR272522] exhibited remarkable resilience under adverse environmental conditions and displayed potent antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis*. These findings highlight the probiotic potential of LAB from donkey milk, suggesting their applicability in biopreservation and functional food formulations.

Keywords: Donkey milk, Probiotics, Lactic acid bacteria, Antimicrobial activity, Functional foods

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INTRODUCTION

Donkeys (*Equus asinus*), among the earliest domesticated equines, play a crucial role in agriculture and transport, particularly in economically disadvantaged communities (Press Information Bureau, Government of India, 2022). Donkey milk has been traditionally valued for its perceived health benefits, including cognitive development and therapeutic effects on liver disorders, respiratory ailments, and appetite loss (Grubb, 2005; Garhwal, *et al.,* 2022). Notably, its biochemical composition closely resembles human breast milk and exhibits anti-inflammatory, antioxidative, and antimicrobial properties (Bhardwaj, *et al.,* 2019; Cirrincione, *et al.,* 2021). Despite these attributes, the microbial diversity of donkey milk remains largely unexplored, limiting its potential applications in functional food formulations.

Over the past decades, the extensive use of antibiotics in livestock production to enhance growth rates and prevent infections has led to a significant rise in antibiotic resistance, posing

severe threats to human and animal health as well as environmental safety (Sharma *et al.,* 2013b). Consequently, there is an urgent need for effective alternatives that improve animal productivity and reduce mortality while maintaining environmental sustainability and consumer health. Among the various substitutes for antibiotics, probiotics have emerged as a widely accepted strategy due to their natural origins and beneficial effects on feed supplementation. Probiotics have been shown to enhance growth performance, improve meat quality, optimize nutrient absorption, strengthen immune responses, and inhibit pathogen colonization in livestock (Quigley, *et al.,* 2013; Bhardwaj, *et al.,* 2019).

Lactic acid bacteria (LAB) represent the predominant group of probiotic microorganisms, characterized by their ability to confer health benefits to the host. LAB strains have been extensively isolated from diverse sources, including fermented foods (Liu, *et al.,* 2016; Wang, *et al.,* 2021), raw milk (Carminati, *et al.,* 2015), the gastrointestinal tract (GIT), and the vaginal microbiota (Cirrincione, *et al.,* 2021). These strains have been incorporated into food fermentation processes, animal feed additives, and therapeutic treatments due to their ability to enhance immunity, improve gut barrier function, and reduce pathogen colonization (Cavallarin, *et al.,* 2015).

Given the increasing interest in strain-specific probiotic properties and the unique biochemical composition of donkey milk, it represents a promising but underexplored source of LAB with potential applications in functional nutrition. In this study, the aim was to isolate and characterize LAB from donkey milk, evaluating their probiotic attributes, including acid and bile salt tolerance, antimicrobial activity, and resilience under adverse environmental conditions. Two strains, identified as *Bacillus paramycoides strain RED9* and *Brevibacillus borstelensis strain 3.1*, exhibited superior probiotic traits and were selected for detailed molecular characterization. These findings provide insights into the probiotic potential of donkey milk-derived LAB and their prospective applications in functional food formulations, animal feed, and biopreservation.

MATERIALS AND METHODS

Sample Collection

Milk samples were obtained from the Jenny Dairy Unit, National Research Centre on Equines, Hisar, Haryana (29°11'13.7"N 75°42'03.9"E) between October 2022 and May 2023. Ten milk samples were manually collected under aseptic conditions. The udders were cleaned with distilled water and dried with sterile towels before collection. The first three milk streams were discarded and subsequent samples were collected in sterilized 250 mL containers, transported at 4–6°C, and analyzed immediately.

Isolation and Identification of Bacterial strains

Donkey milk samples were plated on selective media for total bacterial count analysis. LAB were isolated using de Man, Rogosa, and Sharpe (MRS) agar under anaerobic conditions at 37°C for 72 h, acidified MRS (pH 5.7) at 30°C for 72 h, and M17 agar at 45°C for 48 h. Phenotypic identification was performed based on colony morphology, Gram staining, and biochemical characterization following Bergey's Manual of Systematic Bacteriology (Garrity, 2009).

