Production and Shelf-Life Enhancement of phospholipids enriched instant Lassi powder from Desi Chhaach

FINAL PROGRESS REPORT

SUBMITTED TO

Scientific Engineer (B-I) for Secretary, EC

Haryana State Council for Science, Innovation & Technology (Dept. of Science & Technology, Haryana), Bays 35-38, Sector 2, Panchkula, Phone no-0172-2563439

From

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Performa for submitting Half Yearly Progress Report of the Project

1) **Title of the Project:** *Production and Shelf-Life Enhancement of phospholipids enriched instant Lassi powder from Desi Chhaach (buttermilk)*

2) **Date of start of Project:** 05.03.2021

3) **Name of Principal Investigator and address:** Dr. Ganga Sahay Meena, Scientist (Sr. Scale), Dairy Technology Division, ICAR-National Dairy Research Institute, Karnal, Haryana-132001

4) **Name of Co-Investigators (if any):** Dr. Ashish Kumar Singh, Head, Dairy Technology division

5) **Place of work:** Dairy Technology Division, ICAR-National Dairy Research Institute, Karnal, Haryana-132001

6) **Objectives:**

i. To standardize the production process of phospholipids rich *Lassi* powder from *Desi Chhaach*

ii. Characterization of manufactured powder and evaluation of its storage stability

iii. Conversion of developed powder into Sweet and Spiced variants of instant *Lassi* & Cost analysis

7) **Time schedule of Project indicating year-wise activities and financial requirements:**

Objectives	Activities	**Proposed Time line:** Duration (2 Years)			
		Mar 21-Oct 21 (8M)	Nov 21-Jun 22 (8M)	Jul 22-Dec 22 (6M)	Jan 23-Feb 23 (2M)
		0-8M	9-16M	17-22M	23-24M
Objective 1	• Recruitment of project staff & equipment's procurement • Physico-chemical analysis of Desi Lassi, Concentration employing vacuum evaporation, NF & RO; • Selection of spray and vacuum tray drying parameters Production of Instant Desi Lassi powder				
Objective 2	• Characterization of instant *desi lassi* powder for its physic-chemical, reconstitution, functional and rheological properties as well as microstructure using advanced analytical techniques such as SEM, FTIR, Master Sizer & DSC. • Evaluation of storage induced changes in its various properties at 30±1 ^{0}C up to 6 months				
Objective 3	• Developments of methods for manufacturing of sweet and spicy variants of Lassi from instant Desi Lassi powder. • Compositional and sensory analysis of developed products				
	• Cost analysis, data analysis, report writing & submission				

Approved financial requirements (INR, in lakh)

Items	First year	Second year	**Total**
Salaries/wages (**1 office assistant**)	1.5	1.8	**3.30**
Consumable	1.0	1.0	**2.00**
Travel	0.25	0.50	**0.75**
Other Costs (Equipment's)/ Contingencies	7.23	1.0	**8.23**
Total	**9.98**	**4.30**	**14.28**
Overheads (Institutional charges)	0.50	0.21	**0.71**
Grand Total	**10.48**	**4.51**	**15.00**

8) **(a) Summary of research work done so far viz-a-viz time schedule:** Please see attached ANNEXURE A for detailed project progress

Objective 1: To standardize the production process of phospholipids rich Lassi powder from *Desi Chhaach* (buttermilk). **Completed.**

Objective 2: Characterization of manufactured powder and evaluation of its storage stability. **Completed.**

Objective 3: Conversion of developed powder into Sweet and Spiced variants of instant Lassi & Cost analysis. This bill be completed by 04.03.2023.

(b) Expected date of completion of the project: 04.03.2023

9) **Report of work done so far** (please indicate time versus activity schedule) :

Objectives & Activities	**Time line**		
	April 21-Sept. 21 06 months	October 21-March 22 06 months	April 22-Sept. 2022 06 months
• Recruitment of project staff • Physico-chemical analysis of *desi* chhaach (buttermilk), Concentration employing vacuum evaporation, NF & RO; • Selection of spray and vacuum tray drying parameters Production of *desi* chhaach (buttermilk) powder			
• Production of instant *desi* chhaach (buttermilk) powder and It's detailed and characterization for physic-chemical, reconstitution, functional and rheological properties as well as microstructure using advanced analytical techniques such as SEM, FTIR, Master Sizer & DSC.			
• **Production of instant *desi* chhaach (buttermilk) powder using Nanofiltration (NF) Process and its detailed and characterization**			

10) **Staff position:** Recruited 1 Office assistant from 5.8.2021 is continuing.

11) **Details of Head wise & year wise expenditure:** Funds were utilized in wages and consumables. Detailed UC will be submitted in Final completion report.

12) **List of equipment purchased (if any): Already provided**

(a) Name of equipment:

(b) Date of receipt:

(c) Date of Installation:

13) **Difficulties/problems faced in implementation of project and suggestions for remedies:** NIL.

14) List of publications indicating grant no. of HSCST along with reprints related to the project: 1 (Published research paper in SCI journal.

Manik, S., Meena, G. S., Singh, A. K., Khetra, Y., Singh, R., Arora, S., & Vishweswaraiah, R. H. (2023). Valorization of Sour Buttermilk (A Potential Waste Stream): Conversion to Powder Employing Reverse Osmosis and Spray Drying. Membranes, ***13*****(9)**, 799.

- **Technology Development: A technology for the "Production of instant *desi* lassi powder employing Nanofiltraion" has been developed.**

Any other relevant information: NIL

Signature of PI

Date: - 19/12/2023

Place: - ICAR-NDRI, Karnal

ANNEXURE A

PROJECT PROGRESS

Project Title: Production and Shelf-Life Enhancement of phospholipids enriched instant Lassi powder from *Desi Chhaach*

Project objectives

1. To standardize the production process of phospholipids rich *Lassi* powder from *Desi Chhaach*
2. Characterization of manufactured powder and evaluation of its storage stability
3. Conversion of developed powder into Sweet and Spiced variants of instant *Lassi*

Objective wise progress

Objective 1: To standardize the production process of phospholipids rich *Lassi* powder from *desi chhaach.*

Activity 1: Sample collection and physico-chemical analysis of *desi chhaach.*

For each trail fresh *desi chhaach* samples were procured from various farmers located in the vicinity of ICAR- National Dairy Research Institute, Karnal, Haryana. Such farmers routinely produce *desi chhaach* using traditional method via churning of curd. Samples meeting the organoleptic criteria were collected in early morning, pooled and brought to ICAR-NDRI, Karnal in pre-sterilized SS cans (40 Kg) at the earliest. For each trail 150-160 Kg sample was used.

Just after receiving the pooled sample at ICAR-NDRI, Karnal, it was immediately subjected to physico-chemical analysis. The total solids (TS), fat, protein, ash contents and pH values were determined. Thereafter, *desi chhaach or desi* buttermilk *(***DBM**) was pre-heated to 40±1 ºC, filtered through muslin cloth and subjected to cream separation to obtain separated *desi* buttermilk (**SDBM**) which was further concentrated to obtain *desi* buttermilk concentrate (**DBMC**). A systematic process flow chart related to processing of *desi* buttermilk employing Nanofiltration is shown in Figure 1.

The chemical composition and physical properties of *desi* buttermilk and separated *desi* buttermilk are shown in Table 1. The concentration of milk constituents i.e. TS, fat, protein and ash contents of *desi* buttermilk was relatively lower (Table 1) than that of whey containing TS in the range of 6.1-6.9%.

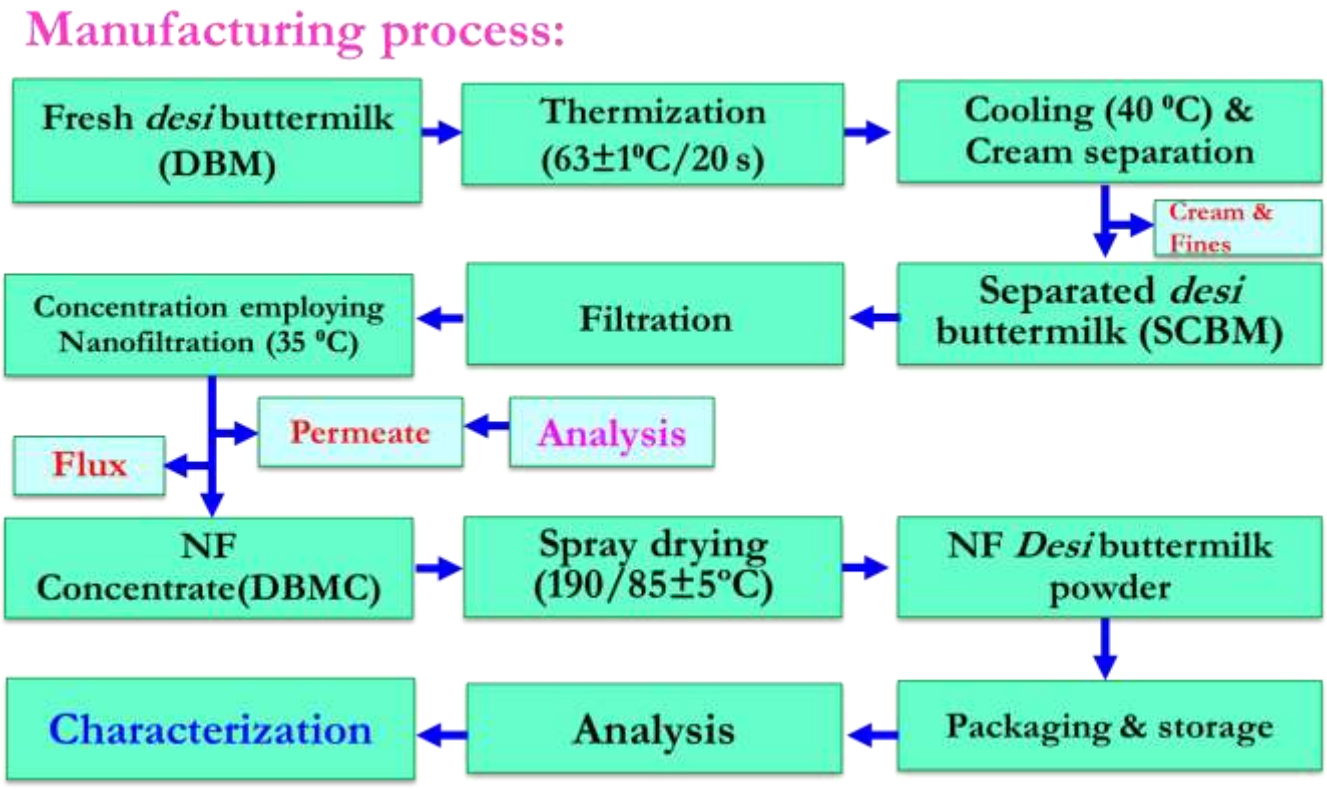

Figure 1: Processing of *desi* buttermilk employing NF as a method of concentration.

Table 1: Physico-chemical properties of *desi buttermilk*, separated *desi* buttermilk and *desi* buttermilk concentrate

Chemical composition					
Buttermilk samples	Total solids (%)	Fat (%)	Protein (%)	Ash (%)	pH at 20 ºC
DBM	3.56±0.02^{b}	0.48±0.01^{b}	1.52±0.00^{b}	0.30±0.01^{b}	3.73±0.01^{b}
SDBM	3.25±0.01^{c}	0.15±0.02^{c}	1.58±0.01^{c}	0.32±0.01^{b}	3.73±0.00^{b}
DBMC	19.09±0.02^{a}	0.88±0.01^{a}	9.59±0.04^{a}	1.41±0.01^{a}	4.07±0.01^{a}
Physical properties					
Buttermilk samples	ζ-potential (mV)	η at 20 ºC & 100 $s^{-1,}$ (mPa.s)	Color values		
			L*	a*	b*
DBM	6.63±0.38^{b}	9.34±0.25^{b}	79.49±0.01^{b}	-2.04±0.03^{b}	7.49±0.08^{b}
SDBM	10.38±0.40^{a}	4.41±0.01^{c}	79.41±0.04^{c}	-2.14±0.07^{b}	7.28±0.10^{c}
DBMC	6.82±0.12^{b}	953.75±1.01^{a}	83.15±0.01^{a}	0.36±0.03^{a}	12.65±0.02^{a}

Mean± SE (n=3), means with different superscripts are significantly different ($p<0.05$) with each other. Where DBM: *desi* buttermilk, SDBM: separated *desi* buttermilk, DBMC: *desi* buttermilk concentrate, η: apparent viscosity.

This is attributed to the amount of water added in curd during its traditional method of churning. This is well established that to maintain the desired temperature of *dahi* or curd during churning, hot or cold water is usually added in every house and the same has resulted in dilution of curd that decreased the concentration of milk constituents simply by dilution phenomena. Thus, *desi* buttermilk had 0.48% % fat, 1.52% protein and 0.30% ash content. It's pH was 3.73 and attributed to its uncontrolled/longer incubation period.

The separation of *desi* buttermilk results in removal of fat and suspended curd particles. A significant decrease ($p<0.05$) was observed in fat content, pH value and L* value of separated *desi* buttermilk compared to that of ordinary *desi* buttermilk as shown in Table. The obtained NF concentrate had 5.36- and 5.42-times higher TS (concentration) than *desi* buttermilk and separated buttermilk respectively.

Activity 2: Concentration of *desi chhaach* employing Nanofiltration process.

Nanofiltration (NF) is a pressure driven membrane process which falls between RO and UF processes based onn membrane pore size. It is well known as demineralization process as well. During concentration of desi buttermilk, the NF plant was operated at a constant temperature of 35±1 ºC and 20 bar pressure. The pictures of NF plant is shown in Figure 2 and technical details are given hereunder. The chemical composition of NF retentate/concentrate is shown in Table 1.

Figure 2: Image of the RO plant used for the concentration of *desi* buttermilk.

