Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 6 Issue 9 September 2023

Research Article

Biochemical and Antimicrobial activity of Potential Probiotics Isolated from Halari Milk

Ankur Kumari[1], Anuradha Bhardwaj[2]*, Parvati Sharma*[1], Yash Pal[2], Varij Nayan[3], Prashant Singh[2] and Ashish Sindhu[1]

[1]*Department of Zoology, Chaudhary Bansi Lal University, Bhiwani, Haryana, India*

[2]*National Research Centre on Equine, Hisar, India*

[3]*Central Institute for Research on Buffaloes, Hisar, India*

***Corresponding Author:** Parvati Sharma and Anuradha Bhardwaj, Department of Zoology, Chaudhary Bansi Lal University, Bhiwani, Haryana and National Research Centre on Equine, Hisar India.

DOI: 10.31080/ASMI.2023.06.1295

Received: August 07, 2023

Published: August 18, 2023

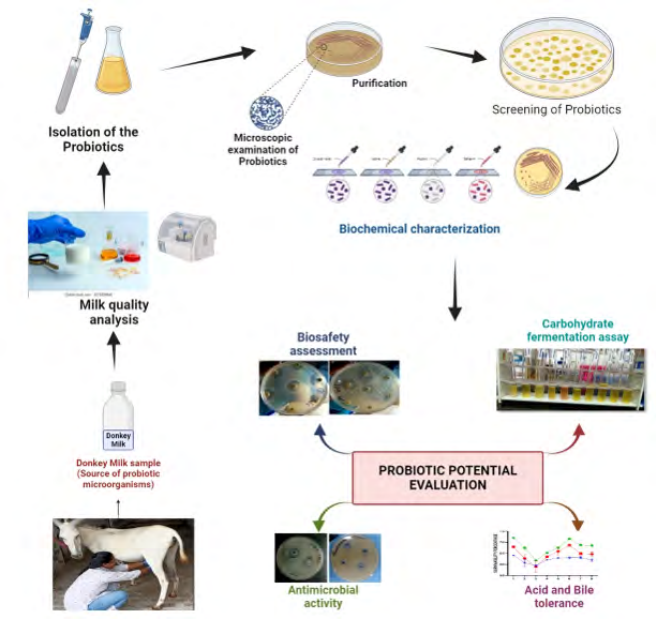
Abstract

The probiotic-enriched food product that not only satisfies nutritional needs but also boost the immune system, is now appalling situation. Non bovine milk, contains various bioactive components which are potentially involved in the immune system functioning, bone strengthening and for balancing gut flora with reliable probiotics. Among these non-bovine milk sources, therapeutic properties of Donkey milk are researched since antiquity, but the microbiological profile has gotten less attention. Therefore, current study emphasized the significance, isolation, biochemical identity, and antimicrobial efficacy of probiotics found in donkey milk. A total of 35 isolates were collected from donkey milk samples and eight potent isolates were chosen based on screening and further evaluated for their significant probiotic activity like, resistance for acidic conditions, high bile salt concentration, antibiotic susceptibility and antibacterial activity against pathogenic bacteria of these isolates was examined. *Lactococcus lactis subsp cremoris* and *Enterococcus faceium* are found to resistant against *in vitro* stress conditions and exhibited maximum antimicrobial activity against *Staphylococcus aureus, Pseudomonas* and *Bacillus subtilis* strains. In line with the findings of the present study, these potent DM probiotics can be employed as bio preservatives to prevent the proliferation of pathogenic or food-spoiled bacteria.

Keywords: Donkey Milk; Probiotics; Bacteria; Biochemical Test; Antimicrobial Activity

Introduction

The Donkeys (*Equus asinus*) are one of the earliest domesticated equine species of the Equidae family [1,2]. *E. africanus asinus*, an African wild ass, is thought to be the ancestor of the donkey. In India, majority of Donkey population play an important role as pack animal for moving and building supplies especially, by poor, landless socially and economically deprived people. Among all donkey breeds, Halari Donkey is one of the important livestock in the semi-arid landscape of Saurashtra's Jamnagar and Dwarka district of the state Gujarat [3,4]. The Bharwad and Rabari pastoralists' communities use Halari donkey as beast of burden [4]. Since ancient times, these pastoralists and local people are using Halari donkey milk (DM) for feeding newborn babies, as a nutritional source for newborns indicates the potential health benefits it may offer like promoting brain development, improves voice, and is a good remedy for cough, liver disorders and appetite loss etc. [4]. Newborns with cow milk protein allergy (CMPA) are recommended to take milk from other animals as goat, horse, and donkey milk for human milk substitute. Donkey milk is gaining international acceptance and interest over the past ten years and demand as substitute for human milk has increased [3]. Due to the special characteristics, donkey milk seems to be a great



Graphical abstract

replacement for people who are intolerant to severely hydrolyzed milk-based formulas as well as for kids with CMPA [4]. Donkey milk resembles human milk chemically and in terms of organoleptic qualities [5]. Donkey milk is most similar to human milk in terms of its high lactose, low protein, and even lower amount of fat content with significant anti-inflammatory, anti-allergic, anti-oxidative [5], and antibacterial characteristics [6]. Though, microbial diversity of DM is well reported, but probiotic diversity which exhibit high ant oxidative, anti-bacterial, and auto-aggregation properties with immuno-modulatory effects in host [7] is less explored. According to previous studies, the probiotic microorganisms of *Lactobacillus paracasei, Lactococcus lactis*, and *Carnobacterium maltaromaticum* are more prevalent in donkey milk and exhibit beneficial benefits *in vivo*, particularly antibacterial, antioxidant, and other properties like human milk in consistency [7]. Now a day many strain specific functional features of probiotics have been investigated but additional excellent strains with improved effectiveness are always needed [8].