Genomic DNA Extraction and Quantification

Following the *in vitro* characterization of candidate probiotic isolates from donkey milk, eight isolates exhibiting superior probiotic potential were selected for molecular identification. Genomic DNA was extracted from the selected bacterial strains using a commercial DNA extraction kit, following the manufacturer's protocol. The purity and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer, and integrity was confirmed by agarose gel electrophoresis. The extracted DNA was stored at −20°C for subsequent analysis.

PCR Amplification of the 16S rRNA Gene

The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCATGGCTC3′) and 1492R (5′-TACGGTTACCTTGTTACGACT-3′). The PCR reaction mixture (50 µL total volume) consisted of 25 µL of 2× Taq Master Mix (containing buffer, polymerase, and dNTPs), 1 µL of forward primer, 1 µL of reverse primer, 2 µL of

ultrapure water, and 1 µL of genomic DNA as the template. Amplification was performed in a thermal cycler under the following conditions: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 45°C for 2min., and extension at 72°C for 2 min. A final extension was carried out at 72°C for 10 min, and the reaction was held at 4°C for long-term preservation.

Probiotic Characterization

Acid and bile salt tolerance were assessed at pH 2.0 and 0.3% bile salt concentration (Vlková, *et al.,* 2006; Aspri, *et al.,* 2017). Antimicrobial activity *against S. aureus and B. subtilis* was evaluated using the agar well diffusion method. Antibiotic susceptibility was determined using the disc diffusion assay with amikacin (5 µg), cefazolin (5 µg), chloramphenicol (30 µg), ampicillin (10 µg), and tetracycline (10 µg) (Zhang, *et al.,* 2016).

Statistical Analysis

All experiments were conducted in triplicate, with data expressed as mean ± standard deviation (SD). Differences were analyzed using one-way ANOVA (GraphPad Prism v8.0.2), with statistical significance set at $P<0.05$.

RESULTS AND DISCUSSION

Bacterial Isolation and Identification

A total of 25 bacterial isolates were initially obtained, of which five representative strains were selected based on distinct colony morphology and biochemical characteristics. All isolates were Gram-positive and catalase-negative, indicative of their classification within Lactic Acid Bacteria (LAB) and Firmicutes as shown in table 1. Morphological analysis revealed three coccus-shaped strains (DM1, DM3, DM4) and two bacilli (DM2, DM5). Biochemical assays demonstrated uniform oxidase, citrate, catalase and methyl red negative, supporting a facultative anaerobic metabolic profile (Garrity, 2009; Sharma, *et al.,* 2013a). Temperature tolerance assessments indicated that strains DM1, DM2, DM4, and DM5 exhibited growth even at 10°C, whereas DM3, DM4, and DM5 tolerated 45°C.

All isolates demonstrated growth in 2% NaCl, while only DM4 exhibited tolerance at 6% NaCl, suggesting differential osmotolerance. Carbohydrate fermentation assays revealed that glucose and lactose were metabolized by all strains except DM5, which exhibited a non-fermentative phenotype for glucose (Cavallarin, *et al.,* 2015). Most strains utilized trehalose and galactose, except for DM4 (galactose-negative) and DM5 (trehalose-negative), while sucrose metabolism was absent in DM4. The ability to ferment arabinose, mannose, ribose, maltose, and D-xylose varied among isolates, with not determined (ND) values in some cases (Garrity, 2009; Zhang, *et al.,* 2016; Kleerebezem, *et al.,* 2003). These findings indicate substantial metabolic diversity, adaptive capabilities and identified isolates as *Lactococcus lactis subsp. lactis, Enterococcus faecalis, Enterococcus lactis, Bacillus paramycoides strain RED9, and Brevibacillus borstelensis strain 3.1.*

Molecular identification of bacterial isolates

The genomic composition of all bacterial isolated from donkey milk was analyzed by the agarose gel electrophoresis (AGE) to measure the genome size compared with the reference. In AGE, the isolated DNA samples were loaded on 0.8% gel along with the standard ladder (1kb) with known DNA size range (fig.1).