Details of the NF plant

- Supplier: M/s Permionics Membranes Pvt. Ltd., Vadodra; Cost: Rs. 6,99,300/-

- Membrane material: Poly Amide-AFC40 pH:3-11, pH range : 1.5-9.5, Operating pressure Max. 60 Bar, Temperature: 60 ºC,
- Membrane area: 0.9 m^2
- Module: Tubular
- Pump: Triple plunger type with SS contact surface

▪ **Change in permeate flux during Concentration of separated *desi* buttermilk**

The change in permeate flux during entire concentration process was recorded. It was observed that with increase in percent volume reduction, the flux exhibited a gradual decrease as shown in Figure 3. Using the following equation, the mean flux (Liters per square meter per hour, L/m^2/h) during concentration of separated *desi* buttermilk employing NF calculated as 27.77 LMH and observed to higher than that recorded for RO (15.07 LMH) process.

$$Flux\ mean = Final\ \ flux + [0.33 \times (initial\ flux - final\ flux)]$$

$$= 5.33 + [0.33 \times (73.33\text{-}5.33) = 27.77\ \text{LMH}$$

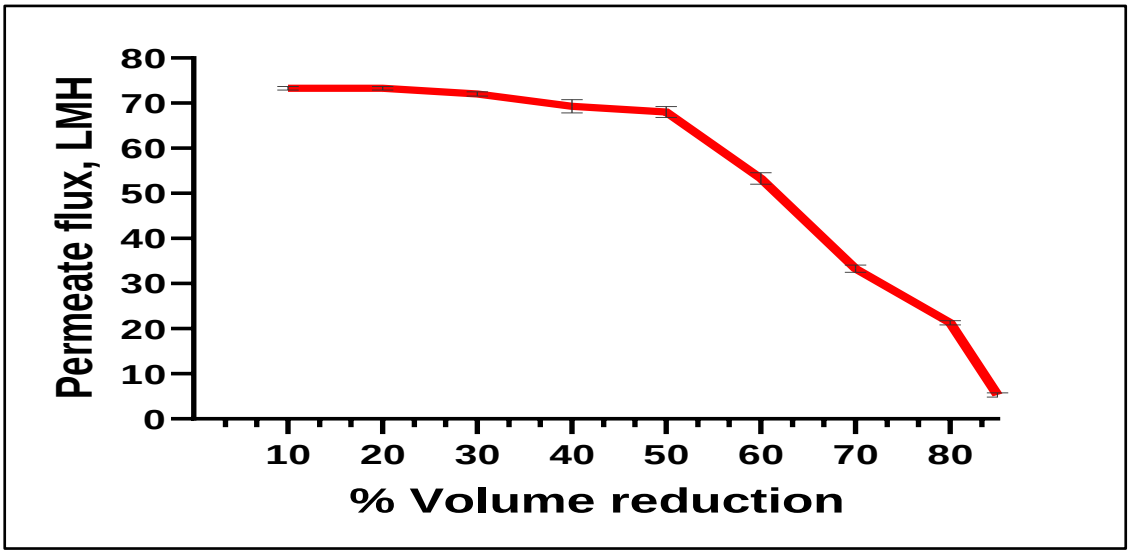

Figure 3: Change in permeate flux as a function percent volume reduction ratio.

▪ **Suitability of NF process for the concentration of separated *desi* buttermilk**

- ✓ The potential of NF process in dewatering of separated *desi* buttermilk (SDBM): Basis on mass transfer
- Quantity of separated *desi* buttermilk used for the trial =159 Kg
- Total solids present in separated *desi* buttermilk = [159×3.25 (%TS as per Table 1) / 100] = 5.1675Kg

- Quantity of water present in separated *desi* buttermilk = 159-5.1675= 153.8325 Kg
- Quantity of NF permeate obtained through 84.89% volume reduction of SDBM = (159×84.89)/100 =134.98 Kg
- Quantity of *desi* buttermilk concentrate (DBMC) obtained = Total feed (159 Kg)-Total permeate (134.98 Kg)=24.02 Kg
- Amount of water present in DBMC = Total amount – TS present in DBMC
  = 24.02- [24.02×19.09(% TS, as per Table 1)/100]=24.3-4.585418= 19.434582 Kg
- Amount of water removed during NF conc.= Total amount – TS present in permeate
  = 134.98-[134.98×0.43(% TS)/100]=134.98-0.580414=134.399586 Kg
- Hence, percent water removed in NF concentration of initial water present in SDBM
  = [134.399586 (permeate generated) ×100]/153.8325 (water present in SDBM) = 87.37%
- Hence, 87.37% water present in separated *desi* buttermilk was successfully removed by NF
- Therefore, NF seems to be an economic dewatering process as it merely consumes pumping energy
- Thus, in nutshell, NF is highly suitable for the concentration of separated *desi* buttermilk

Hence, it is emphasized that 87.37% water present in separated *desi* buttermilk was successfully removed by NF process and the same was higher than 72.62% water removal by RO concentration. Hence, NF process was even more economical than RO during concentration of *desi* buttermilk.

Activity 3: Drying of *desi chhaach* concentrate

Manufacturing of *desi* buttermilk powder (DBMP) using spray drying

As another approach, *desi* buttermilk concentrate obtained through NF concentration was subjected to spray drying. The used pilot scale single stage spray drier was equipped with a feed pump (positive displacement type, controlled by VFD) and a centrifugal (rotary) atomizer and two cyclone separators. To achieve < 5% moisture content, the inlet air temperature was maintained at 190±5 ºC while outlet air temperature was maintained at 85±5 ºC by control feed rate. During spray drying, the RPM of centrifugal atomizer were kept constant at 22000. The resultant powder was referred as *desi* buttermilk powder (DBMP). It was observed that spray drying is a suitable technique for the conversion of *desi* buttermilk concentration in to *desi* buttermilk powder.

Objective 2:

Characterization of manufactured Lassi powder and evaluation of its storage stability.

Activity 1: Determination of physico-chemical, reconstitution, functional properties, microstructure & rheological properties of *Lassi* powders.

The obtained buttermilk powder was subjected to detailed characterization for its various powder properties as discussed in the following section. The image of manufactured DBMP is shown in Figure 4 while its chemical composition is shown in Table 2.

▪ **Chemical composition of desi buttermilk powder (DBMP)**

Table 2 clearly indicates that based on TS, DBMP had < 5% moisture content and the same is in accordance with the FSSAI (2011) standards prescribed for skim milk powder (SMP) and whole milk powder (WMP). The fat content (4.69%) of DBMP is higher than that of SMP (1.05%) but near to the fat content (6.38%) of sweet cream buttermilk powder (SCBMP).

Figure 4: Manufactured *desi* buttermilk powder (DBMP).

Table 2: Chemical composition of *desi* buttermilk powder, pH and zeta potential of its reconstituted solution

Sr. No.	Parameters	*Desi* buttermilk powder (DBMP)
1	Total solids (%)	98.04±0.03
2	Fat (%)	4.60±0.03 ***(4.69)***
3	Protein (%)	49.09±0.08 ***(50.07)***
4	Lactose (%)	36.37±0.05 ***(37.09)***
5	Ash (%)	7.25±0.03 ***(7.40)***
6	pH (10% w/v solution), at 20 ºC	4.13±0.01
7	ζ-potential (mV) of reconstituted DBMP solution (1000 times dilution)	-2.45±0.33

Mean ± S.E. (n=3), the values in **bold font** are on dry matter basis.

The protein content of manufactured *desi* buttermilk powder was 50% protein, 37% lactose and 7.40% ash contents, respectively. The protein, lactose and ash contents of SMP were reported as 40.29%, 48.15 % and 7.76%, respectively by Pal & Mulay (1983). Thus compared to SMP, DBMP had higher protein and lower lactose and ash contents.

▪ **Physical and reconstitution properties of *desi* buttermilk powder (DBMP)**

Table 3 shows the physical and reconstitution properties of the manufactured *desi* buttermilk powder. Reconstitution properties of powders are governed by their chemical composition and physical properties, which directly influence their sensory attributes and functional properties as well. The water activity of this powder was 0.25 and the same lies in 0.2-0.3 water activity normally obtained for dried powders.

Table 3 : Physical and reconstitution properties of *desi* buttermilk powder (DBMP)*

Physical and reconstitution properties		*desi* buttermilk powder
Air contents (mL 100 g^{-1} powder)	Interstitial air	78.73±2.37
	Occluded air	77.86±1.15
Densities (g mL^{-1})	Loose bulk density (LBD)	0.49±0.01
	Packed bulk density (PDB)	0.62±0.01
	Particle density (PD)	1.25±0.02
	Angle of repose, (ɵ°)	32.10±1.36
Flowability	Hausner ratio (H_R)	1.26±0.01
	Compressibility index (CI)	26.58±0.81
Porosity (%)		48.80±0.9
Wettability (s),	**Instant soluble**	**03.00±0.00**
Dispersibility, %		82.07±0.84
Solubility, %		46.64±0.69
Color values	*L**	74.14±0.05
	*a**	2.17±0.20
	*b**	25.87±0.44
Water activity (a_w)		**0.266±0.00**
Hydroxymethylfurfural (HMF) µmol/Kg of powder		1166.15±1.17
Free fat (% of total fat)		1.23±0.01
2-thiobarbituric acid, TBA (µg/mL)		0.15±0.00

*Mean ± S. E. (n=9)

Carr index (1965) relates angle of repose with powder flowability. It was observed that based on angle of repose, the manufactured powder was exhibited **excellent flowability** as powder with Ɵ =25–30◦ falls under excellent flow category as per USP standards. This powder

exhibited 51.36% porosity. Amaladhas and Emerald (2017) reported that milk powders produced from feed containing higher viscosity have increased densities, but decreased porosity values. Wettability refers to the inherent ability of milk powder particles to absorb water and get wet. **Furthermore, the wetting index of this powder was 3 seconds only which is even half of that normally observed for spray dried SMP.** Thus, any powder which shows wetting time < 15 seconds is considered as instantly soluble. **Hence, the manufactured was also instantly soluble.** . The powder was brownish in color that could be attributed to prolonged heating of milk by farmers and changes in its color during spray drying as witnessed by its HMF content as well. The free fat of this powder was quite low. Bulk density of milk powders is quite important from cost (packaging material and storage space) and market point of view. Its loose and packed bulk density values were 0.49 and 0.62 g/mL which is accordance with bulk density values of SMP (0.543 g/ mL) but higher than the bulk density of SCBM (0.345 g/mL) as reported by Pal & Mulay (1983). The variation in particle size of DBMP is shown in Table 4

Table 4: Particle size analysis of *desi* buttermilk (DBM), separated *desi* buttermilk (SDBM), *desi* buttermilk concentrate (DBMC) and NF *desi* buttermilk powder (DBMP)

Parameters	DBM	SDBM	DBMC	DBMP
SSA, m^2/kg	1200±0.00^{c}	1321.58±0.00^{b}	1324.50±0.00^{a}	1199.20±0.80^{c}
Span (%)	5.31±0.00^{b}	5.22±0.01^{b}	3.74±0.03^{c}	99.98±0.46^{a}
d_{10}, µm	0.22±0.00	0.199±0.00	0.199±0.00	0.208±0.00
d_{50}, µm	0.71±0.00^{a}	0.61±0.00^{d}	0.62±0.00^{c}	0.69±0.00^{b}
d_{90}, µm	4.01±0.00^{b}	3.40±0.00^{b}	2.54±0.02^{c}	69.60±0.32^{a}
$D_{3,2}$, µm	0.5±0.00^{a}	0.45±0.00^{b}	0.45±0.01^{c}	0.50±0.00^{a}
$D_{4,3}$, µm	1.88±0.00^{b}	1.54±0.00^{c}	1.05±0.01^{d}	15.53±0.01^{a}

Mean ± SE, n=3; abcd indicates different superscript are significantly($p<0.05$) different

- **Functional properties of *desi* buttermilk powder (DBMP)**

Protein rich powders delivers desirable attributes to the food formulations owing to their unique functional properties. The functional properties *desi* buttermilk powder is shown in Table 5. Solubility of dried powders is vital for the full expression of other functional properties of these

powders. The solubility of *desi* buttermilk powder was determined in terms of percent (Table 2) and as insolubility index. The insolubility Index of this powder was 22 mL/ 100 mL and the same could be regarded as good for a product manufactured from highly acidic feed material and contain higher protein and low lactose content. Due to low pH and destabilized proteins, its heat stability is very poor.

Table 4 : Functional properties of *desi* buttermilk powder (DBMP)*

Insolubility index (mL/ 100 mL)	**22.00±0.28**
Apparent viscosity (mPa.s) at 20 ºC	3.08±0.56
Water binding capacity (g per g of protein)	**4.20±0.02**
Oil binding capacity (g per g of protein)	2.44±0.00
Foaming capacity (%)	25.00±0.7
Foam stability (%)	18±1.4
Emulsion activity (%)	31.35±0.35
Emulsion stability (%)	80.15±0.02

*Mean ± S. E. (n=9)

Water binding (WB) refers to holding of water in a 3-D structure of food (Patil et al. 2018). It is collectively influenced by pH, temperature, salts, ionic strength as composition of proteins (Schuck 2013). The water binding capacity of this powder was excellent (4.20 g per g of protein). It is because water binding of fully soluble proteins is lesser than that of partially soluble protein (Singh 2011). Casein micelles have been reported to bind large quantities of water (2–4 g of H_2O g^{-1} protein) in their native state (Singh 2011). Its oil binding capacity was 2.77 g per gram of protein. Zayas (1997) reported that insoluble and hydrophobic proteins exhibit better oil binding capacity and vice-versa. The foaming capacity and foam stability of this powder were 18 and 31 % and could be attributed to its lower pH. Further, Zayas (1997) reported that soluble proteins have been reported to form most stable foams that can interact and even form thick viscous films.

Rheological properties and particle size analysis

The rheological properties i.e. flow curve and change in viscosity as function of rise in temperature was determined and depicted in Figure 5 . A typical shear thinning behavior (i.e. decrease in viscosity with increase in shear rate) was observed in all samples. Similarly, rise in temperature also decreased the apparent viscosity of test samples as shown in Figure 6.