Therefore, present study highlights the probiotic diversity of Halari donkey milk and its antimicrobial potential against selected pathogens. The isolated strains demonstrated high stress tolerance, which indicates their potential survival and functionality in various environments. Moreover, these strains exhibit potent antimicrobial activity against specific pathogens, suggesting their possible application as natural alternatives to antibiotics. These findings open new avenues for further research into the development of functional foods and nutraceuticals using donkey milk-derived probiotics. Additionally, this research reaffirms the significance of traditional knowledge surrounding Halari donkey milk, as it aligns with modern scientific evidence, further validating its potential as a valuable resource for human health.

Materials and Methods

Sample collection

The milk samples were collected from Jenny Dairy Unit, National Research Centre on Equines, Hisar, Haryana (29°11'13.7"N 75°42'03.9"E) as shown in figure 1.

Figure 1: Sample collection site, Halari Jenny Dairy, National Research Centre on Equines, Hisar, Haryana.

Particularly, sampling was conducted from October, 2022 to May 2022, and a total of ten milk samples were collected by manual milking from apparently healthy Jennies. The milk samples were obtained directly from the udder. The udder was washed with distilled water before collection and dried with single-service towels [10,11]. The initial three streams of milk were discarded. Then milk samples were collected in sterilized 250 mL containers, kept in aseptic ice box and immediately transported to laboratory at 4-6 °C. The samples were subjected to microbiological analysis same day, after 24hrs incubation at 37°C and low temperature treatment at 4°C.

Isolation, enumeration and identification of LAB

Firstly, raw pure milk samples were directly used to count total bacterial colonies then serial dilutions of each sample in physiological solution (0.85% NaCl, w/v) were prepared and plated in duplicate on plate count agar plates. Then, milk dilutions were cultured on MRS agar (Himedia, Mumbai) in anaerobic conditions for 72h at 37 °C, on acidified MRS (pH 5.7) in anaerobically for 72h at 30 °C and on M17 agar for 48h at 45 °C and then for 4d at 20°C plates. After incubation at 37°C for 48 hours under anaerobic condition individual different colonies were phenotypically selected [12,13]. Colony enumeration was conducted after incubation and was recorded as CFU per liter of milk.

All isolates were microscopically investigated for their morphological characteristics *i.e.* cellular arrangement, Gram staining and cell morphology. Based on their capacity to perform particular metabolic reactions, biochemical tests were performed for differentiation of different bacterial species. Catalase test, Oxidase test, starch hydrolysis, lipid hydrolysis, gelatine hydrolysis, Carbohydrate Fermentation, motility test, H_2S test, Methyl Red Test, test Voges-Proskauer and citrate test etc. as per Bergey's Manual of Systematic Bacteriology Volume 3 [14] as follow.

Gram staining

Gram staining was used for differentiating bacteria into Gram-positive and Gram-negative groups based on the structure of their cell walls. Firstly, bacterial cells were stained for 1 min with crystal violet stain. Then after with iodine solution, quick washing with alcohol, and finally counter stain safranin for 30 sec. Gram-positive bacteria retain the crystal violet stain, appearing purple, while Gram-negative bacteria lose the crystal violet stain and take up the safranin counter stain, appearing pink [14].

Oxidase test

The oxidase test is used to identify bacteria that possess the enzyme cytochrome c oxidase. Few drops of the tetramethyl-p-phenylenediamine dihydrochloride reagent solution containing a redox indicator were applied to a bacterial sample, and results were observed within few seconds [14].

Arginine hydrolysis test

This test assesses a bacterium's ability to hydrolyze arginine using the enzyme arginase. The breakdown of arginine produces ammonia, which raises the pH of the medium. A pH indicator was used to detect the increase in pH, indicating a positive test result. a pure bacterial culture was inoculated in arginine hydrolysis broth with a sterile loop and incubated at 37^0C for 30 hrs. After incubation, color changes around the bacterial growth were reported [14].

Citrate test

The citrate test evaluates whether bacteria can utilize citrate as the sole carbon source. The medium contains citrate as the only carbon source and a pH indicator was inoculated with bacterial isolates and incubated at 37^0C for 30 hrs and after incubation changes in medium were reported [14].

Catalase test

The catalase test determines whether bacteria produce the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen. Bacterial isolates were exposed to few drops of 3% hydrogen peroxide, and changes were observed immediately to avoid false results [14].

Motility test

This test examines whether bacteria are motile or non-motile. Bacteria are inoculated into a semisolid agar medium, and motile bacteria will spread out from the point of inoculation, creating a diffuse growth pattern. Non-motile bacteria will only grow in the area of the inoculation [14].