Amplification of 16S rRNA gene

The genomic DNA samples of all probiotic strains with 16SrRNA gene amplification, using 27 F and 1492 R primers were analyzed by the 1.5 % agarose gel electrophoresis (AGE) to measure the PCR product size compared with the reference ladder (100bp). The 1300 bp PCR products size were obtained as shown in fig. 2. The consensus sequences of bacteria obtained after sequencing were submitted to NCBI Genbank Database with accession number *Bacillus paramycoides strain RED9* [OQ746321] and *Brevibacillus borstelensis strain 3.1*[OR272522].

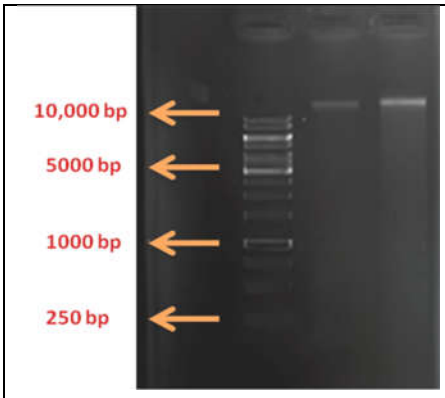

Figure 1: Agarose gel electrophoresis (0.8 %) with 1 kb ladder with multiple bands in lane 1st and isolated genomic DNA of probiotic strains in lane 2 & 3

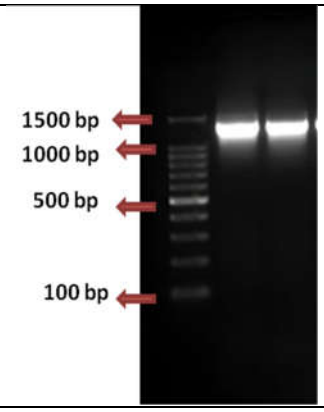

Figure 2: 1.5% Agarose gel electrophoresisof PCR products with100bp ladder in 1st lane, showing the size of PCR products in lane 2 & 3.

Table 1: Phenotypic and biochemical characteristics of LAB isolates of Donkey milk

Characteristics	DM1	DM2	DM3	DM4	DM5
Cellular morphology	Coccus	Bacillus	Coccus	Coccus	Bacillus
Gram staining	(+)	(+)	(+)	(+)	(+)
Oxidase test	(-)	(-)	(-)	(-)	(-)
Citrate test	(-)	(-)	(-)	(-)	(-)
Catalase test	(-)	(-)	(-)	(-)	(-)
Methyl red test	(-)	(-)	(-)	(-)	(-)
Growth at 10^{0}C	(+)	(+)	(-)	(+)	(+)
Growth at 45°C	(-)	(-)	(+)	(+)	(+)
Growth at 2% NaCl	(+)	(+)	(+)	(+)	(+)
Growth at 6% NaCl	(-)	(-)	(-)	(+)	(-)
Glucose fermentation	(+)	(+)	(+)	(+)	(-)
Trehalose fermentation	(+)	(+)(+)	(+)	(-)	(+)
Arabinose fermentation	(+)	ND	(-)	ND	(-)
Mannose fermentation	(+)	ND	(+)	ND	ND
Galactose fermentation	(+)	(+)(+)	(+)	(-)	(+)
Lactose fermentation	(+)	(+)	(+)	(+)(+)	(+)
Maltose fermentation	(+)	ND	(+)(+)	(-)	(+)
Ribose fermentation	(+)	(+)	(+)	(+)	(-)
Sucrose fermentation	ND	(+)	(+)	(-)	(+)
D-Xylose fermentation	ND	(-)	(+)	ND	(-)

- (+) = positive reaction, (-) = negative reaction

Probiotic Potential Assessment

All isolates demonstrated carbohydrate fermentation ability, with variable fermentation rates and exhibited the highest survivability at pH 2.0 and 0.3% bile salt concentration (Fig.3).

