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Exploration and Insights of Potential Probiotics Diversity of Donkey (*Equusafricanusasinus*) Milk†

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Abstract: In this scientific era non-bovine milk is gaining more international acceptance for research and commercialization. As bovine milk is reported as hypersensitive to infant's serum due to high concentration of the caseins proteins which manifest the allergic reaction and act as potent allergen.To overcome these problems for infants, elders and immunocomprised people alternative nutritious and healthy drink is in demand. Among non-bovine milk, donkey milk consumption is increased since last decade. The nutritious components of donkey milk are comparable to human milk. Besides of high lysozymal the well adapted potential probiotics species of donkey milk are identified and categorized on the basis of their relative abundance. *Lactobacillus paracasei*, *Lactococcus lactis* and *Carnobacterium maltaromaticum* are more abundant while the genera *Leuconostoc*, *Enterococcus* and *Streptococcus* are least. Composition of these species also varies in raw milk and fermented milk. These strains also exhibit antimicrobial, antioxidant, anti-proliferative properties in culture.From literature it was found that the species isolated from fermented milk *Enterococcus faecium DM33* exert greatest antioxidative and most potent antimicrobial activity.It was also reported that this strain shows highest ACE-inhibitory activity with *Lactobacillus casei DM214* fermented milk.In view of this study, it seemed that donkey milk is very healthy, beneficial and immune booster for the digestive system of human being.

Keywords: probiotics; donkey milk; nutritious; antioxidant; anti allergic

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1. Introduction

Advancements in health sector is always most promising area specially preferment in dairy products. In last decade donkey milk is capturing international attention and increased market demand because of presumed health benefits. Donkey milk is very closely similar to human milk and exhibit anti-allergic, anti-oxidative, antimicrobial, anti-proliferative & anti-diabetic activities. Donkey milk production differs greatly from that of conventional dairy species, especially in terms of milk supply. Recent studies on donkey lactation curves showed that individual milk yield ranged between 1.54 and 1.73 kg/day on specialized farms [1] which generally raise animals in semi extensive conditions and care about their wellness. In comparison with bovine milk, donkey's milk contains less fat, protein and inorganic salts but more lactose content which is fast energy source. Moreover, high lactose content suggests use of donkey milk for probiotic purpose [2] because it is an ideal substrate for a correct development of intestinal lactobacilli and makes donkey's milk an ideal matrix for the preparation of probiotic drinks following the incubation

with *Lactobacillus rhamnosus*strains [3]. Isolated probiotics from donkey milk (*Lactobacillus paracasei*, *Lactococcus lactis* and*Carnobacterium maltaromaticum)*modulates gastrointestinal flora and stimulates the immune system [4] Fermented donkey milk enriched with probiotics is one of the most significant advancement in nutrition sector in order to deliver probiotics efficiently in host system [6] Thus, donkey milk (DM) is the one of the most demanding and healthy non-ruminant dairy product.

In this paper, donkey milk samples from local areas of southern Haryana are selected to explore biochemical properties and existence of probiotic in donkey milk.

2.Material &Methods

2.1. Collection of Samples

Milk Samples were collected from donkey farms and local tribal communities of village Luhari jattu (District Bhiwani), village Budaak (District Hisar), village Badopal (District Fatehabad) etc.Southern Haryana region. Milk samples were collected in sterile sample vials and during sample collection firstly udder/teats wiped with 70% ethanol or spirit dipped cotton by wearing sanitized gloves then few drops of milk were discarded& Collected milk a sterile vials [7](After collection, samples were stored at very low temperature in ice basket then transported to laboratory and stored in freezer or at −20 °C in lab for further use.

2.2.Identification of Biochemical Properties of DM Samples

The biochemical properties of milk samples i.e., SNF, fat content, density, CLR, proteins, temperature, water content etc. were studied by milk analyzer ultra.

2.3.Selective Culture of DM Probiotics

To reduce lysozymal activity in DM and increase bacterial population DM samples were stored at 20 °C for 24 h then used for culture [8] Selective culture of donkey milk probiotics was done on MRS agar and total no. of bacterial viable counts at optimum value of milk was standardized by pouring 200µL, 300µL and 400µLneat milk. 300µLmilk is standardized for sufficient bacterial culture.Then milk samples were prepared by serial dilution (85:15*v/v*) in saline solution and peptone as [9] protocol.Then cultures were incubated at 37°C for 48 h. in anaerobic conditions. Well defined glossy colonies were obtained and then inoculated in broth for different morphological, biochemical and confirmatory tests isolates were also preserved in 30% glycerol stock solution and kept at−80 °C for further analysis.