Hippurate hydrolysis

The hippurate hydrolysis test is used to identify bacteria capable of hydrolyzing hippurate (a derivative of glycine). The bacterial isolates were inoculated in hippurate containing broth and incubated at 37^0C for 30 hrs and after incubation changes in medium were reported [14].

Methyl red test

This test evaluates the ability of bacteria to perform mixed acid fermentation of glucose. After incubation in a glucose-containing medium, the addition of methyl red indicator showed the pH of the medium [14].

Growth at different temperatures

Bacterial isolates were inoculated in broth medium and incubated at temperatures ranges i.e. 10°C-45°C for 30 hrs and after incubation turbidity in medium was reported can be tested for their ability to grow at various temperatures. Growth at extreme temperatures helps identify thermophilic bacteria capable of surviving and growing in extremely hot conditions [14].

Amygdalin fermentation

This test examines the ability of bacteria to ferment amygdalin, a glycoside found in some seeds and plants. After bacterial inoculation and incubation at 37^0C for 24 hrs in medium supplemented with amygdalin, the fermentation of amygdalin was observed whether produces acids, including hydrogen cyanide (HCN), which reacts with ferric ions in the medium to form a dark precipitate [14].

Lipid hydrolysis test

This test determines whether bacteria can hydrolyze lipids (fats). The bacteria were grown on a medium containing lipids, and the breakdown of lipids results in the formation of fatty acids. A pH indicator, such as spirit blue agar, changes color from green to blue when fatty acids are present [14].

Starch hydrolysis test

This test evaluates the ability of bacteria to produce amylase, an enzyme that hydrolyzes starch. Bacteria were grown on a starch-containing medium, and after incubation, the medium is flooded with iodine. If the bacterium can hydrolyze starch, a clear zone will form around its growth, indicating a positive result [14].

Growth at different NACL concentrations

This test assesses the ability of bacteria to tolerate varying levels of salt (NaCl). Bacteria were tested for growth at 2% NaCl and 6% NaCl, which helps identify their halotolerance or halophilic properties [14].

Voges-proskauer test

The Voges-Proskauer test detects the production of acetoin from the fermentation of glucose. After incubation of each pure bacterial isolate in glucose-containing medium, specific VP reagents were added, and a positive result is indicated by the development of a red color [14].

Gelatin hydrolysis test

This test examines whether bacteria can produce gelatinase, an enzyme that hydrolyzes gelatin. Pure bacterial colony was inoculated in agar plates containing gelatin and incubated overnight at room temperature [14].

H_2S test

This test detects the production of hydrogen sulfide (H_2S) by bacteria during the metabolism of sulfur-containing amino acids. The results were observed after incubating bacterial isolates in medium containing a sulfur source at room temperature for 24hrs [14].

Indole test

The indole test detects the ability of bacteria to produce indole from the amino acid tryptophan. After incubation in a medium containing tryptophan, Kovac's reagent was added [14].

Screening for probiotic potential of isolated LAB

The probiotic properties were screened by different tests like low pH tolerance test by regulating the pH (2.0) of the medium to and bile salt tolerance (0.3%) test by modifying protocol [14]. Then growth rate was observed by viable counts by plating 100 µL of experiment culture on MRS agar plates. All the experiments were technically performed independently in triplicates. After calculating log cfu/mL survivability was counted as

% survivability = (viable log count at time t/viable log count at t = 0) × 100

Antimicrobial activity assay

The antimicrobial activity of selected acid bile tolerant strains was determined by the agar well diffusion assay by modifying [14]. More accurately, in freshly prepared lawns of overnight growth of the indicator strains in BHI agar (HiMedia), wells were aseptically punched. Then, 20 µL of each sample were added in the wells. Incubation was carried out at 37^0C for 24h. Inhibition of the indicator strain's growth around the wells suggested the presence of antimicrobial activity of the used sample. The indicator strains used were, *Staphylococcus aureus, Pseudomonas* and *Bacillus subtilis* diameters of inhibition zones were measured using calipers

Antibiotic susceptibility tests

Each of acid-bile tolerant lactic acid bacteria was selected examining sensitivity against the antibiotics Amikacin (5 µg), Cefazolin (5 µg), Chloramphenicol (30 µg), Ampicillin (10 µg) and Tetracycline (10 µg) by the disc diffusion method as described by Zhang., *et al*. 2016 [15]. Overnight grown bacterial culture of each acid-bile tolerant bacteria was swabbed evenly over the MRS agar plates with a sterile cotton swab under aseptic laboratory conditions. After, proper drying of agar surface the antibiotic discs were placed over culture surface and then for proper antibiotics diffusion plates were incubated for 30 min. at 4°C and then final anaerobic incubation at 37°C for 48 h. The results were observed and calculated by the measuring the diameter (mm) of zone of inhibition of each antibiotics using callipers [16] and expressed as susceptible, S (≥12 mm); Less sensitive LS (10 mm), and resistance, R (≤8 mm).

Statistical analysis

All experiments were performed in triplicate and average values were represented as mean ± standard deviation (SD). The significant differences among treatments for pH resistance and bile salt concentration were evaluated by RM one-way ANOVA using Graph Pad Prism software (v8.0.2).