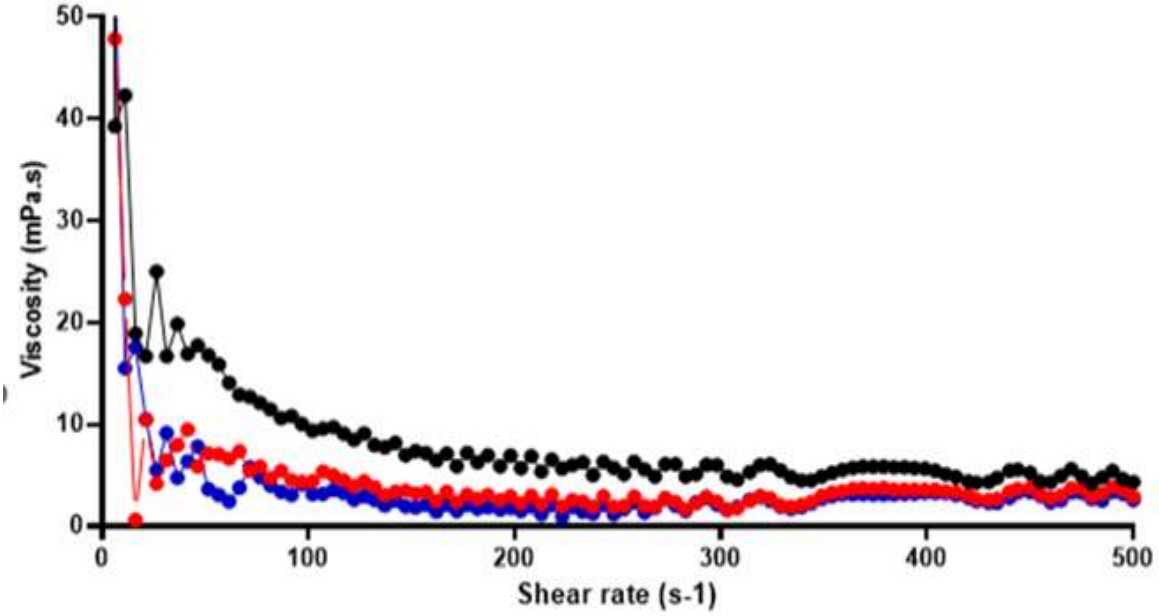

Figure 5: Change in apparent viscosity as a function of shear rate *desi* buttermilk **(black line**), separated *desi* buttermilk concentrate (**redline**) and reconstituted powder solution (**blue line).**

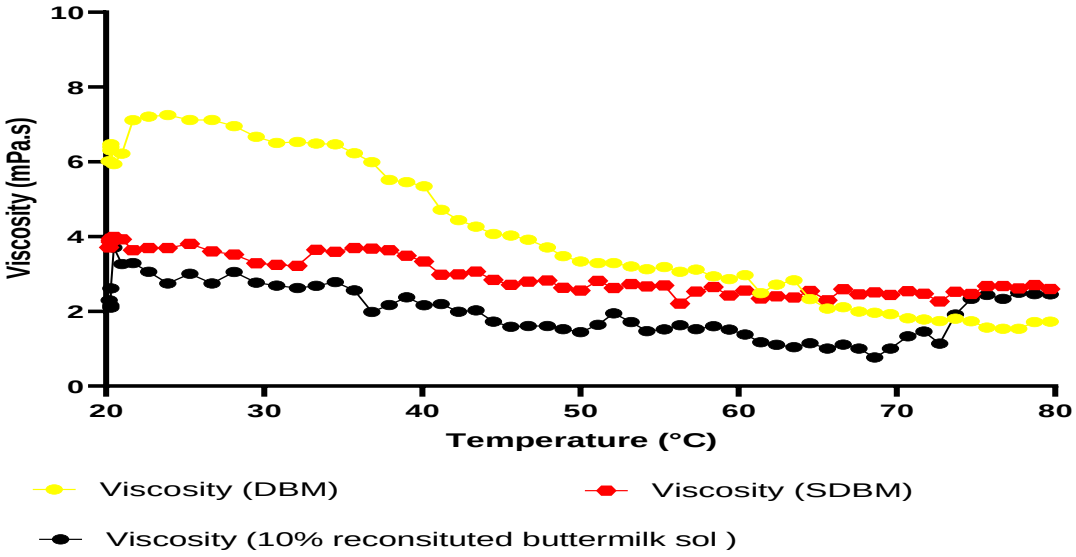

Figure 6: Change in apparent viscosity as a function rise in temperature.

Microstructure of Buttermilk Powder

The microstructure of the manufactured buttermilk powder was studied using Scanning electron microscopy (SEM). The SEM micrograph of buttermilk powder are shown in Figure 7. The buttermilk powder was observed to have particles of variable size which was also advocated by its particle size analysis shown in Table 4.

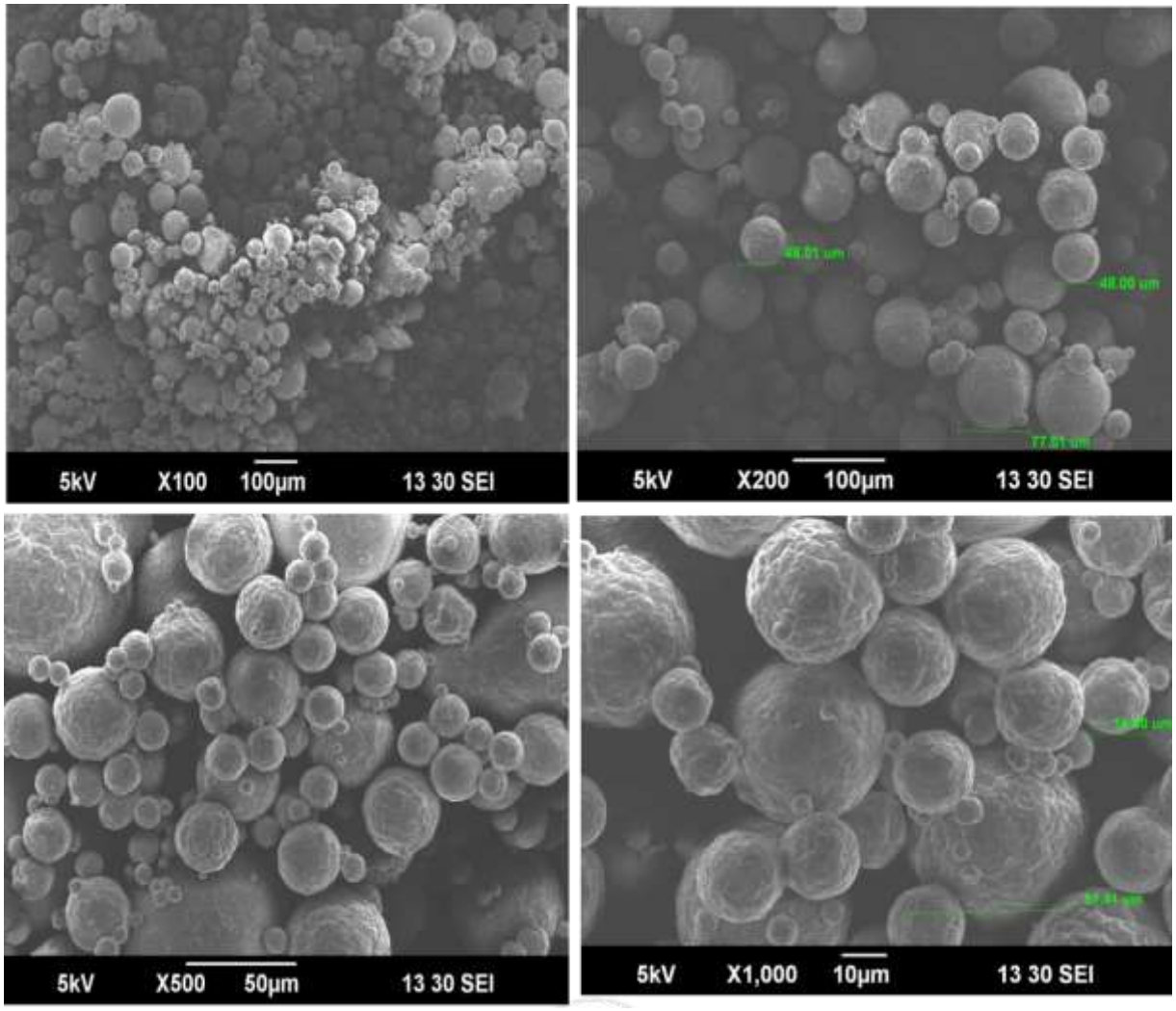

Figure 7: SEM micrograph of buttermilk powder at 100, 200,500 and 1000×.

Fourier transform infrared (FTIR) Spectroscopy

The *desi* buttermilk, separated buttermilk, NF concentrate and *desi* buttermilk powder were subjected to FTIR analysis and resultant FTIR spectra are shown in Figure 8. Powder samples shows different pattern in its spectra.

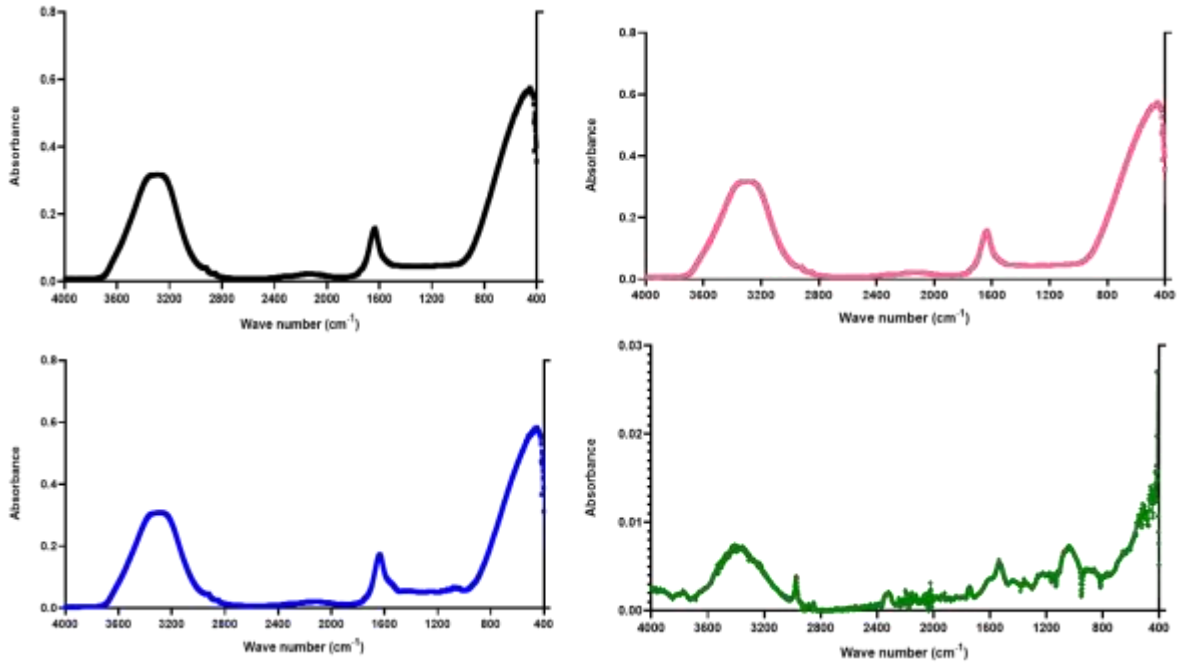

Figure 8: FTIR Spectra of *desi* buttermilk (black line), Separated buttermilk, (pink line), Desi buttermilk concentrate (blue line), and desi buttermilk powder (Green line)

Buffering Index of Buttermilk powder

Buffering capacity of reconstituted buttermilk solutions was determined using a method described by Van Slyke (1922). The pH of reconstituted buttermilk powder solution varied in 2–10 pH range with 0.5-unit increments. The pH values were changed with the addition of acid and base and relative changes in its dB/dpH values are shown in Figure 11. The buffer index of reconstituted buttermilk powder solution showed an increase during its acidification (up to pH 2) and then decreased during its alkalinisation (up to pH 10). Salaun et al. (2005) reported that total buffering capacity of milk powders receives 5%, 20%, 35% and 40% contribution from whey proteins, colloidal calcium phosphate, casein and soluble minerals, respectively.

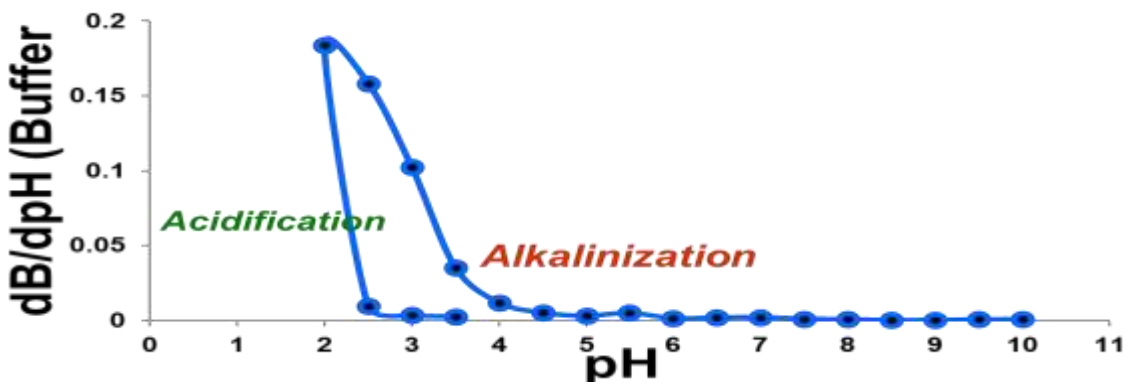

Figure 8: Buffer index of acidification-alkalization with 0.1 N (i.e. 1.0×10^{-4} g equivalent per m^3) HCl and 0.1 N (i.e. 1.0×10^{-4} g equivalent per m^3) NaOH solution of reconstituted powder (0.5% protein solution).

Activity 2: Storage stability of *Lassi* powders.

The shelf life of milk is extended by its conversion to powder form using drying technique. Milk powder has a shelf life of ~ 0.5 to 2 years when stored at room temperature (~ 30 °C). Maintaining the quality and functional attributes of milk powder during storage is an essential criterion for dairy powders which governs its future use (Shinde, 2018). In this investigation also, the DBMP was developed using spray drying and resultant powder was stored at 30 °C for 6 months. Its various properties were checked at constant interval of 1 months to evaluate its storage stability (table 5). Physical properties such as a_w, acidity (% LA), colour values (*a** and *b**) are significantly ($p<0.05$) increased while pH and *L** value in colour were significantly ($p<0.05$) decreased during the progression of storage period. Flowability showed a significant ($p<0.05$) increase as shown in Table 5. Similarly chemical changes such as HMF, tyrosine and TBA were significantly ($p<0.05$) increased during the entire storage period. The solubility of the fresh DBMP decreased from 46 % to 43% after 6-month storage.

Objective 2:

Developments of methods for manufacturing of sweet and spicy variants of Lassi from instant Desi Lassi powder

Activity 1: Sensorial analysis of developed products and Conversion of developed powder into plain, sweet and spiced variant of *lassi*

Sensory parameters along with the nutritional properties decide the consumer preference regarding food products. Sensory analysis is one of the mandatory steps before introducing a product in the market (Routray and Mishra, 2012). The developed DBMP was also subjected to sensory evaluation by a trained panel of judges. DBMP samples (5%, w/v) under the test were evaluated for sensory attributes viz. appearance of package, appearance of powder, appearance of reconstituted buttermilk prepared from powder, body and texture of reconstituted buttermilk and flavour of reconstituted buttermilk by a panel of judges using a sensory evaluation score card (Bureau of Indian Standards, IS:10030 –1981). The judges were suitably trained to acquaint them with sensory attributes of the product to be tested. DBMP samples were reconstituted by weighing 5 g of the sample and making the volume to 100 mL in a volumetric flask using RO water. The water and powder were vigorously mixed for 90 s using a magnetic stirrer. Here, the cumulative score (sum of all sensory attributes) assigned to DBMP

by judges was 81.67 (Figure 9). According to above discussed BIS method, milk powder with ≤59, 60-79, 80-89, ≥ 90 cumulative score have been graded as Poor, Fair, Good and Excellent. Hence as per this method, developed DBMP was graded as "Good" with 81.67 score by the trained panel of judges.