2.4.Identification of Bacterial Isolates

Microbial isolates were identified by different morphological studies like gram staining properties, shape and size, motility test, spore staining etc. physiological test like KOH test and catalase test then biochemical characteristics of all tests was reported based on Bergey's Manual [10] Further the in vitro analysis of probiotic properties like tolerance to low pH (2.0), tolerance against bile salt (0.3%), carbohydrate fermentation test was done according to modified [11] protocol. 1–1 mL of MRS brothtubes were adjusted at pH 2.0 & 0.3 % bile salt concentration by adding 1 M HCl and ox gall [12]. These tubes were incubated with 200µLof 48 h grown bacterial culture for 2–3 hrs. at37 °C anaerobically. Then viable counts were noted by plating 80µLof culture on MRS agar plates. All the experiments were technically performed independently in triplicates. After calculating log cfu/mL survivability was counted as

$$\% \text{ survivability} = (\text{viable log count at time t/viable log count at t} = 0) \times 100$$

3.Result and Discussion

The biochemical analysis of fresh milk samples were recorded as shown in fig. 1 and fig.2 . There are many environmental and genetic factors like lactation stage, health, maternal age and type of feed, breed, frequency and milking completeness etc. which directly or indirectly influence gross composition of milk. Donkey milk contains less protein, fats and inorganic salts but more lactose concentration comparable to human milk [13], In our study we reported that milk samples collected from local areas contain less SNF & lactose concentration.

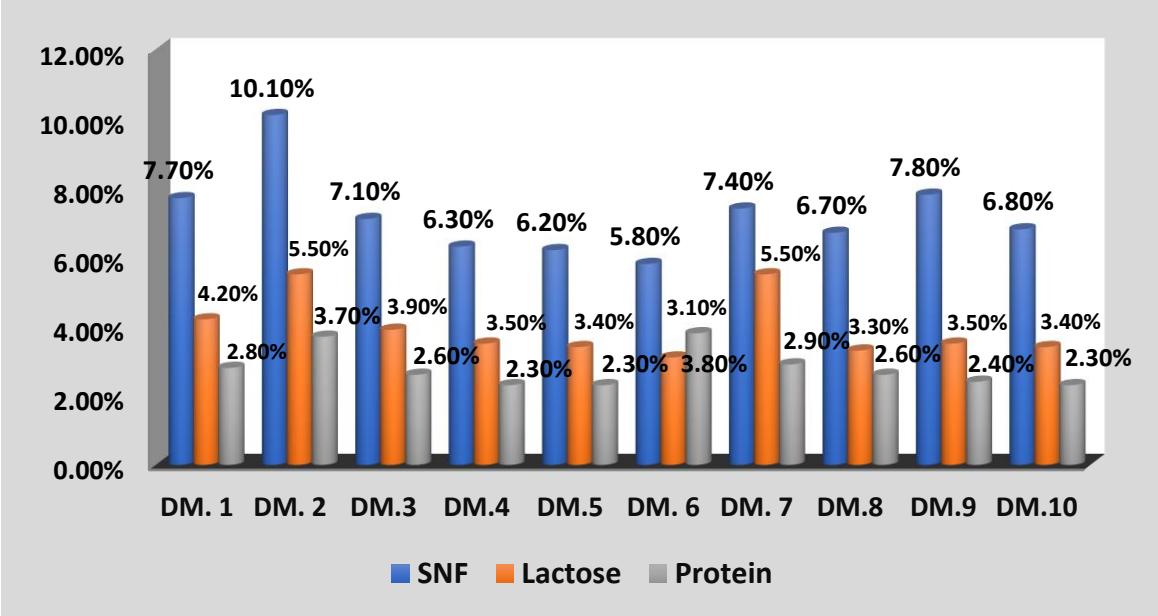

Fig. 1. Biochemical profiles of donkey milk samples collected from farms and local areas.