Results

Bacterial strain identification

The bacterial colonies from raw donkey milk samples were reported 28, 25, 17, 12 and 22 on NA, PCA, MRS, LBS and GM17 culture media respectively as shown in table 1. Number of bacterial isolates on NA and PCA showed some unwanted colonies but on MRS, LBS and GM17culture media only white or creamy rounded colonies were reported. Therefore, MRS and GM17 culture media were selected to examine the probiotic bacterial count of DM as these are very selective probiotic culture media. The average bacterial population of raw donkey milk on MRS was reported high than other media. Hence, MRS media for raw milk samples was preferred further for probiotic culture. Among all microbial colonies 08 different LAB colonies were isolated and enumerated by different selective growth media for analyzing their potential probiotic properties.

S.No	Growth Media	Bacteria	Temperature/time	Number	References
1	NA	Total microbes	37ºC/ 24hrs	28	ISO,2013
2	PCA	Aerobic	37ºC/ 48hrs	25	ISO,2013
3	MRS	Lactobacilli	30-37ºC/ 48-72hrs	17	ISO,2004b; ISO,2004a and Carminati., *et al*., 2014.
4	LBS(5.5)	Acid tolerant lactobacilli	30-37ºC/ 48-72hrs	12	ISO,2004b; ISO,2004a and Carminati., *et al*., 2014.
5	GM17	Lactococci & enterococci	30-37ºC/ 48hrs	22	ISO,2004b; ISO,2004a and Carminati., *et al*., 2014

Table 1: Methods used in microbe enumeration in this study.

NA: Nutrient Agar; PCA: Plate Count Agar; MRS: Man Rogosa Sharpe; LBS: Lactobacillus Selective Agar and M17: M17 Agar

Characteristics	DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8
Cellular morphology	**Coccus**	**Coccus**	**Coccus**	**Coccus**	**Coccus**	**Rod**	**Rod**	**Coccus**
Gram staining	+	+	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-	-	-
Arginine Hydrolysis	+	+	+	+	+	+	+	+
Citrate test	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-
Motility test	-	-	-	-	-	-	-	-
Hippurate hydrolysis	+	+	+	+	+	+	+	+
Methyl red test	-	-	-	-	-	-	-	-
Growth at 10^0C	+	+	-	+	+	+	+	+
Growth at 35^0C	+	+	+	+	+	+	+	+
Growth at 45^0C	-	-	+	+	+	+	+	+
Amygdalin fermentation	+	+	+	+	+	+	+	+
Lipid hydrolysis test	-	-	-	-	-	-	-	-
Starch hydrolysis test	-	-	-	-	-	-	-	-
Growth at 2% NaCl	+	+	+	+	+	+	+	+
Growth at 6% NaCl	-	+	-	+	-	-	+	-
Voges Proskauer test	-	-	-	-	-	-	-	-
Gelatin hydrolysis test	-	-	-	-	-	-	-	-
H_2S test	-	-	-	-	-	-	-	-
Indole test	-	-	-	+	-	-	-	-

Table 2: Morphological and biochemical characteristics of LAB isolates of Halari Donkey milk.

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

The morphological and biochemical characteristics of all the bacterial isolates (DM1-DM8) are shown in table 2 which depicts that DM1-DM5 and DM8 isolates showed cocci shaped, gram positive cellular morphology whileDM6 and DM7 showed rod shaped, gram positive cellular morphology. All these isolates were biochemically very active and showed negative results towards catalase, Voges Proskauer test, Gelatin hydrolysis test, H_2S test, Indole test and oxidase reagents but positive results for Amygdalin fermentation, Hippurate hydrolysis and arginine hydrolysis tests. The confirmed isolated colonies from DM1- DM8 were identified as *Lactococcus lactis subsp. Lactis, Lactococcus lactis subsp cremoris, Enterococcus faceium, Enterococcus faecalis, Enterococcus lactis, Lactobacillus brevis, L. salivarius and Leuconostoc cremoris etc.* respectively as per Bergey's manual [17]. Some differential growth patterns of strains were also observed as *Lactobacillus* strains having luxuriant growth only at 37- 45^0C no growth at low temperature while the *Lactococcus* and *Enterococcus* strains were cocci shaped gram positive shows a characteristic growth at low temperature range i.e., at 4 - 10^0C also at higher NaCl concentration i.e. 8% (w/v) only *Enterococcus* strains and *L. salivarius* showed characteristic growth while other strains showed no growth at this concentration [14,16].

DM1-DM8 isolates were capable of metabolizing all the carbohydrates used in study as shown in figure 2. The detailed carbohydrate fermentation profile of each isolate is shown in table no.3 only Leuconostoc bacteria fermented all the test sugars except xylose sugar other bacteria exhibited a differential fermentation rate.