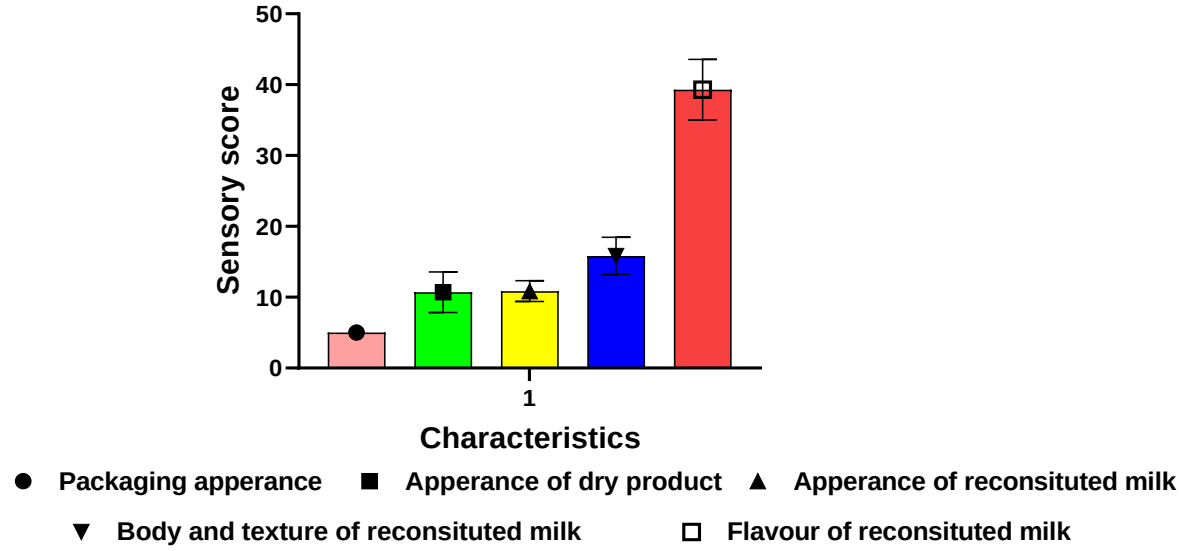

Figure 9: Sensory attributes of DBMP powder

Furthermore, it was converted into plain, sweet and salted variant of instant lassi and subjected to sensory analysis using 9-point hedonic scale. Here a variation in sugar level (1.2:1, 1.5:1 and 1.8:1 of TS, Figure 10), salt level (0.1:1, 0.15:1 and 0.2:1 of TS, Figure 11) and spice level (0.2:1, 0.3:1 and 0.4: of TS, Figure 12) were used for conversion of sweet, salted and spiced variant of *lassi*. The 1.5:1 sugar level, 0.15:1 salt level and 0.2:1 spice level were selected based on sensory scores. The overall acceptability of all these samples were rated as Like moderately. So that, the developed DBMP powder was successfully converted into plain, sweet and spiced variant of *lassi*.

Table 5 : Storage stability of *desi* buttermilk powder (DBMP)

Parameters		Storage period at 30 °C						
		0 day	1st month	2nd month	3rd month	4th month	5th month	6th month
a_w		0.27±0.00^{f}	0.27±0.01^{c}	0.29±0.00^{b}	0.30±0.00^{ef}	0.32±0.00^{de}	0.35±0.1^{d}	0.39±0.02^{a}
Acidity (% LA)		1.31±0.01^{d}	1.34±0.00^{c}	1.35±0.01^{c}	1.36±0.00^{c}	1.37±0.00^{bc}	1.39±0.00^{ab}	1.40±0.00^{a}
pH		4.12±0.00^{a}	4.08±0.00^{ab}	4.06±0.00^{b}	4.02±0.01^{c}	3.97±0.00^{d}	3.92±0.01^{e}	3.87±0.01^{f}
Color value	*L**	74.14±0.03^{a}	73.48±0.03^{b}	73.03±0.02^{c}	72.32±0.01^{d}	72.30±0.03^{d}	72.12±0.03^{e}	71.82±0.01^{f}
	*a**	2.16±0.11^{ef}	2.28±0.03^{de}	2.46±0.03^{d}	2.75±0.03^{c}	3.35±0.02^{b}	3.49±0.02^{ab}	3.70±0.01^{a}
	*b**	28.74±0.02^{g}	29.94±0.03^{f}	30.23±0.03^{e}	30.71±0.07^{d}	31.38±0.07^{c}	31.80±0.04^{b}	32.03±0.03^{a}
Solubility (%)		46.64±0.69^{a}	46.35±0.63^{a}	46.08±0.60^{a}	46.57±0.32^{ab}	45.31±0.24^{ab}	44.78±0.28^{ab}	43.41±0.24^{b}
Flowability (AOR)		33.50±0.78^{c}	34.20±0.15^{c}	34.73±0.30^{bc}	36.57±0.20^{b}	38.62±0.21^{a}	39.08±0.54^{a}	40.20±0.20^{a}
HMF (µmol/kg)		1167.43±0.67^{g}	1255.89±1.36^{f}	1373.58±2.96^{e}	1441.28±1.12^{d}	1495.38±2.03^{c}	1543.98±3.00^{b}	1598.46±5.92^{a}
Tyrosine (g/100g)		27.54±0.05^{g}	44.00±0.35^{f}	50.20±0.32^{e}	58.00±0.08^{d}	62.20±0.36^{c}	69.43±0.27^{b}	76.75±0.15^{a}
TBA (ug/g)		0.15±0.00^{g}	0.25±0.00^{f}	0.33±0.00^{e}	0.39±0.01^{d}	0.42±0.00^{c}	0.46±0.00^{b}	0.49±0.00^{a}

Mean ± SE, n=3; ^{abcdefg} indicates different superscript are significantly($p<0.05$) different from each other in colum wise

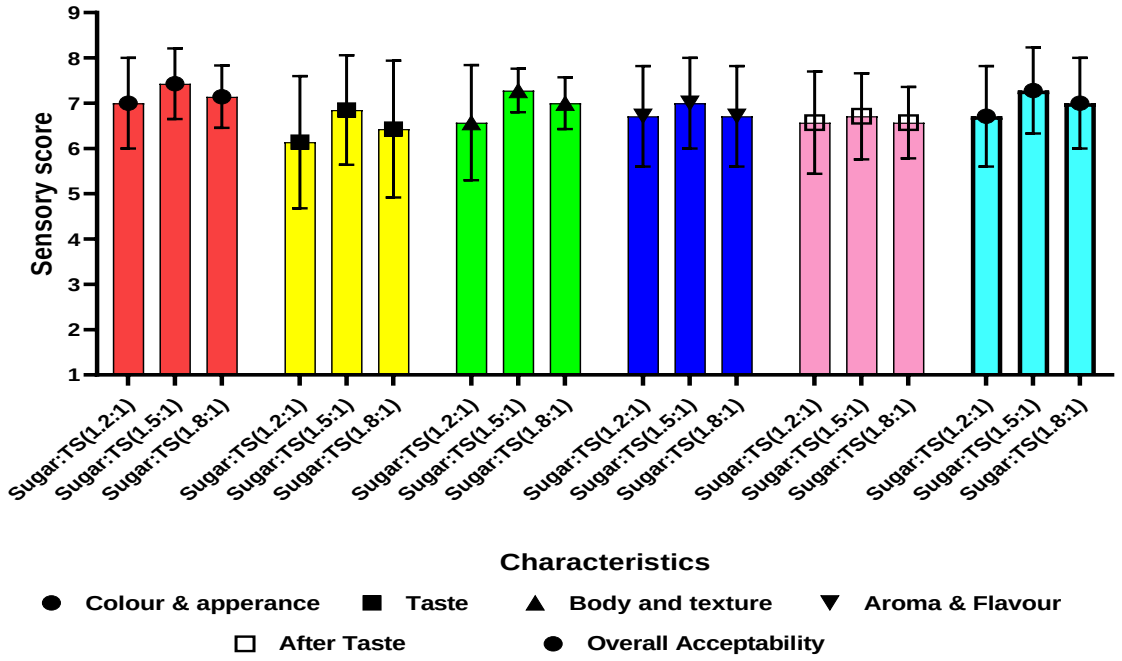

Figure 10: Conversion of sweet variant of instant *lassi*

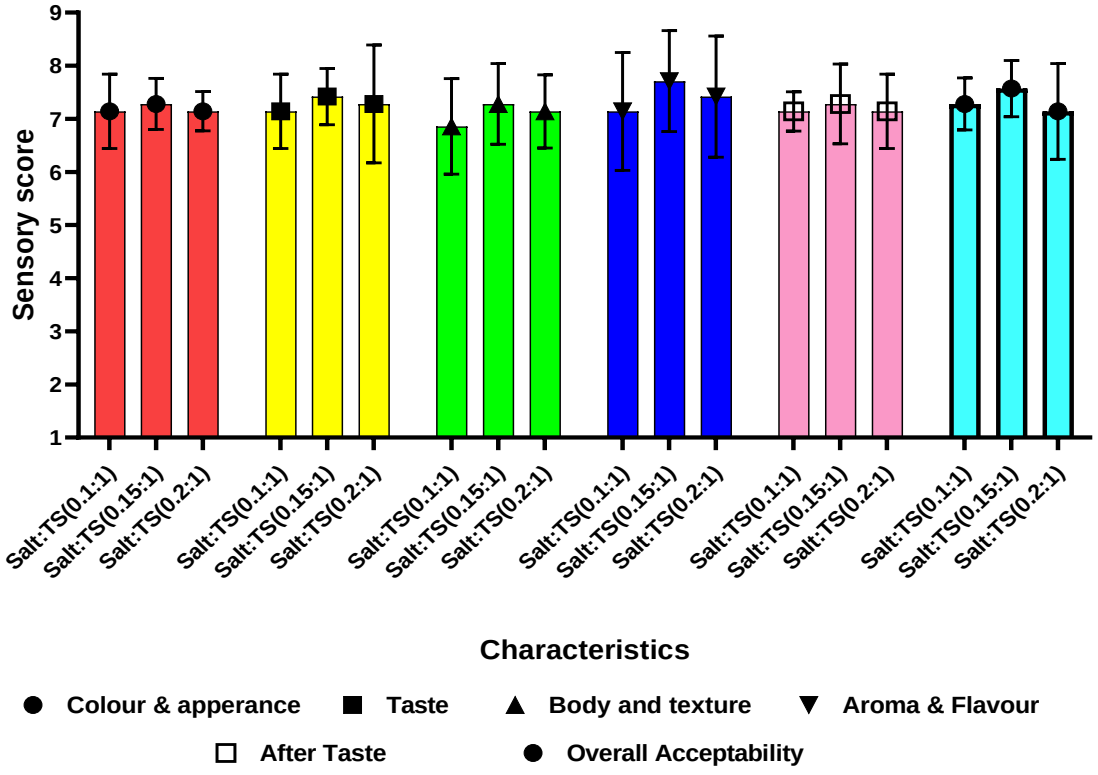

Figure 11: Conversion of salted variant of instant *lassi*

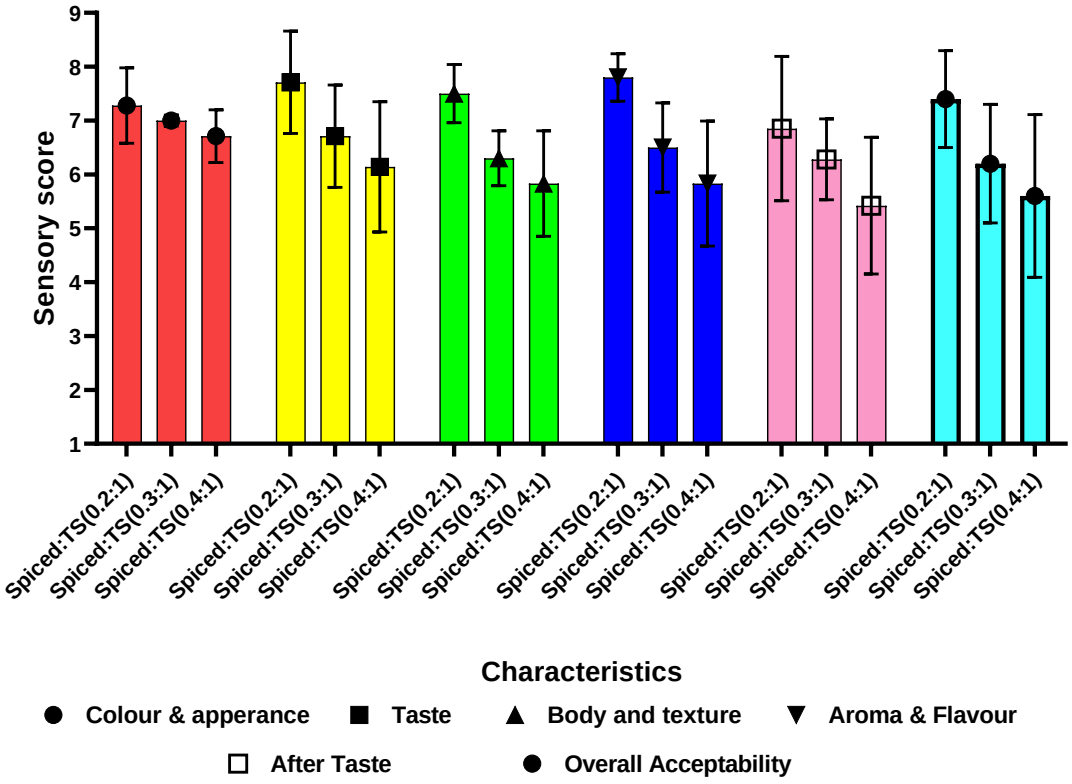

Figure 12: Conversion of spiced variant of instant *lassi*

Activity 2: Estimation of production cost for developed DBMP

Yadav *et al.* (2015) reported that for any newly developed product, evaluation of techno-economic feasibility is purposefully an important pre-requisite, before the launch of the product in the market. Therefore, it was logical to estimate expenses required for the manufacture of DBMP. The cost of production of DBMP was estimated as per the guidelines reported by Meena et al. (2017). The calculated cost for DBMP per 500g pack was Rs. 362.96 and 1 kg pack size would be Rs. 725.91, respectively. It is even lower than then the cost of protein rich powders having similar protein content (~50%).