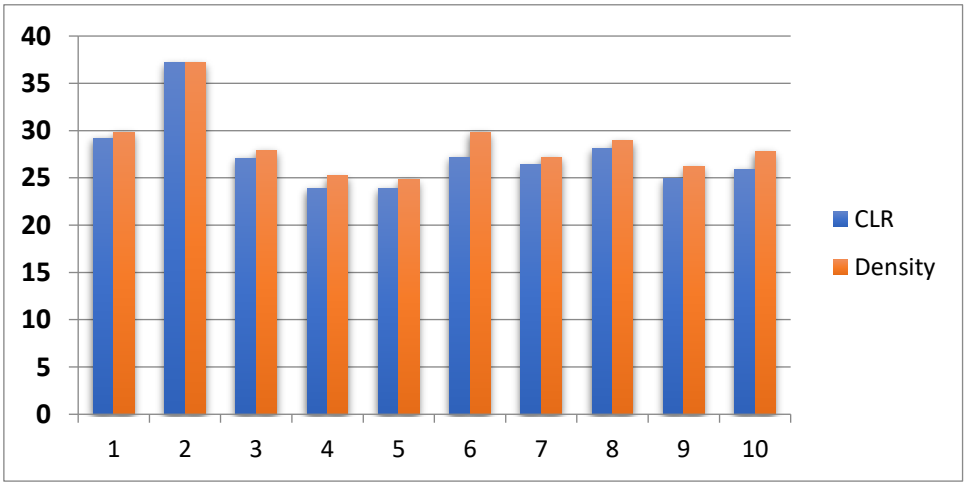

Fig.2 . Donkey milk samples density and CLR values.

The average value of protein, lactose, fat, solid non-fat (SNF) dry matter, ash & total solids ranges from 1.22–1.87%, 6.01–6.78%, 7.23–8.65%, 0.343–0.438% and 8.37–9.50%respectively).The average fat content on donkey milk ranges from 0.5–1.7% or negligible [15-16]. Likewise, in our study we have also reported insignificant fat concentration in our samples. But DM2 which was collected from farm area contained high (10.10%) SNF & lactose content (5.50%) while DM 5 collected from rural area of showed least SNF (5.80%) content and DM 6 collected from rural area contains least (3.10%) lactose amount. Sample

Density and CLR values in both types of samples are not varying so significantly and DM 2 possesses high density and CLR values.

The microbial population in fresh raw milk is comparatively less than other non-bovine milk due to presence of more natural antimicrobials such as lysozyme or lactoferrin.But, in this study, we have identified total 25 colonies and after combining biochemical tests of probiotic potential.The total viable bacterial count ranged between 1.8 to 2.8 log cfu/mL. Donkey milk exhibit differential microbial composition as reported less than 4 log cfu/mL and [17] reported high bacteria count 5 log cfu/mL. Among all bacterial isolates total 08 species as mentioned in table 1 DM.1(a), DM.2(a), DM.3(a), DM.4(a), DM.5(a), DM.5(c), DM.6(b), DM.8(a) are *Lactococcus lactis*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides*, *Lysinibacillus sphaericus*, *Enterococcus faecalis*, *Lysinibacillus fusiformis Brevibacillus choshinensis & Enterococcus durans* respectively.

Table 1. Morphological and Biochemical Characteristics of bacterial Isolates of Donkey milk on MRS agar.

Bacterial Isolates	Characteristics on MRS Agar Plates	Microscopic Characteristics(Gram Staining)	Log cfu/ ml	KOH Test	Catalase Test	Growth at (6%NaCl)
DM.1(a)	Small, smooth,	Gram (+)ve, single	2	(−)ve	(−)ve	(−)ve
DM.2(a)	Small, flat, creamy colour	Gram (+)ve, cocci, short chains	2.3	(−)ve	(−)ve	(+)ve
DM.3(a)	Small, white	Gram (+) ve, cocci	2.5	(−)ve	(−)ve	(+)ve
DM.4(a)	Medium, rounded, creamy	Bacilli, gram (+)ve, non−spore	1.8	(−)ve	(−)ve	(−)ve
DM.5(a)	Very small, glossy	Cocci, gram (+)ve,	2.9	(−)ve	(−)ve	(+)ve
DM.5(c)	Circular, medium, off−white	Gram (+)ve, cocci, straight, chain	2.6	(−)ve	(−)ve	(+) ve
DM.6(b)	Large, pale yellow	Gram (+)ve, bacilli	1.9	(−)ve	(−)ve	(−)ve
DM.8(a)	Medium, glossy white	Gram (+)ve, bacilli, tapering ends	2.8	(−)ve	(−)ve	(+)ve

We have also reported more prevalence of coccus shaped bacteria than bacilli. [18)]demonstrated that coccus shaped lactic acid bacteria are more lysozyme resistant than lactobacilli. *Lactococcus lactis* first time in DM were isolated by & also reported their prevalence as second most abundant bacterial species in DM of their study. *Lysinibacillus sphaericus, Brevibacillus choshinensis & Lysinibacillus fusiformis* species are gram positive, rod shaped endospore forming bacteria exhibiting high chemical resistant potential. These species were also isolated from raw cow milk. [19]also reported presence and theri potential in commercial probiotic formulations [20] *Lactobacillus paracasei* species are bacillus shaped, mesophilic & lysozyme resistant bacteria.