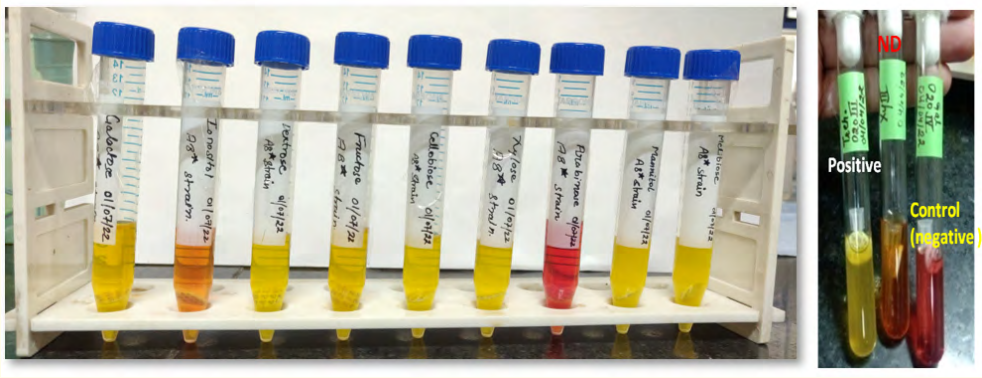


Figure 2: Carbohydrate fermentation ability of isolated bacterial strains.

Red color = negative result, yellow color = positive result and orange color = not determined.

Carbohydrates	***Lact. lactis***	***Lact. cremoris***	***E. faceium***	***E. lactis***	***E. faecalis***	***L. brevis***	***L. salivarius***	***Leuconostoc cremoris***
Glucose Fermentation	+	+	++	+	+	+	-	+
Trehalose Fermentation	+	-	+	++	+	-	+	+
Arabinose Fermentation	+	+	--	ND	-	ND	-	+
Mannose Fermentation	+	+	ND	ND	+	ND	ND	+
Galactose Fermentation	+	+	+	++	+	-	+	+
Lactose Fermentation	+	+	+	+	+	++	+	+
Maltose Fermentation	+	-	ND	ND	++	-	+	+
Melibiose Fermentation	-	-	+	+	-	+	-	+
Mannitol Fermentation	ND	ND	ND	-	+	-	-	+
Raffinose Fermentation	+	-	+	+	+	+	-	+
Ribose Fermentation	+	-	+	+	+	+	-	+
Sucrose Fermentation	ND	-	-	+	+	-	+	+
D-Xylose Fermentation	ND	-	-	-	+	ND	-	-
ND= not determined, + = positive reaction and -= negative reaction.								

Table 3: The carbohydrate fermentation ability of all bacterial isolates.

Probiotic potential characterization

The isolated strains i.e. *Lactococcus lactis subsp. Lactis, Lactococcus lactis subsp cremoris, Enterococcus faceium, Enterococcus faecalis, Enterococcus lactis, Lactobacillus brevis, L. salivarius and Leuconostoc cremoris* (DM1-DM8) showed good survivability at low pH calculated by formula as given in material and methods. The results are shown in figure 3 with the standard

mean error values of triplicate values of survivability rate of all isolates. Among all strains *Lactococcus lactis subsp cremoris* showed highest survivability against acidic conditions.

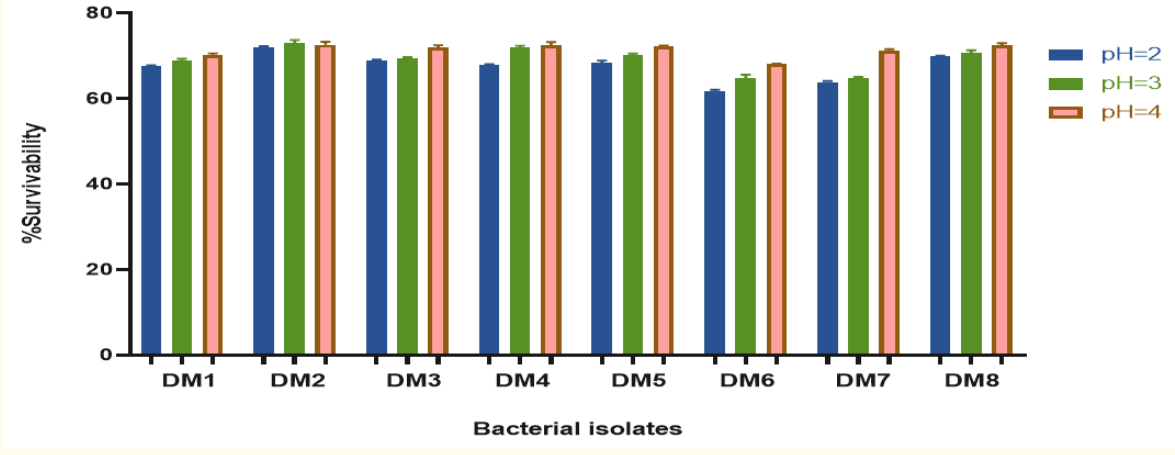


Figure 3: Relative analysis of survivability rate of different probiotic strains of Halari Donkey Milk (DM1- DM8) at different pH viz., pH = 2, pH = 3 and pH = 4. The survivability rate of all bacterial isolates was found significantly higher ($P < 0.05$) at pH=4 as compared to other pH conditions.