Table 8: detailed break-up cost of DBMP (Capacity 2132.46 kg/day)

	Sr. No	Cost component	Rates	Per annum (Rs.)
A	**Manufacturing Cost**			
	a	**Direct product cost**		
	i	Raw materials	Cost of *desi* buttermilk	300000000.00
	ii	Annual packaging material requirement (2753.87*300*2) = 1652322+0.5% (8261.61) i.e. Total 1660583.61 pouches.	Rs.5 per pack for packing of powder	83302920.00
	iii	Manpower cost	Cost of manpower	10008000.00
	iv	Power and utilities	Cost of electricity, steam, fuel oil, RO water, refrigeration.	53297686.54
	v	Laboratory charges	0.2% of cost of raw material	600000.00
	vi	Cleaning and sanitizing material	0.1% of cost of raw material	300000.00
	Sub-total (a)Total			**447508605.54**
	Fixed charges			
	i	Interest on total fixed capital and working cost	12% of total fixed capital and working capital	19986000.00
	ii	Depreciation on capital investment		**11348750.00**
	iii	Insurance and taxes	2% of the capital investment	3331000.00
	Sub-total (b)			**34665750.00**
	Manufacturing cost (A=a+b)			**482174356.54**
B	**MARKETING AND DISTRIBUTION**			
	i	Plant overhead cost	15 % of manufacturing cost	72326153.48
	ii	Marketing and distribution	10% of manufacturing cost	48217435.65
Total (b)				**120543589.13**
Total cost (Rs.) Per annum (A+B)				**602717945.67**
Total cost of DBMP (per 500g pack=2512284)				**Rs. 362.96/-**
The cost of DBMP (per kg)				**Rs. 725.91/-**

Conclusion

The use of Nanofiltration process for concentration of *desi* buttermilk was observed to be more efficient than Reverse Osmosis. NF could raise the TS of *desi* buttermilk up to 19 %. Thereafter, rise in viscosity of retentate negatively influenced the flux. The subsequent spray drying of NF concentrate produced *desi* buttermilk powder. This powder has been characterized in detail for its physico-chemical and functional properties. Furthermore, developed powder was stable till the studied 6-month storage. Developed powder was successfully converted into plain, sweet variant of lassi. During estimation of cost for developed powder, the cost for 500g and 1 kg pack would be Rs. 362.96/- and Rs. 725.91/, respectively. In nutshell, a process was developed for the valorization of *desi* buttermilk to powder employing nanofiltration and spray drying as well converted into different variant of instant *lassi* powders.

Achievements:

i. Presented **flash presentation** on "**Conversion of Traditionally Produced Sour Buttermilk (a by-product) into a New Generation Dairy Ingredient Employing Reverse Osmosis and Spray Drying**" in 2023 Dairy Science and Technology Symposium. University College Cork, Ire land. June 19-22, 2023.

ii. Oral presentation on "**Processing, Preservation and Value Addition in *desi* buttermilk (a potential domestic waste)"** was presented and **won 2nd prize** in" **Agri-Food systems' Transformation through engineering Innovations & International Symposium on Engineering Interventions for making millets a global food**" Organised by Indian Society of Agricultural Engineers, New Delhi & UAS, Raichur at UAS, Raichur on November 6-8, 2023.

iii. **Best poster award** on "**Value addition & preservation of desi buttermilk**" In National Seminar on "Innovations and advances in valorization of functional dairy products, organized by Dept. Dairy Science and Food Tech. IAS, BHU, Varanasi on Nov., 25-26, 2022.

iv. A poster on "**Processing, Preservation and Value Addition in *desi* buttermilk"** was presented and **won 2nd prize** in VII Convocation of NADS (I) & National Dialogue on "Innovation in Reshaping the Indian Dairying" organized at Pandit Deen Dayal Upadhayay Pashu Chikitsa Vigyan Vishwavidyalaya Evm Go Anusandhan Sansthan (DUVASU) Mathura (U.P.) on October 29, 2022.

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Valorization of Sour Buttermilk (A Potential Waste Stream): Conversion to Powder Employing Reverse Osmosis and Spray Drying

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Abstract: Reverse osmosis (RO) is known for the economic dewatering of dairy streams without any change in phase. At the household level, surplus milk is fermented and churned to obtain butter, which is subsequently heated to obtain clarified milk fat (*ghee*). The production of 1 kg *ghee* generates 15–20 kg sour buttermilk (SBM) as a by-product that is mostly drained. This causes a loss of milk solids and environmental pollution. The processing, preservation and valorization of SBM are quite challenging because of its low total solids (TS) and pH, poor heat stability and limited shelf life. This investigation aimed to transform SBM into a novel dried dairy ingredient. SBM was thermized, filtered, defatted and concentrated at 35 ± 1 °C, employing RO up to 3.62× (12.86%). The RO concentrate was subsequently converted into sour buttermilk powder (SBMP) by employing spray drying. SBMP was further characterized for its physicochemical, reconstitution and functional properties; rheological and morphological characteristics; and amino acid and fatty acid profiling, along with FTIR and XRD spectra. SBMP was "instant soluble-3 s" and exhibited excellent emulsion stability (80.70%), water binding capacity (4.34 g/g of protein), flowability (28.36°) and antioxidant properties. In nutshell, a process was developed for the valorization of sour buttermilk to a novel dairy ingredient by employing reverse osmosis and a spray-drying process.

Keywords: sour buttermilk; reverse osmosis; domestic waste stream; valorization; dairy ingredient; spray drying; amino acid and fatty acid profiling

Citation: Manik, S.; Meena, G.S.; Singh, A.K.; Khetra, Y.; Singh, R.; Arora, S.; Vishweswaraiah, R.H. Valorization of Sour Buttermilk (A Potential Waste Stream): Conversion to Powder Employing Reverse Osmosis and Spray Drying. *Membranes* **2023**, *13*, 799. https://doi.org/10.3390/membranes13090799

Academic Editors: Alfredo Cassano, Margarita Terzyiska and Mariya Dushkova

Received: 2 August 2023
Revised: 13 September 2023
Accepted: 14 September 2023
Published: 17 September 2023

1. Introduction

The reduction of food loss and waste is extremely essential from a global perspective, as the number of people affected by hunger has gradually increased since the year 2014, and millions of tons of wholesome food is lost or wasted everyday worldwide. Globally, around 14% of food produced is lost between harvest and retail, and 17% of total food production is wasted (i.e., 11% in households, 5% in food service and 2% in retail) [1]. The dairy industry plays a key role in serving the food demands of people but also causes significant pollution, as it generates a significant amount of effluent, whose efficient treatment is mandatory prior to its disposal into the environment. Around 4–11 million tons of dairy waste residue is produced each year worldwide in the form of solid waste and effluents [2,3]. Furthermore, every volume of milk that is processed generates an effluent to the tune of 1–3 times. It accounts for an annual waste of 3.739–11.217 million cubic meters [3,4].

The unorganized sector in India produces fermented milk products, consuming 14% of the nation's total milk production [5]. This sector regularly produces *ghee* to preserve the milk fat, using an indigenous process consisting of the fermentation of whole milk, followed by its churning. In the Indian subcontinent, the production of 1 kg of *ghee* also generates 15–20 kg of sour buttermilk (SBM) [6,7]. Usually, SBM is brownish in color, as the

milk is subjected to prolonged heating prior to its inoculation with undefined starter culture, causing uncontrolled fermentation. It generally contains 3.8% TS, 0.8% fat, 1.29% protein, 1.2% lactose, 0.44% lactic acid and 0.4% ash [8]. It contains large size curd particles and is characterized by a nonhomogeneous consistency (prone to settling and accumulation of watery portion on the top) due to its higher acidity. Curd deposition is frequently observed when kept undisturbed [9,10]. The key factors posing numerous challenges in its utilization include its limited shelf life, high acidity, low heat stability, huge bulk with low TS content and lack of proper collection and processing system [11]. These are the probable reasons for the non-inclusion of SBM in mainstream dairy processing. Therefore, it is drained into the environment.

RO is termed as a concentration or dewatering membrane process. It can selectively separate solutes with a molecular weight of more than 150 Daltons [12]. Recent developments improved the functionality of RO membranes, endowing them with characteristics such as acid resistance, high retention, antifouling and ultralow pressure [13]. Liquid dairy streams such as milk, whey and buttermilk can be efficiently concentrated without any compositional change (i.e., ratio of milk constituents remains same) by the RO process [14]. The feed handling flexibility, operational simplicity and affordable cost make it suitable for use at the farm-to-industrial scale. Sweet cream buttermilk (SCBM) was successfully concentrated by employing RO from its initial 8.2% total solids to 21% after a 2.56-fold concentration [15].

Spray drying is rapid and suits large-scale production. Drying prevents product deterioration during storage by reducing the water activity. Additionally, it lowers the transportation cost and simplifies its application in a variety of food formulations. The drying process brings numerous structural and physicochemical changes that impact the handling and rehydration characteristics of dairy powders and their shelf life [16]. Tamime et al. [17] recommended the pumping of acidified buttermilk concentrate into a spray drier at 43 °C, with a recommended inlet temperature between 175 to 195 °C and a low outlet air temperature to control powder discoloration.

For the inclusion of SBM into the main dairy stream, the required scientific method is not available. Therefore, the current investigation aimed to develop a method for the processing, preservation and valorization of SBM, along with detailed characterization.

2. Materials and Methods

2.1. Procurement, Analysis, Pretreatments and Concentration of SBM

Analytical-grade chemicals procured from Sigma Aldrich (Bengaluru, India) and HiMedia Laboratories (Mumbai, India) were used in this investigation. For each trial, fresh SBM samples produced via the traditional method were procured from the countryside farmers (29.6857° N, 76.9905° E; Karnal, India) in the early morning. Thereafter, SBM samples were pooled and then thermized (63 °C/20 s), cooled (40 ± 1 °C) and subsequently defatted using centrifugal separation (Model: Kamdhenu KD-600; Make: Sinhal Metal India Pvt. Ltd., New Delhi, India). The obtained defatted sour buttermilk (DSBM) was filtered through a muslin cloth and concentrated by employing the RO process, maintaining a 30 ± 5 bar pressure and 35 ± 1 °C temperature to obtain sour buttermilk concentrate (CSBM) with maximum possible TS (Figure 1). RO plant was supplied by Peterson Candy International Ltd., Reading, UK. A total membrane area of 0.9 m^2 (polyamide, AFC 99) was installed in this plant (length—1500 mm; height—800 mm; depth—700 mm; weight—70 kg). The plant was equipped with Type B1 tubular module of 1.2 m in length. It also had a shroud and heat exchanger (length—0.6 m) made of SS 316. A triple plunger pump (contact surface-SS, 4 kW motor; flow, 22 liters/min), which can generate 70 bar pressure, was used. The reported flux range of this plant was 15–60 LMH ($L/m^2/h$). Its hold-up volume was 6.50 L. The plant can be operated at a maximum of 70 bar of pressure and can withstand 3–11 pH and 70 °C.

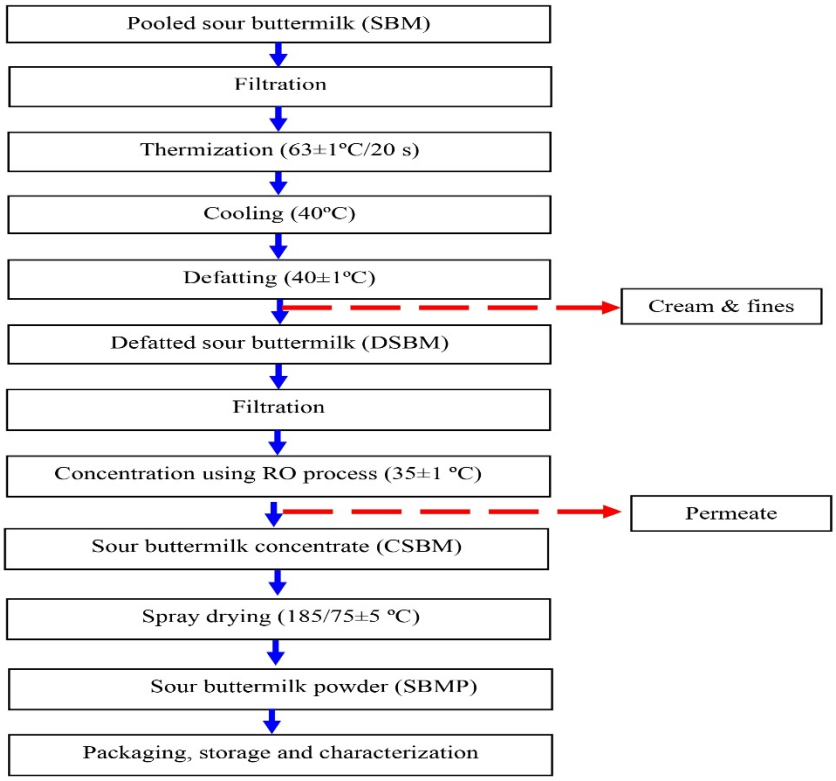

Figure 1. Production process of sour buttermilk powder (SBMP).

The change in permeate flux was recorded and expressed as a function of percent VRR and concentration factor (*CF*), as shown in Figure 2. The flux mean (*FM*) was calculated from initial flux (*IF*) and final flux (*FF*) values using following formula earlier described by St-Gelais et al. [18].

$$FM = FF + [0.33 \times (IF - FF)] = 10.67 + [0.33 \times (24 - 10.67)] = 15.07\ LMH$$

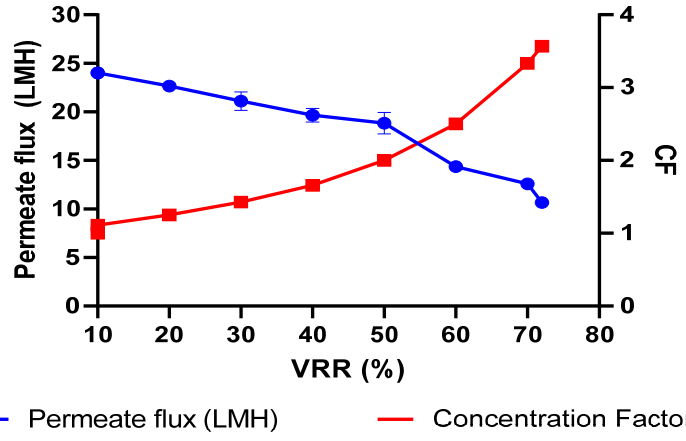

Figure 2. Change in permeate flux as a function of percent volume reduction ratio and concentration factor.