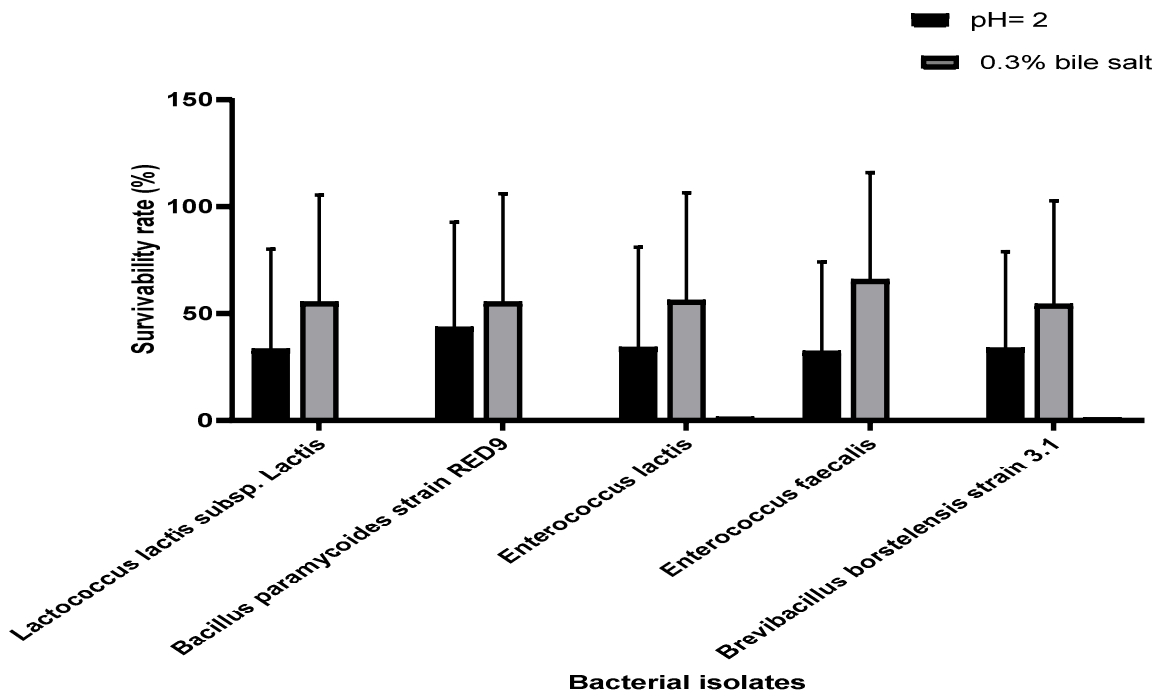

Figure 3: Survivability of Probiotic Strains from Donkey Milk Under Acidic (pH 2) and Bile Salt (0.3%) Conditions.

The survivability of different probiotic strains isolated from donkey milk was assessed under acidic conditions (pH 2) and 0.3% bile salt, revealing a significantly higher survival rate across all isolates ($P<0.05$). Notably, *Bacillus paramycoides strain RED9* and *Brevibacillus borstelensis strain 3.1* exhibited superior tolerances at pH 2 compared to other strains, indicating their enhanced resilience to acidic environments and potential as robust probiotic candidates. Statistical analysis using RM one-way ANOVA confirmed a significant difference ($P<0.05$), highlighting the adaptability of these strains under gastrointestinal conditions