At pH 3 and pH 4 *Enterococcus faecalis, Lactococcus lactis subsp cremoris* and *Leuconostoc cremoris* showed higher tolerance than other strains and *Lactococcus lactis subsp cremoris* and *Leuconostoc cremoris* showed higher survivability rate at pH2 than other strains. Therefore, tolerance against low pH shows *Lactococcus lactis subsp cremoris* possesses greater survivability at different low pH range and presumptively a potential probiotic candidate. RM one way ANNOVA test was performed and the data was analysed having significant value $P<0.05$.

All the isolated strains showed good survivability at bile concentration as shown in fig4 with their mean standard error values. DM1-DM8 is denoted by 1-8 numbers respectively.

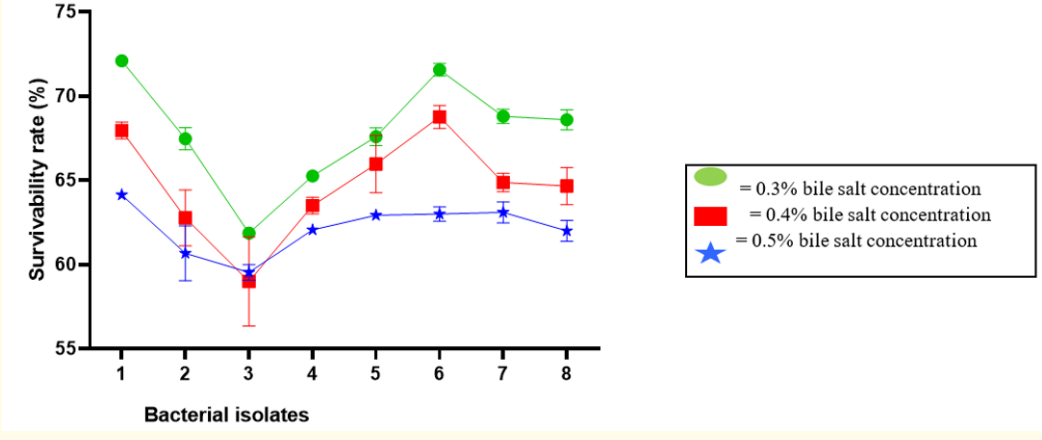


Figure 4: The Relative analysis of survivability rate of different probiotic strains of Halari Donkey Milk (DM1- DM8) at different bile salt concentration viz., 0.3%, 0.4% 0.5%. The survivability rate of all bacterial isolates was found significantly higher ($P < 0.05$) at bile salt concentration 0.3% as compared to higher concentration.DM1 and DM6 showed higher survivability at all concentrations than other strains. RM one-way ANNOVA test was performed and the data was analysed having significant value $P < 0.05$.

Among all strains *Lactococcus lactis subsp cremoris* and *Enterococcus faceium* exhibited highest resistance against 0.5.% bile concentration similar to previous studies [18,19].

Antibiotic susceptibility test

The methods recommended by CLSI (Clinical and Laboratory Standards Institute) [20] were followed when conducting antibiotics disc susceptibility tests (CLSI 2015). All of the studied LAB isolates grew uniformly over MRS and M17, and inhibitory halos were clearly discernible as shown in figure 5.

The summary of the findings ofthe susceptibility profile of isolates against the antibiotics discs used during experiment as shown in table 4.

All the isolated strains were sensitive towards tetracycline, ampicillin, and Chloramphenicolbut least sensitive towards Cefazolin and Amikacin.

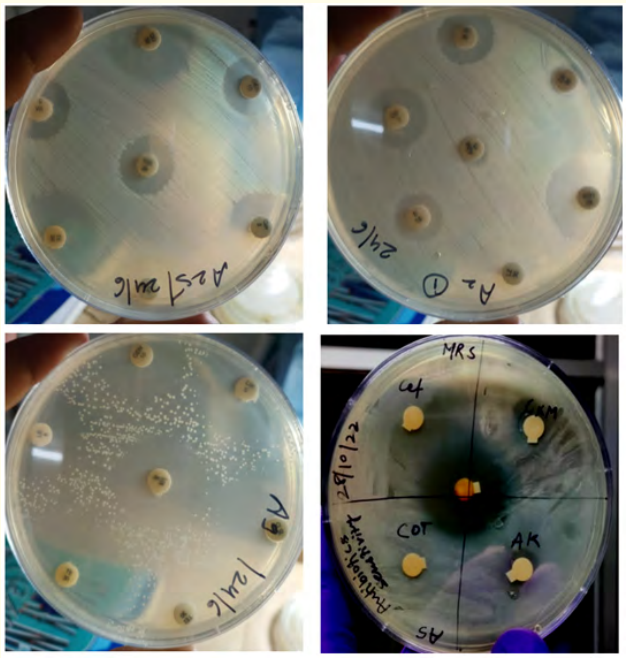


Figure 5: Antibiotic susceptibility activity of probiotics against selected antibiotics.

Antibiotics	Disc potency(µg)	DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8
Tetracycline	10	S	S	S	S	S	S	S	S
Ampicillin	10	S	S	S	S	S	S	S	S
Chloramphenicol	30	S	S	S	S	S	S	S	S
Cefazolin	5	R	R	R	LS	R	LS	R	R
Amikacin	5	LS	LS	LS	LS	LS	S	S	S

Table 4: Antibiotic susceptibility profile of probiotics isolates DM.