2.2. Spray Drying of CSBM

A single-stage spray drier (Jektron Pvt. Ltd., Pune, India) equipped with rotary atomizer was used for the spray drying (185/75 ± 5 °C of RO concentrate to obtain SBMP powder. This non-agglomerated SBMP was immediately packed and sealed in metalized polyester-LDPE laminates, which were stored at 4 ± 1 °C till further analysis. Sour buttermilk powder (SBMP) was prepared (Figure 1) and analyzed in triplicate

2.3. Analysis of Chemical Composition and Determination of Physical Properties of Different Buttermilk Samples and SBMP

Total solids (TS), protein, ash and fat contents of SBM, DSBM, CSBM and SBMP samples were determined by adopting solids in milk and powder (AOAC official method of analysis 925.23 and 927.05), Kjeldhal (AOAC official method of analysis 991.20), ash in milk and powder (AOAC official method of analysis 945.46, 930.30) and Mojonnier (AOAC official method of analysis 989.05) methods as per AOAC [19], while the Lane Eynon method [20] was used to estimate their lactose content. The free fat of SBMP was determined by adopting the method reported by Hall and Hedrick [21]. Hydroxymethylfurfural and 2-thiobarbituric acid were measured by adopting the method described by Keeney and Bassette [22] and Hegenauer et al. [23], respectively. A calibrated pH probe (Eutech, Cyberscan 1100, Thermo Scientific, Waltham, MA, USA) was used to measure the pH of different buttermilk samples and reconstituted 10% (*w/v*) SBMP solution, while their acidity values were determined as per the IS: SP:18 method [24]. The ζ-potential of these samples was measured by adopting the method described by Mahadev and Meena [25], using Zetasizer Nano ZS, (Malvern, UK) at 25 °C. The color values of the above samples were recorded using Hunter Lab model color Flex® (Mini-Scan XE plus, Hunter Associates Laboratory Inc., Reston, VA, USA). The water activity (a_w) of SBMP was determined in Aqua Lab (Model Series 3 TE; supplied by M/s Decagon Devices, Pullman, WA, USA).

2.4. Determination of Bulk and Flow Properties of SBMP

The interstitial air content (IAC), occluded air content (OAC) and particle density (PD) of SBMP were determined as per the Niro Atomizer [26] method, while the loose bulk density (LBD), packed or tapped density (PBD), porosity and flowability (in terms of angle of repose, θ°) were determined by adopting the methods reported by Sjollema [27]. The Hausner ratio (HR = PBD/LBD) and Carr index (CI) = [(PBD − LBD) × 100/LBD] were calculated using LBD and PBD values of SBMP.

2.5. Determination of Reconstitution and Functional Properties of SBMP

The method described by Muers and House [28] and American Dry Milk Institute [29] were used to determine the wettability and dispersibility of SBMP, respectively. The solubility index for SBMP was measured by adopting the method reported by Schuck et al. [30]. Briefly, 10 g SBMP was reconstituted and mixed for 90 s in 100 mL water at 24 °C, using a solubility index mixer (LABINCO L295, Breda, The Netherlands), and then centrifuged in 50 mL centrifuged tubes at 160× *g* for 10 min. The sediment volume obtained in mL after the second centrifugation was defined as the *insolubility index*. The *solubility index* (*SI*, %) was calculated from the following equation, as reported by Schuck et al. [30].

$$Solubility\ index\ (SI),\ \% = 100 - (2 \times insolubility\ index)$$

The water binding capacity (WBC), oil binding capacity, emulsification capacity, emulsification stability, foam stability and capacity of SBMP were determined by adopting the method reported by Shilpashree et al. [31]. The buffering capacity of SBMP (0.5% protein solution) was determined by adopting the method reported by Mann and Malik [32].

2.6. Determination of Rheological Properties of SBM, DSBM, CSBM and Reconstituted SBMP Samples

The flow curve of SBM, DSBM, CSBM and reconstituted (10% *w/v*) SBMP samples was determined using a rheometer (model; MCR52; make: Anton Paar, Graz, Austria) with an attached cup and bob (CC27) probe between 0 to 500 s^{-1} shear rate at 20 °C. Change in apparent viscosity ($\eta_{Appa.}$ mPa.s) of these samples as a function of rise (5 °C min^{-1}) in temperature was also recorded at a constant (100 s^{-1}) shear rate, as reported by Patil et al. [33].

2.7. Analysis of Particle Size Distribution of SBMP

A particle size analysis of SBMP was performed using Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK) as per the method outlined by Mahadev and Meena [25]. This analysis provided values for specific surface area (SSA), particle size distribution (d_{10}, d_{50} and d_{90}), De Broukere ($D_{4,3}$) and Sauter ($D_{3,2}$) means; in addition, the span or dispersion index (span index = $(d_{90} - d_{10})/d_{50}$) was also calculated. The refractive index (1.334) and density (1.30 g cm^{-3}) of SBMP were determined and subsequently used for its particle size analysis.

2.8. Analysis of Antioxidant Properties of SBMP

The ABTS activity of SBMP was estimated according to the method reported by Salami et al. [34], while its DPPH and FRAP were determined by adopting the method reported by Zhang et al. [35] and Benzie and Strain [36], respectively. Total phenolic component and flavonoids present in SBMP were estimated by adopting the method described by Sharma et al. [37].

2.9. Fatty Acids Profiling of SBMP

A gas chromatography (GC) unit (model: GC-FID 2010 plus, make: Shimadzu, Japan) was used to estimate the fatty acid profile of SBMP. Fatty Acid Methyl Ester (FAME) derivatization was carried out with 20 mg of extracted fat from SBMP sample as per the ISO 15884 [38] method.

(All methods from the Sections 2.3–2.9 are discussed in brief in Supplementary Materials Section S1.)

2.10. Amino Acids Profiling of SBMP

SBMP powder was passed through a sieve (80 mesh size) to screen for a uniform size. A total of 30 mg of SBMP was weighed in three replicates and transferred into the PTFE tube of a microwave digestion system. Then, 8 mL of 6 N hydrochloric acid was transferred into a PTFE tube. A SBMP powder sample was dissolved in 0.5 mL phenol, 1.0 mL of Nor-leucine internal standard (1000 ppm) and 0.5 mL of 0.1 N hydrochloric acid. Then, it was subjected for digestion, using microwave at 850 W. The digestion was achieved in two steps: ramping up the temperature to 160 °C for 15 min and then cooling the digested samples for 15 min.

Before pre-column derivatization, digested samples were diluted to 100 mL with 0.1 N hydrochloric acid solution. For pre-column derivatization, digested samples were mixed with 100 µL of borate buffer and 1.0 mL of 9-Fluorenylmethy1 chloroformate (FMOC-Cl, 0.4% solution) and kept undisturbed for 2 min. A total of 4.0 mL of n-pentane was added to the solution and then vortexed for 45 s. The upper layer was discarded, and the lower layer was transferred to the HPLC 1.5 mL vial. Amino acid profiling was conducted using reverse-phase UHPLC (Thermofisher Scientific Dionex Ultimate 3000, Waltham, MA, USA) with a C18 column (Acclaim T* 120, 5 pm, 120 A, (4.6 × 250 mm)) and photo diode array detector (Ultimate 3000 Diode Array) in the UV range. Mobile phase A consisted of 1800 mL of buffer and 200 mL of organic phase, while mobile phase B consisted of 200 mL of buffer and 1800 mL of organic phase. Here, the buffer was tetra-methy-l-ammonium chloride and sodium acetate trihydrate with a pH of 0.5, and the organic phase was mixture of acetonitrile and methanol in the ratio of 49:1. The gradient program used for the quaternary RS pump compartment is shown in Supplementary Table S1. The flow rate of the eluant was 1.0 mL/min, with a run time of 75 min. The response of the monitor was monitored at 265 nm, and data were acquired and processed by Chromeleon (6.8 SR1 5b; Build 4981).

2.11. FTIR Spectra of SBMP

An FTIR analysis of SBMP was conducted by its direct contact into Diamond crystal cell Attenuated Total Reflectance crystal of Shimadzu IR Affinity-1. According to Patil et al. [39], absorption spectra of SBMP was recorded between 4000 and 400 cm^{-1} wavenumbers at a

4 cm^{-1} resolution and a 0.2 cm s^{-1} scan speed, as per Patil et al. [39] Background run was taken before placing SBMP to the diamond crystal, as reported by Upadhyay et al. [40].

2.12. XRD Spectra of SBMP

X-ray diffraction (XRD) spectra were recorded for SBMP, using an X-ray diffractometer (model: MiniFlex II, make: Rigaku, Japan) equipped with a graphite reflected beam monochromator and PC-Automatic Powder Diffraction software version (APD, 3.0). It was operated in reflection mode at 40 kV and 50 mA. SBMP was slightly pressed on aluminum trays, using a 10 mm wide spatula (sample layer, 15 mm × 20 mm × 1.5 mm), and exposed to CuKα radiation (l = 0.15418 nm) at diffraction angles (2θ) from 10 to 80° (step size, 0.02°; time per step, 2.5 s). The divergence slit for the primary beam was 1°, and the divergence and receiving slits for the diffracted beam were 1° and 0.2 mm, respectively. The peak was searched for in APD software to locate the peaks in XRD patterns by detecting the minima from the second derivative of the diffractogram.

2.13. Microstructure of SBMP

The microstructure of SBMP was examined by scanning electron microscopy (EVO 50, Carl ZEISS Special Edition, Cambridge, UK), as per Shilpashree et al. [31].

2.14. Statistical Analysis

The results obtained during the processing of SBM in this investigation were subjected to a one-way analysis of variance (ANOVA), with a 5% level of significance (α = 0.05). Means were compared using Tukey's HSD as a post hoc test in the IBM SPSS program, version 25. Wherever applicable, a descriptive statistics analysis was also performed and reported for SBMP parameters.

3. Results and Discussion

3.1. Chemical Composition of Buttermilk Samples and Concentration of DSBM

The concentration of DSBM from 1× to 3.62× (72% VRR) in the RO process gradually decreased the flux (Figure 2) and resulted in a 15.07 Lm^{-2} h^{-1} mean flux value, which corresponded to the critical flux of CSBM, and this was the reason for terminating the RO process at 3.62×. The concentration polarization and fouling of the RO membrane could explain such a reduction in flux. It is a well-established fact that the permeate flux decreases with the increase in the concentration factor or percent VRR due to an overall increase in the TS and viscosity of the RO concentrate.

The chemical composition and other physical properties of SBM, DSBM and CSBM are shown in Table 1. The acidity and pH values of pooled SBM were the values reported by Padghan et al. [9] for a similar product. Defatting significantly ($p < 0.05$) decreased the TS, fat, L^*, a^*, b^* and η_{Appa} of SBM as compared to DSBM. The dewatering of DSBM in the RO process significantly ($p < 0.05$) enhanced all of its chemical constituents and physical properties in CSBM (Table 1). Contrary to that, after a 3.62× concentration of DSBM, a significant ($p < 0.05$) decrease was observed in the ζ-potential of CSBM which could be attributed to its lower pH. A significant ($p < 0.05$) decrease in the pH of CSBM over DSBM was attributed to the concentration of lactic acid (LA) by RO. Li et al. [41] also successfully concentrated the LA present in nanofiltration (NF) permeate by employing RO. Govindasamy-Lucey et al. [15] also used RO to dewater sweet cream buttermilk (SCBM) containing 8.2% initial TS and achieved 21% TS after its 2.56× concentration.

Defatting significantly ($p < 0.05$) decreased the TS, as well as the η_{Appa}, of DSBM as compared to SBM. This could have been attributed to the removal of milk fat and suspended milk solids. Opposite to this, the concentration of DSBM in RO significantly ($p < 0.05$) increased the TS and η_{Appa} of CSBM. The η_{Appa} of SBM, DSBM and CSBM exhibited a gradual decrease with the increasing shear rate and temperature, as demonstrated in Figure 3a,b, respectively.

Table 1. Chemical composition and physical properties of SBM, DSBM and CSBM samples.

Parameters		Sour Buttermilk (SBM)	Defatted Sour Buttermilk (DSBM)	Concentrated Sour Buttermilk (CSBM)
TS (%)		4.13 ± 0.14 ^{b}	3.55 ± 0.12 ^{c}	12.86 ± 0.31 ^{a}
Fat (%)		0.72 ± 0.29 ^{a}	0.18 ± 0.17 ^{b}	0.67 ± 0.28 ^{a}
Protein (%)		1.88 ± 0.19 ^{b}	1.91 ± 0.10 ^{b}	6.93 ± 0.39 ^{a}
Lactose (%)		1.09 ± 0.33 ^{b}	1.15 ± 0.24 ^{b}	4.08 ± 0.30 ^{a}
Ash (%)		0.23 ± 0.12 ^{b}	0.28 ± 0.13 ^{b}	0.98 ± 0.09 ^{a}
Acidity (% LA)		0.70 ± 0.08 ^{b}	0.70 ± 0.06 ^{b}	2.44 ± 0.15 ^{a}
pH, 20 °C		3.95 ± 0.07 ^{a}	3.95 ± 0.07 ^{a}	3.28 ± 0.09 ^{b}
ζ-potential (mV) (100× dilution), 25 °C		11.20 ± 6.15 ^{a}	13.85 ± 1.14 ^{a}	8.88 ± 2.14 ^{b}
Color values	*L**	76.38 ± 0.20 ^{c}	76.93 ± 0.22 ^{b}	80.39 ± 0.44 ^{a}
	*a**	−1.96 ± 0.04 ^{b}	−2.18 ± 0.15 ^{c}	0.12 ± 0.85 ^{a}
	*b**	8.37 ± 0.50 ^{b}	8.19 ± 0.45 ^{c}	14.12 ± 0.06 ^{a}
Apparent viscosity ($\eta_{Appa.}$), 100 s^{-1} (mPa s), at 20 °C		14.10 ± 1.22 ^{b}	8.47 ± 0.32 ^{c}	90.66 ± 2.08 ^{a}

Mean ± SD ($n = 3$) with different superscripts; $^{a–c}$ are significantly different ($p < 0.05$) from each other column-wise.