Table 2. Results of Carbohydrate fermentation tests of bacterial Isolates of DM grown in MRS broth.

Bacterial Isolates	Te	Ce	Ga	Mb	Su	Xy	Ma	Mo	Rf
DM.1(a)	P	N	P	N	P	N	N	N	P
DM.2(a)	N	P	N	P	P	P	P	P	P
DM.3(a)	P	N	P	P	P	P	P	N	N
DM.4(a)	N	N	N	P	N	N	N	N	P
DM.5(a)	P	P	N	N	P	P	P	P	N
DM.5(c)	P	P	P	P	P	P	P	P	P
DM.6(b)	P	P	N	P	N	P	P	P	P
DM.8(a)	P	N	P	N	P	N	N	N	P

Keys: P= Positive, N = Negative: Te =Trehalose,Ce = Cellobiose, Ga= Galactose,Mb=Melibiose, Su= Sucrose, Xy= Xylose, Ma= Maltose, Mo=Mannose, Rf= Raffinose.

Among all species only *Enterococcus faecalis*can reduce all sugar base used in experiment while *Leuconostoc mesenteroides*can reduce all sugars except cellobiose as shown in table 2.

All species showed good survivability at low pH and bile salt concentration.Among all bacterial species *Enterococcus faecalis* exhibited very high survivability and *Lactobacillus paracasei* showedleast tolerance against low pH and *Brevibacillus choshinensis* low survivability rate at bile salt concentration as shown in Chart 3.The physiological conditions of human gastrointestinal tract (GI tract) vary with age & gender and normally stomach pH is 1.5–2.5 while bile concentration lies in the range of 0.3–0.5% [21]. To withstand against GI tract probiotics must resist pH changes and bile salt concentration and these species showed good resistance against these changes.

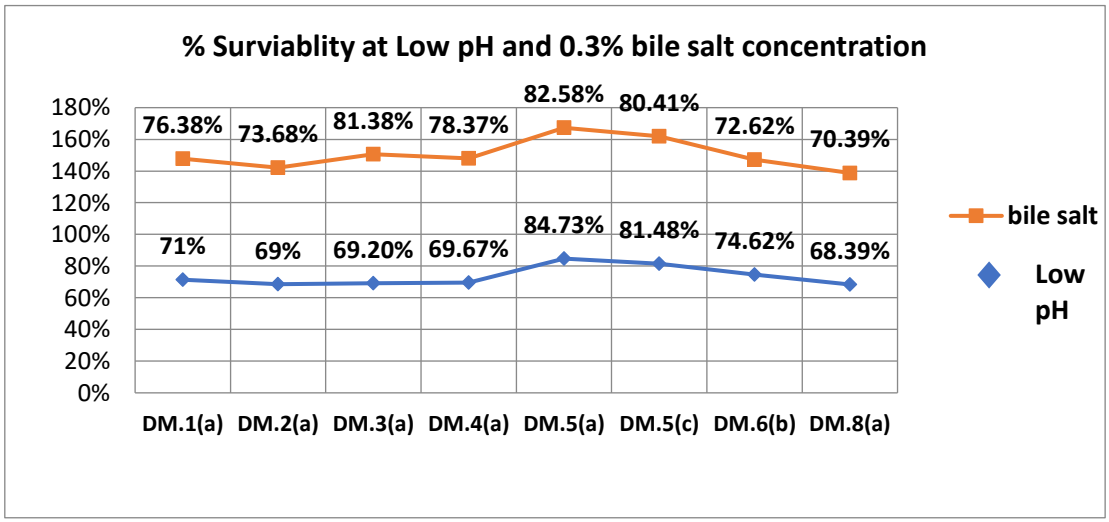

Fig. 3.% Survivability rate of bacterial isolates of donkey milk at 0.3% bile salt concentration and at low pH.

4.Conclusions

The biochemical characteristics i.e., lactose, SNF, proteins and fat content of donkey milk from local areas and farms of southern Haryanan found no significant difference in fat content but lactose content was less in field samples than farm milk samples. But, lactose content was enough to support the lactic acid bacteria growth as reported by their selective growth on MRS agar. *Lactobacillus paracasei* &*Enterococcus faecalis* species were in predominantly present in donkey milk samples collected from farm sites while in local field samples *Lactococcus lactis* and *Lysinibacillus sphaericus* species were dominant. The

probiotic potential of all the bacterial isolates was reported by observing their good survivability rate against low pH and bile salt concentration.

Data Availability Statement: data supporting this paper was generated by Department of Zoology, Chaudhary Bansi Lal University and ICAR-NRCE, Hisar, and are available from the corresponding author upon request.

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