S= Sensitive, R= Resistant and LS= Least Sensitive

Antibacterial activity

The antimicrobial activity of each isolate was assessed by diameter of zone of inhibition (ZOI) against pathogenic bacteria. All these experiments were also performed in triplicates as per CLSI (Clinical and Laboratory Standards Institute) followed (CLSI 2015). The average range of ZOI was ranging in between 12 to 15mm as shown in figure 6.

Lactococcus lactis subsp cremoris displayed highest antagonistic activity against *Staphylococcus aureus* and *Bacillus subtilis* with the inhibition zone ranged from 14 mm in diameters. However *L. salivarius* showed a minimum inhibition zone of diameter ranging from 08 to 12 mm against the indicator microorganisms.

Discussion

New probiotic strains which can withstand gastrointestinal stresses and have antimicrobial activity against pathogens as well as immunomodulating and anti-inflammatory properties may be

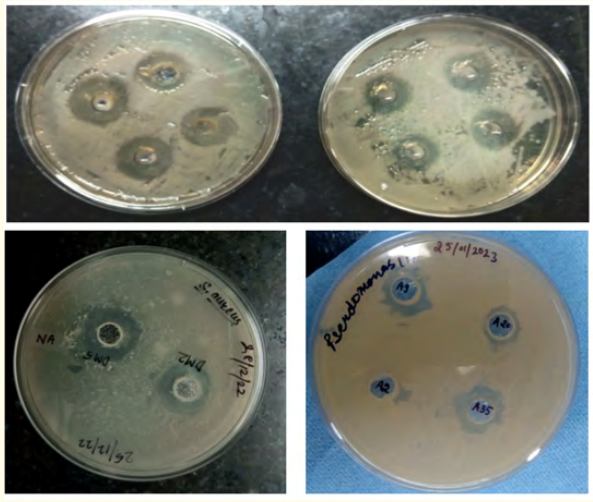


Figure 6: The zone of inhibition of DM isolates against pathogenic bacteria.

used to treat a variety of illnesses and balance the microbiota in the lower gastrointestinal tract, particularly in cases of inflammation. In our study, among 8 selected strains coccus-shaped bacteria are prevalent corresponded to Lactococcus and Enterococcous, constituting the most abundant group in DM. As per findings [21] the abundance of cocci in DM is due to the higher lysozyme resistance of coccus-shaped LAB than lactobacilli. Though *L. lactis*, has been also widely described in cow, goat, sheep, buffalo and human milk [22]. It is interesting to note that the authors found only coccus-shaped LAB; in particular *Enterococcus faecalis*, and *Enterococcus faecium* as the dominant species showed high tolerance toward acidic gastric conditions and high bile salt concentrations that are important factors in all probiotic products because transit through the acidic conditions of the stomach enables high probiotic activity in the desired part of the gut. The physiological conditions of human gastrointestinal tract (GI tract) vary with age and gender and normally stomach pH is 1.5-2.5 while bile concentration lies in the range of 0.3-0.5%. During probiotic strain selection strains must have ability to withstand against harsh conditions i.e., high bile concentration and low pH (mimicking GI tract conditions [17]. Recently it was found that the resistance of several commercially available probiotic products to simulated gastric juices and demonstrated high sensitivity and number reduction in acidic conditions, concluding that strong enteric protection of probiotic bacteria is needed [18]. Prominent candidates for probiotic use are LAB that can overcome stresses prevailing in the human gastrointestinal tract, especially in non-encapsulated, orally administered form where it is essential for bacteria to retain viability during adverse conditions of pH in the stomach compartment and resist toxicity of bile salts in the small intestine [23-26].Therefore, strains investigated in this work have great potential as part of probiotic products even in non-encapsulated form [27-30] . Both isolated strains showed great antimicrobial activity of a natural (acidic) supernatant against the tested pathogens.

Conclusion

In our study, we have isolated the ample of microbes but only 08 potent probiotic were selected for study. Among isolated probiotics *Lactobacillus, Enterococcus, Lactococcus* and *Leuconostoc* species were more effective than other isolated colonies. All isolates exhibited good survival ability rate against low pH and bile salt concentration but *Lactococcus lactis subsp cremoris and Enterococcusfaceium* were the most resistant to these harsh conditions. Hence, these two species are strong probiotic candidates also confirmed by literature. In future, DM probiotics will be the most interesting research area and a novel source for fermented food products with highly efficient probiotics.

Bibliography

1. Hemming F. "Usage Of 17 Specific Names Based on Wild Species Which Are Pre-Dated by Or Contemporary with those Based on Domestic Animals (Lepidoptera, Osteichthyes, Mammalia): Conserved". The Bulletin of Zoological Nomenclature 60 (2003): 81-84.

2. Grubb P. "Order Perissodactyla. In Wilson, D.E.; Reeder, D.M. Mammal Species of the World: A Taxonomic and Geographic Reference" (3rd edition) (2005): 62265494.

3. Garhwal R., et al. "Systematic Review of the Bioactive Components, Nutritional Qualities and Potential Therapeutic Applications of Donkey Milk". Journal of Equine Veterinary Science 115 (2022): 104006.