3.2. Characterization of SBMP

3.2.1. Chemical Composition and Physical Properties of SBMP

The production process of SBMP is outlined in Figure 1. Its chemical composition, physical, reconstitution, functional and antioxidative properties are shown in Table 2. Because of the lack of works in the scientific literature on powder manufactured from sour buttermilk (that too produced using traditional method), a the direct comparison of the results of the manufactured SBMP is not possible. Hence, its chemical composition and other powder properties were compared with related (lassi powder, yoghurt powder, buttermilk powder, skim milk powder (SMP) and whole milk powder (WMP)) products. Its TS (97.819%) content was in accordance with the TS content of *lassi* powder (94–97%; [42]), yoghurt powder (≥95%; [43]), SMP (97%; [44]), WMP (97.75%; [45]) and buttermilk powder (97%; [46]). SBMP had a markedly higher protein (53.64%, shown in Table 2) content over that which was reported for yogurt powder (35–38%; [43]), buttermilk powder (30–33%; [46]), SMP (34–37%; [44]) and WMP (24.5–33%; [45]). The fat content of SBMP was also higher than the fat contents (1.2–2.4%) of lassi (2.4%; [42]) and skim milk yoghurt powders (1–1.5%; [43]), respectively. Opposite to this, the lactose content of these powders (45–52% for yoghurt powder [43], 30–33% for buttermilk powder [46], 34–37% for SMP [44] and 24.5–27% for WMP [46]) was higher than that of SBMP (Table 2). Not much of the ash content of SBMP and yoghurt powders (6.8–8%; [43]) was comparable.

The reconstitution and flow properties of milk powders exhibit an inverse relationship with their free fat content. Powders also become prone to oxidation with an increase in their free fat content [47]. The lower fat content of CSBM was responsible for the lower free fat (1.23%) content of SBMP. The free fat content of WMP has been reported to be 2–3% [48] and <10% [49]. The Maillard reaction is a non-enzymatic browning reaction that occurs between milk sugar and proteins during the heating and drying of milk and even continues during the storage of milk powders. This also leads to a nutritional loss and change in color of milk and milk powders [50]. Hydroxymethylfurfural (HMF) compounds indicate the severity of the Maillard reaction [51]. The protein-to-lactose ratio and severity of the heat treatment greatly influence the formation of HMF in milk powder [52,53].

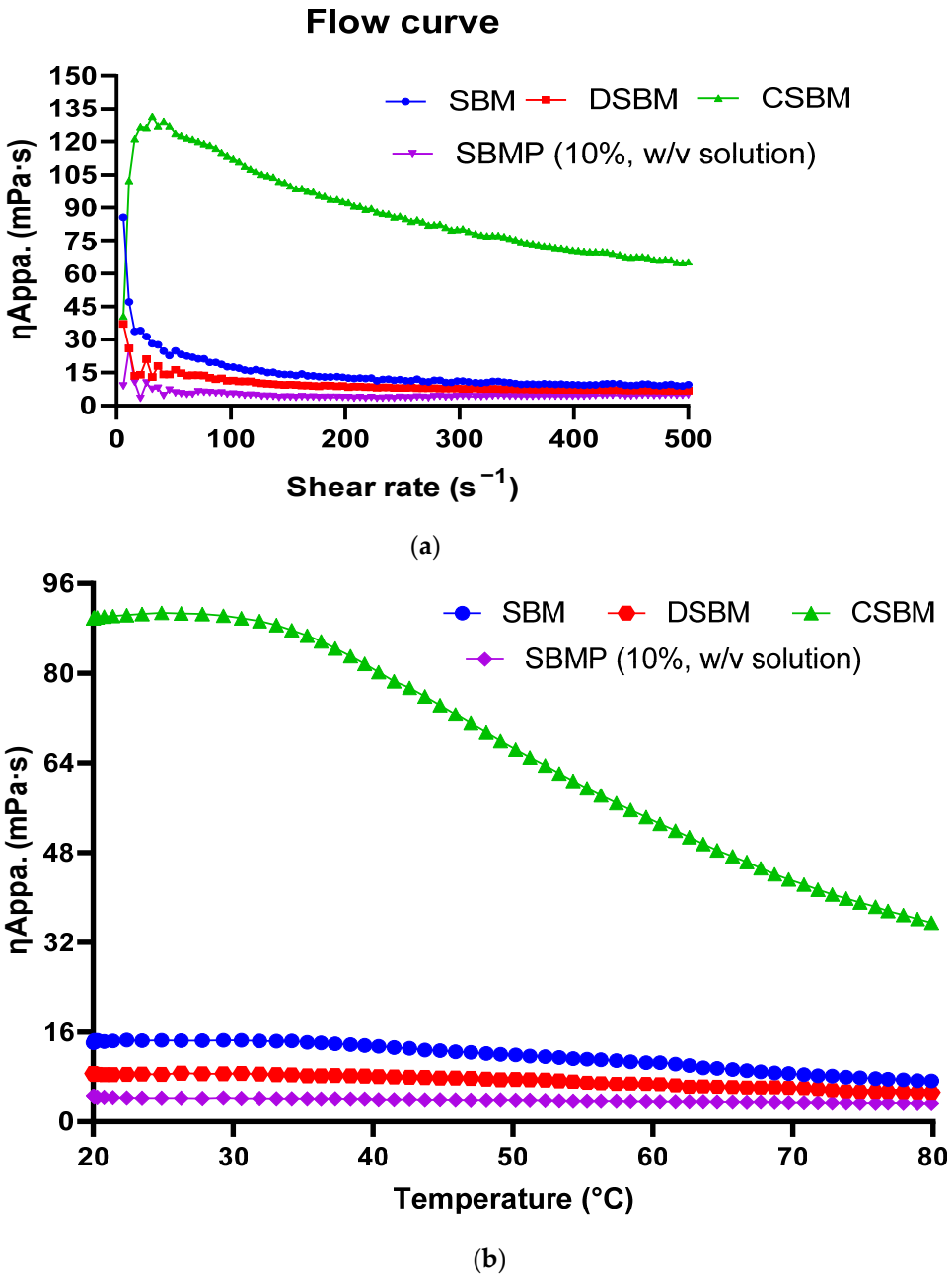

Figure 3. Change in viscosity of sour buttermilk (SBM), defatted sour buttermilk (DSBM), concentrated sour buttermilk (CSBM) and reconstituted (10% w/v) sour buttermilk powder (SBMP) samples as a function of increase in (**a**) shear rate and (**b**) temperature.

The higher HMF content of SBMP was mainly attributed to the prolonged heat treatment offered to milk from which SBM was produced. According to Sert et al. [54], the HMF content of SMP and WMP was 1.180 µmol/L and 0.715 µmol/L, respectively. The higher protein content, use of a higher processing temperature and low pH of the concentrate might have collectively contributed to a higher HMF content in SBMP. Stapelfeldt et al. [55] reported that the TBA value measures the lipid oxidation and determines the formation of secondary oxidation products (e.g., carbonyls). The formulated oxidative products further govern the sensory attributes of dairy powders. SBMP had a lower TBA value (Table 2) owing to the lower fat content of DSBM and CSBM (Table 1).

Table 2. Physicochemical, reconstitution, functional and antioxidative properties of SBMP (Mean ± S.D.; n = 3 independent trails).

	Parameters		SBMP
Chemical composition and physical properties	TS (%)		97.82 ± 0.18
	Fat (%)		5.04 ± 0.23
	Protein (%)		53.64 ± 0.31
	Lactose (%)		31.34 ± 0.60
	Ash (%)		7.48 ± 0.12
	Free fat (% of total fat)		1.23 ± 0.13
	Hydroxymethylfurfural (μmol/kg of powder)		1085.17 ± 1.61
	2-thiobarbituric acid, TBA (μg/mL)		0.12 ± 0.03
	Acidity (% LA)		1.80 ± 0.12
	pH (10% w/v solution), at 20 °C		4.22 ± 0.10
	ζ-potential (mV) (1000× dilution)		−0.18 ± 0.17
	Water activity (a_w)		0.25 ± 0.04
	Color values	L^*	74.69 ± 0.27
		a^*	1.65 ± 0.15
		b^*	26.62 ± 0.14
Bulk and flow properties	Interstitial air content (cm^3 100 g^{-1} powder)		74.62 ± 3.68
	Occluded air content (cm^3 100 g^{-1} powder)		8.41 ± 0.57
	Loose bulk density (g cm^{-3})		0.53 ± 0.10
	Packed bulk density (g cm^{-3})		0.66 ± 0.14
	Particle density (g cm^{-3})		1.30 ± 0.16
	Porosity (%)		59.36 ± 0.31
	Flowability (angle of repose, θ°)		28.36 ± 0.48
	Hausner ratio (HR)		1.23 ± 0.01
	Compressibility index (CI)		18.83 ± 0.60
Reconstitution and functional properties	Wettability (s)		03.00 ± 0.00
	Dispersibility (%)		73.74 ± 0.70
	Solubility index (mL per 100 mL reconstituted)		71.50 ± 0.20
	Water binding capacity (g per g of protein)		4.34 ± 0.63
	Oil binding capacity (g per g of protein)		2.77 ± 0.46
	Foaming capacity (%)		22.18 ± 2.82
	Foam stability (%)		14.32 ± 0.90
	Emulsion capacity (%)		32.05 ± 0.24
	Emulsion stability (%)		80.70 ± 0.79
	$\eta_{Appa.}$(mPa s), at 100 s^{-1} and 20 °C		4.09 ± 0.74
Particle size distribution	d_{10}		33.43 ± 0.11
	d_{50}		69.17 ± 0.32
	d_{90}		130.33 ± 2.89
	$D_{3,2}$		91.23 ± 3.38
	$D_{4,3}$		4.26 ± 0.01
	Span (%, dispersion index)		1.06 ± 0.01
	SSA (m^2 kg^{-1})		65.83 ± 2.47
Antioxidant properties	ABTS (% RSA)		50.65 ± 0.47
	DPPH (%)		120.19 ± 0.42
	FRAP (μM/mL)		110.13 ± 0.64
	Total phenolic content (μg/mL)		1699 ± 6.08
	Flavonoids (μg/mL)		0.85 ± 0.12

The acidity of the reconstituted SBMP solution (10%, w/v) was determined and expressed in terms of % LA (Table 2). The acidity of skim milk yoghurt powder and

reconstituted lassi powder was 5–8% LA and 0.52–0.57%, LA, respectively [42,43]. The pH values of reconstituted SBMP, yoghurt powder and lassi powder solutions were 4.22 (Table 2), 4.3–5.3 [43] and 4.63–4.7 [42], respectively. Wade et al. [56] reported that the ζ-potential becomes less negative with the decrease in pH, and the same can easily explain lower the lower ζ-potential value of the SBMP solution, as shown in Table 2. The a_w of SBMP was 0.25, as shown in Table 2. According to Koc et al. [57], lower (<0.25) a_w values ensure powder stability during storage and also prevent microbial growth. For SMP and WMP, a_w values have been reported in the range of 0.1–0.303 and 0.23–0.32 by Szulc et al. [58] and Pugliese et al. [59].

The color of the powder plays an important role in its consumer acceptance and application. The color values of SBMP are shown in Table 2. The *L**, *a** and *b** values of spray dried *dahi* powder, SMP and WMP were 93.6, 3.2 and 15.3 [60]; 96.94, −2.32 and 11.12; and 96.01, −1.74 and 14.45 [59]. As evident by its color values, SBMP was slightly brown in color, and this could be attributed to the formation of brownish pigments by Maillard reactions during the heat treatment of the milk [61] and spray drying of CSBM.

3.2.2. Bulk and Flow Properties of SBMP

The volume difference between the mass of powder particles and volume of the same mass of tapped powder is known as the IAC, while the difference between the volume of the particles of the given mass and the volume of air-free solids is known as the OAC [62]. The IAC and OAC values of SBMP are shown in Table 2. As per Schuck [62], the IAC values of SMP and WMP were 41 and 120 cm^3 100 g^{-1} powder, respectively. The IAC of SBMP falls in between the IAC values of SMP and WMP. The degree of agglomeration and particle size distribution are the major factors which affect the IAC [62]. The OAC content of SBMP was 8.414 cm^3 100 g^{-1} powder (Table 2), which was markedly lower than the OAC content of SMP and WMP [62].

According to Schuck [62], the OAC content of SMP and WMP were 119 and 63 cm^3 100 g^{-1} powder, respectively. The OAC content of SBMP (Table 2) was markedly lower compared to these values. Indeed, 100 g of milk powder has been reported to contain 10–200 mL of OAC, which is influenced by feed properties (pH and protein content) and processing conditions such as the air incorporation/whipping of feed and its foam stability, as well as the type of atomizer used for its spray drying [62]. Zang and Goff [63] also reported that foaming decreases with the decrease in pH of the feed. Hence, severe heat treatment of milk prior to fermentation and the low pH (3.28) of CSBM could collectively explain the lower OAC content of SBMP.

The bulk characteristics of SBMP are shown in Table 2. The bulk characteristics (bulk and tapped densities, porosity and flowability) of a food powder rely heavily on particle size and its distribution [64]. A product with a low bulk density needs a larger packing volume. The LBD of skim milk yoghurt powder, skim milk yoghurt powder with a natural sour taste, normal yoghurt powder, SMP and WMP was 0.60–0.75 g/mL, 0.4–0.7 g/mL [43], 400 kg/m^3 [65] and 431 and 360 kg m^{-3} [62], respectively.

The flowability is the ability of the powder to flow without forming any lump or aggregates [62]. The angle of repose (θ) is most commonly used to describe it. According to Carr [66], powders with θ values up to 35° have been classified as free-flowing. SBMP also exhibited flowability similar (free-flowing) to that of sand/salt. As per HR and CI values, SBMP demonstrated fair (CI, 16–20%; HR, 1.19–1.25) flow characteristics. For yoghurt powder, the reported CI value was 27.93 [65]. Schuck [62] reported that powder particles with larger particle diameters (>90 μm) demonstrate better flow characteristics. Hence, the presence of larger particles and lower fat content could explain the free-flow nature of SBMP.

The porosity of SBMP was 59.36% (Table 2), and the same was higher than the porosity (36.54%) of yoghurt powder [65], as well as that of WMP and SMP (52%) [47]. Fang et al. [67] reported that the presence of higher number of smaller particles causes a decrease in the

porosity of the powder. Hence, the observed variation in particle size could explain the better porosity of SBMP.

3.2.3. Reconstitution and Functional Properties of SBMP

The time taken by one gram of powder sample for the penetration of the still surface of water is known as wettability [62]. Factors such as particle density and size, powder porosity, surface area, surface charge and activity, as well as the presence of moisture-absorbing constituents, collectively influence the powder wettability. As per Kelly et al. [68], the wettability of WMP ranged between 30 and 60 s; meanwhile, SMP being wetted in <15 s deemed it to be an "instant powder". The wettability of yoghurt powder developed by Koc et al. [65] was 374 s. The SBMP powder demonstrated wettability of only 3.00 s, which clearly indicated that it was an "instant powder". As per Pimentel et al. [69], SBM is a good source of phospholipids (115.50 mg per 100 g); hence, the natural presence of surface-active agents (phospholipids particularly lecithin) could explain its excellent wettability.