Effective probiotic strain selection requires resistance to these harsh conditions, including acidic pH and high bile concentrations (Neviani, *et al.,* 1991; Quigley, *et al.,* 2013; Vlková, *et al.,* 2006). Zhang *et al.* (2016) reported that many commercial probiotics exhibit high sensitivity to simulated gastric juices, leading to significant bacterial reduction under acidic conditions, emphasizing the need for resilient probiotic strains that can sustain viability throughout GI transit. Among the identified LAB, Lactococcus lactis has been extensively documented in the milk of various mammals, including cows, goats, sheep, buffalo, and humans (Carminati, *et al.,* 2014; CLSI, 2015; Neviani, *et al.,* 1991). Notably, this study predominantly identified bacillus-shaped LAB, with *Bacillus paramycoides* strain RED9 and *Brevibacillus borstelensis* emerging as the dominant species (Kumari, *et al.,* 2022a,b; Kumari, *et al.,* 2024). These strains demonstrated strong resistance to acidic gastric conditions and high bile salt concentrations, key traits essential for probiotic viability. Such resilience enhances their survival and functionality during gastrointestinal transit, ensuring effective colonization and activity in the gut (Kumari, *et al.,* 2024).

Antibiotic Resistance and Antimicrobial Susceptibility Assessment

The antibiotic susceptibility of *Lactococcus lactis, Bacillus paramycoides, Enterococcus lactis, Enterococcus faecalis,* and *Brevibacillus borstelensis* was evaluated using the disc diffusion method. All strains were susceptible (S) to tetracycline, ampicillin, and chloramphenicol, indicating no resistance to these antibiotics. However,

cefazolin resistance (R) was observed in all strains except *E. faecalis,* which showed low susceptibility (LS), suggesting partial β-lactam resistance.

Antibiotics	Disc potency (µg)	L. lactis	Bacillus paramycoides	E. lactis	E. faecalis	Brevibacillus borstelensis
Tetracycline	10	S	S	S	S	S
Ampicillin	10	S	S	S	S	S
Chloramphenicol	30	S	S	S	S	S
Cefazolin	5	R	R	R	LS	R
Amikacin	5	LS	LS	LS	LS	LS

Additionally, all strains exhibited low susceptibility (LS) to amikacin, indicating reduced aminoglycoside sensitivity. These findings provide insight into strain-specific resistance profiles, essential for assessing their safety and probiotic potential (Kleerebezem, *et al.,* 2003; Jagadeesh, *et al.,* 2015; Papademas, *et al.,* 2015; Gupta, *et al.,* 2015; Gupta, *et al.,* 2017).

The antimicrobial activity of each isolate was evaluated by measuring the zone of inhibition (ZOI) against pathogenic bacteria. All experiments were conducted in triplicate following CLSI guidelines (CLSI, 2015). The average ZOI for *Lactococcus lactis, Bacillus paramycoides, Enterococcus lactis, Enterococcus faecalis,* and *Brevibacillus borstelensis* ranged from 8 mm to 15 mm. Among these, *Bacillus paramycoides* exhibited the strongest antagonistic activity, with an inhibition zone of 14.6 mm against *Staphylococcus aureus* and *Bacillus subtilis*. In contrast, E. lactis showed the lowest inhibition, with a ZOI of 8 mm against the tested pathogens.

Antimicrobial activity assays revealed substantial inhibition zones against *S. aureus* and *B. subtilis*, reinforcing their potential as biopreservative agents.

CONCLUSION

This study highlights the isolation and characterization of probiotic strains from donkey milk, demonstrating their survivability under acidic and bile salt conditions, antimicrobial potential, and antibiotic susceptibility profiles. Among the isolates, *Bacillus paramycoides* and *Brevibacillus borstelensis* exhibited superior tolerance to low pH and strong antagonistic activity against pathogenic bacteria, suggesting their potential as robust probiotic candidates. The antibiotic susceptibility patterns further support their safety for therapeutic and functional food applications. These findings provide valuable insights into the biotechnological potential of donkey milk-derived probiotics, paving the way for further exploration of their mechanisms of action, gut microbiome interactions, and clinical efficacy.

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