4. Press information bureau, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. Saurashtra Maldhari Sammelan (2022).

5. ISO EN. "15214. Microbiology of Food and Animal Feeding Stuffs-Horizontal Method for the Enumeration of Mesophilic Lactic Acid Bacteria-Colony-Count Technique at 30 Degrees C. In ICS, 7 (1998): 300.

6. Rastogi S., et al. "In vitro evaluation of probiotic potential and safety assessment of Lactobacillus mucosae strains isolated from Donkey's lactation". Probiotics and Antimicrobial Proteins 12 (2019): 1045-1056.

7. Cirrincione S., et al. "Donkey Milk Fermentation by Lactococcus lactis subsp. Cremoris and Lactobacillus rhamnosus Affects the Antiviral and Antibacterial Milk Properties". Molecules 26.16 (2021): 5100.

8. Bhardwaj A., et al. "Donkey milk composition and its therapeutic applications". Indian Journal of Animal Research 90.6 (2020): 837-840.

9. Kaushik JK., et al. "Functional and probiotic attributes of an indigenous isolate of Lactobacillus plantarum". PLoS One 4 (2009): e8099.

10. Massouras T., et al. "Microbial Profile Antibacterial Properties and Chemical Composition of Raw Donkey Milk". Animals 10 (2020): 2001.

11. Sharma P., et al. "Isolation and identification of pathogenic bacteria and fungi isolated from skin ulcers of Cirrhinus mrigala". Indian Journal of Animal Research 47.4 (2013): 283-291.

12. Sharma P., et al. "Relative efficacy of two probiotics in controlling the epizootic ulcerative syndrome disease in Mrigal (Cirrhinus mrigala Ham.)". Journal of Fisheries and Aquatic Science 8.2 (2013): 305.

13. Garrity and Jones D. "Bergey's Manual of Systematic Bacteriology". New York: Springer 3 (2009).

14. Kleerebezem M and Hugenholtz J. "Metabolic pathway engineering in lactic acid bacteria". Current Opinion in Biotechnology 14 (2003): 232-237.

15. Zhang B., et al. "Screening of probiotic activities of lactobacilli strains isolated from traditional Tibetan Qula, a raw yak milk cheese. Asian-australas". Journal of Animal Science 29.10 (2016): 1490-1499.

16. Vlková E., et al. "Antimicrobial susceptibility of bifidobacteria isolated from gastrointestinal tract of calves". Livestock Science 105 (2016): 253-259.

17. Aspri M., et al. "Raw donkey milk as a source of Enterococcus diversity: Assessment of their technological properties and safety characteristics". Food Control (2016).

18. Cavallarin L., et al. "A survey on the milk chemical and microbiological quality in dairy donkey farms located in North-Western Italy". Food Control 50 (2015): 230–235.

19. Neviani E., et al. "Characterization of Lactobacillus helveticus strains resistant to lysozyme". Le Lait 71 (1991): 65-73.

20. Clinical and Laboratory Standards Institute (CLSI). "Performance standards for antimicrobial susceptibility testing: twenty-second informational supplement. In: CLSI document M100-S22. Clinical Laboratory Standard Institute, Wayne (2015).v

21. Carminati D., et al. "Biotyping of cultivable lactic acid bacteria isolated from donkey milk". Applied Microbiology 59.3 (2014): 299-305.

22. Quigley L., et al. "The complex microbiota of raw milk". FEMS Microbiology Reviews 37 (2013): 664–698.

23. Kumari A and Sharma P. "The Nutraceutical Characteristics of Donkey Milk: A New Insight for its Potential Probiotics". Journal of Veterinary Medicine and Animal Sciences 4.2 (2021): 1074.

24. Kumari A., et al. "Exploration and Insights of Potential Probiotics Diversity of Donkey (Equus africanus asinus) Milk. 2nd International Electronic Conference on Diversity". IECD (2022).

25. Kumari A., et al. "Isolation and identification of probiotic bacteria from donkey (Equus africanus asinus) milk of South-Western Haryana". International Journal of Innovative Science, Engineering and Technology 9.4 (2022): 2348-7968.

26. Papademas P., et al. "Probiotic, Antimicrobial, Antioxidant and Sensory properties of Fermented donkey milk with Lactobacillus fermentum ME-3 and Lactobacillus acidophilus (ATCC4356)". BAOJ Microbiology 1 (2015): 004.

27. Gupta AK., et al. "Phenotypic Clustering of Indian Donkey Population Belonging to Six Agro Climatic Regions". Journal of Biodiversity and Endangered Species 5 (2017): 201.

28. Gupta AK., et al. "Biochemical profiles of Indian donkey population located in six different agro-climatic zones". Comparative Clinical Pathology (2015).

29. Jagadeesh KS. "Lactic acid bacteria as a source of functional ingredients". South Indian Journal of Biological Sciences 1 (2015): 70-71.

30. Bhardwaj A., et al. "Estimation of antioxidant potential of indigenous Halari and French Poitu Donkey milk by using the total antioxidant capacity and ferric reducing antioxidant power essay". Asian Journal of Dairy and Food Research 4 (2019): 307-310.