Under standard testing conditions, the ability of powder particles to be uniformly dispersed in water is known as the dispersibility and confirms whether the powder is "instant" or not [62]. It is greatly influenced by heat-induced interactions between casein and whey proteins which may lead to the formation of an unstable dispersion [68]. As per Tammie [70], SMP possesses higher dispersibility (≥90%) compared to WMP (≥85%). Ji et al. [71] also reported that the dispersibility of SMP and WMP were 95 and 71%, respectively. SBMP had 73.74% dispersibility, as shown in Table 2. Koc et al. [65] reported that the dispersibility of spray-dried yoghurt powder was 351 s. Schokker et al. [72] reported that dried milk powders containing higher casein content were poorly dispersible and require a longer time for their complete dispersion. Singh and Newstead [73] reported that the dispersibility of milk powders is inversely proportional to the presence of fine particles (<90 µm). The heat-induced interactions between casein and whey proteins, higher HMF content and high protein content could collectively explain the intermediate dispersibility of SBMP (Table 2).

The solubility of milk powders can be expressed using different methods. Table 2 clearly shows that the solubility index of SBMP was 71.50%, which is higher than the reported solubility index value (68.70%) of yoghurt powder [65]. As per Schuck [30], a dairy powder is considered to be soluble if its solubility index is >99%. Lactose and salts are major hydrophilic constituents present in milk powders. The prolonged heat treatment of milk prior to fermentation and higher protein content of SBMP (Table 2) could be responsible for its relatively lower solubility index in comparison to SMP and WMP.

The interaction of proteins with water has been reported to determine the functional properties of proteins in different food systems. According to Zayas [74], the WBC of proteins is collectively influenced by several factors, namely the temperature, ionic strength, pH, concentration of proteins, lipids and salts, presence of hydrophilic polysaccharides, severity of heat treatment and storage conditions. Knightbridge and Goldman [75] reported that the method used for the drying and grinding of milk powders influences their topography, porosity and size and affects their water-holding capacity. The WBC of SBMP was 434 g water per 100 g protein, which was observed to be markedly higher compared to 33–180 g water per 100 g protein present in whey powders. The higher WBC of SBMP could be attributed to its higher protein content (Table 2) and prolonged heating of milk (advocating partial denaturation, dissociation and unfolding of protein) from which SBM and SBMP were produced. The severe heat treatment of whey proteins results in a higher WBC. Furthermore, Wagner and Anon [76] reported an inverse relationship between WHC and solubility. Indeed, denatured proteins result in a stable protein matrix in which a significant amount of water gets entrapped. A similar explanation holds true for the higher WBC of SBMP.

Similarly, the oil-binding capacity (OBC) is the ability to retain and absorb fat. It is influenced by the size of powder particles. Zayas [77] reported that protein powders possessing a low density and smaller particle size can adsorb and retain more oil over

protein powders with a higher density. Hence, the higher LBD, PBD and PD of SBMP (Table 2) could explain its observed OBC.

Foaming capacity is the ability of protein solution to entrap the air bubbles or foam formation at the protein–water interface, whereas foam stability is the ability of milk protein to provide strength to foam lamella and retain the foam in the protein matrix [78]. The protein concentration, degree of denaturation, preheat treatment, ionic strength and lipid content collectively influence the foaming properties [79]. Both the foam capacity and foam stability of SBMP (Table 2) were poor and could be explained by its lower pH, lower ζ-potential and lower solubility index. This is because the foaming capacity of proteins is directly proportional to the net proton charge present on them.

The emulsifying capability (EC) refers to the ability of protein solutions to emulsify at the oil–water interface via adsorbing the oil molecules at their surface [80]. The emulsion stability (ES) is the ability of emulsion droplets to remain distributed without aggregating, flocculating or creaming [81]. Emulsification properties are influenced by several factors, including TS, pH, protein, calcium content and powder particle size [82]. The ability of a protein to act as an emulsifier majorly depends on its amphipathic nature, solubility, extent of surface denaturation and lipid-to-protein ratio [83]. Several factors, such as the presence of MFGM and phospholipids, which are known as natural emulsifiers, as well as lower solubility of SBMP, could be responsible for higher EC and ES (Table 2) values.

The apparent viscosity of reconstituted SBMP solution was measured and is shown in Table 2. In the case of yoghurt powder, the consistency of reconstituted yoghurt solution was thinner than that of original yoghurt [84]. This may be due to extensive heat treatment during spray drying which results in the denaturation of milk protein. This showed poor rheological attributes during rehydration [85]. Hence, similar changes could also explain the three-times-lower apparent viscosity of SBMP (Table 2) compared to SBM (Table 1).

The buffering capacity is the typical property of milk proteins to resist a change in pH value. The buffering capacity of the SBMP solution (0.5% protein) is shown in Figure 4. Salaün et al. [86] reported that casein, whey proteins, colloidal calcium phosphate and soluble minerals contribute 35%, 5%, 20% and 40% of the buffering capacity in milk powders. Hence, its buffering capacity can be explained by its chemical composition, as shown in Table 2.

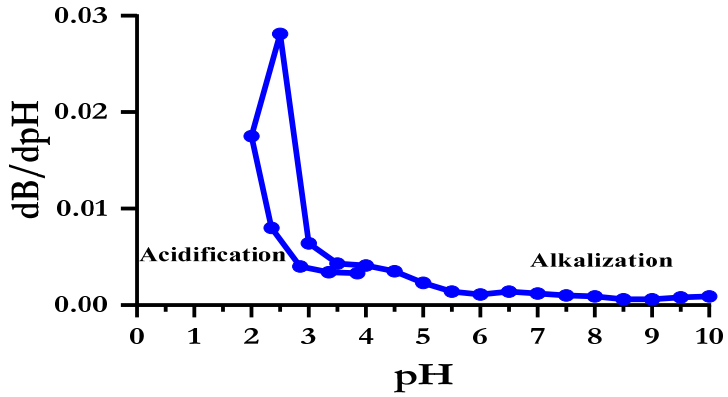

Figure 4. Buffering capacity of reconstituted sour buttermilk powder (SBMP) solution.

3.2.4. Particle Size Distribution of SBMP

The appearance, reconstitution properties, flow characteristics and surface reactivity of milk powder are collectively explained by its particle size distribution. The processing conditions, original feed characteristics and use of different equipment during processing including drying are the key factors that influence particle size distribution [62].

Table 2 clearly represents the particle size distribution of SBMP. A higher number of particles (d_{10}, d_{50} and d_{90}) with a lower specific surface area (SSA) and span were observed for SBMP. The d_{10} of SBMP (Table 2) was higher than that of (27.15 µm) SMP but lower than that of (86.10 µm) WMP [59]. Furthermore, its d_{50} was lower than that of

both SMP (83.87 µm) and WMP (128.76 µm), as reported by Pugliese et al. [59]. Similar findings were also observed for its d_{90}, as shown in Table 2. The d_{90} values of SMP and WMP were 191.55 µm and 287.40 µm, respectively [59]. The $D_{4,3}$ value of SBMP (Table 2) was found to be lower than that of SMP (9.56 µm) and WMP (49.17 µm), whereas the $D_{3,2}$ value was higher than that of SMP (151.33 µm) but found to be lower than that of WMP (86.10 µm) [59], as reported in Table 2. The observed variation in the particle size distribution of SBMP could be attributed to the low total solids and viscosity of feed. The SEM micrograph (Figure 5) of the manufactured SBMP also indicates a porous structure and variability in their particle size, with a significant number of smaller-sized particles.

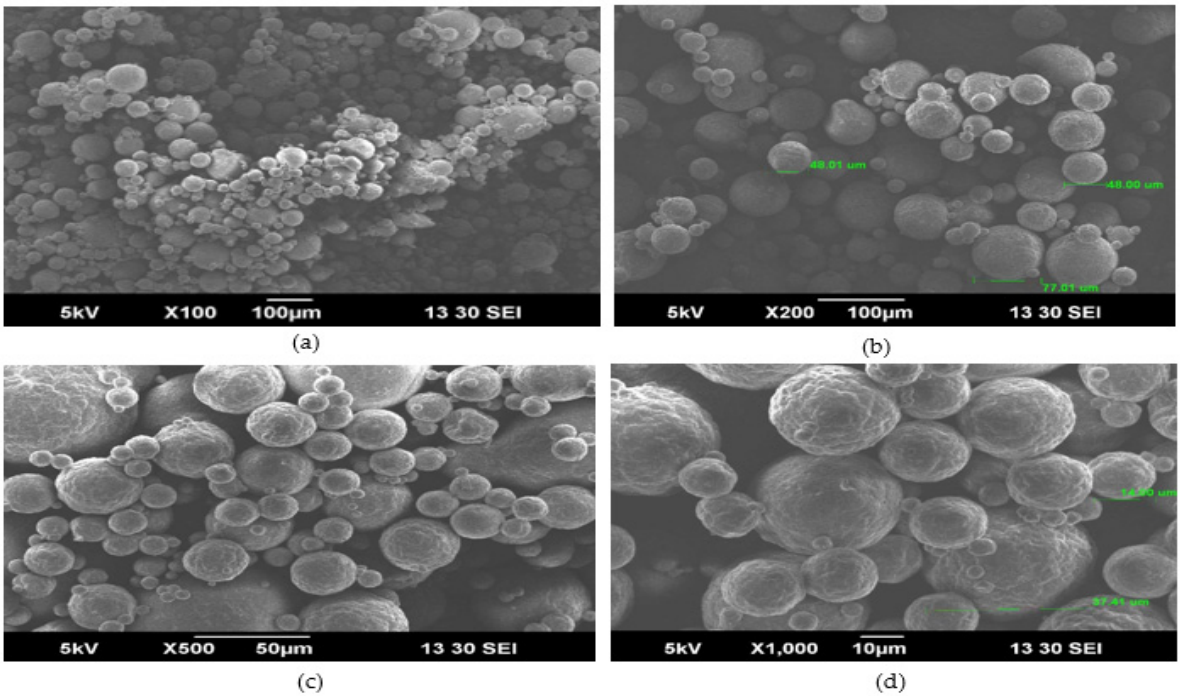

Figure 5. SEM images of sour buttermilk powder (SBMP) at (**a**) 100×, (**b**) 200×, (**c**) 500× and (**d**) 1000×.

3.2.5. Antioxidant Properties of SBMP

The ABTS value of SBMP (Table 2) was lower compared to the reported 57% [87] and 75% RSA [88] values for SMP. Meanwhile, the DPPH value for SBMP (Table 2) was higher than that of (77–94%) WMP [89], (3.26%) buffalo milk powder [90] and (2.87%) cow milk powder [90]. Zivkovic et al. [91] reported that the lipid content, polyphenol level, casein and whey protein contribute to the radical scavenging activity of milk. Padhgan et al. [9] reported that prolonged heat treatment before the fermentation of milk leads to the formation of a brown pigment and also liberates -SH groups. According to Taylor and Richardson [92] and Tong et al. [93], the protein's unfolding and exposure to thiol groups may function as hydrogen donors during thermal treatments, which may result in an increase in antioxidant activity. Sarmadi and Ismail [94] reported that hydrophobic amino acids, including aromatic amino acids, can increase the radical scavenging activity. The FRAP activity of manufactured SBMP (Table 2) was higher than the FRAP values reported by Bhardwaj et al. [95] for Poitu (French breed) donkey milk powder (101.95 µmole/L) and that of Halari (Indian breed) donkey milk powder (74.62 µmole/L). Milincic et al. [96] reported that SMP produced from goat milk and enriched with grape pomace showed the ability to reduce the ferric ions due to the action of free phenolic compounds (such as gallic acids). Hence, the presence of phenolic compounds (Table 2) in SBMP could be responsible for its ferric-reducing antioxidant power.

The total phenolic compound content of SBMP (Table 2) was higher than that of WMP (8.1–9.8 mg/L) [89], SMP (163.75–96.48 µM GE/L) [87], cow milk powder (0.49 mg GAE/g) and buffalo milk powder (3.26 mg GAE/g) [90]. The presence of phenolic compounds at high levels in cattle feed and the microbial activity in milk are liable for the presence of phenolic compounds in milk [97]. This explanation could be true for the observed total phenolic content of SBMP. Additionally, the flavonoid content of SBMP (Table 2) was found to be slightly higher than that of cow milk powder (0.05 mg RE/g) and (0.04 mg RE/g) buffalo milk powder [90]. It is reported that dietary supplementation of dairy feeds with *Scutekkaria baicaolensis* [98], soybean oil or grapes pomace [99], or feeding of fruit [100], could lead to a change in the antioxidant profile in milk and milk products.

3.2.6. Fatty Acid Composition of SBMP

Table 3 and Supplementary Figure S1 reveal the presence of short-, medium- and long-chain fatty acids in SBMP. It contained a major proportion of palmitic acid (46.89%), myristic acid (15.88%) and stearic acid (13.47%). Halder et al. [6] reported that the use of a high temperature during the churning of curd via the traditional method yields a higher proportion of high-melting-point fatty acids over low-melting-point fatty acids. This could explain the observed difference in concentrations of fatty acids in SBMP. Marconi and Panfili [101] also observed a maximum concentration of palmitic acid (33.8%), followed by oleic acid (25.4%) and steric acid (12.7%), in cow milk powder. It is evident in our results also that the saturated fatty acids with a higher melting point that consisted of palmitic, myristic and stearic were markedly higher than the unsaturated fatty acids, such as oleic acid (0.434%), linoleic acid (1.32%) and linolenic acid (0.47%).

Table 3. Fatty acid profiling of sour buttermilk powder (SBMP).

Types	Fatty Acids	% Total Fatty Acids
Short-chain fatty acids	Butyric acid (C4:0)	4.58
	Caproic acid (C6:0)	3.28
	Caprylic acid (C8:0)	1.42
	Capric acid (C10:0)	2.90
Medium-chain fatty acids	Lauric acid (C12:0)	3.57
	Myristic acid (C14:0)	15.88
	Myristoleic acid (C14:1) #	0.87
Long-chain fatty acids	Pentadecylic acid (C15:0) *	1.45
	Palmitic acid (C16:0) *	46.89
	Hexadecenoic acid (C16:1)	2.33
	Margaric acid (C17:0) *	0.68
	Stearic acid (C18:0) *	13.47
	Oleic acid (C18:1) #	0.434
	Linoleic acid (C18:2) #	1.32
	Linolenic acid(C18:3) #	0.47
	Arachidic acid (C20:0) *	0.46

* Saturated fatty acids; # unsaturated fatty acids.

3.2.7. Amino Acid Composition of SBMP

The amino acid profiling of SBMP is shown in Table 4 and Supplementary Figure S2, which reveals the presence of different amino acids in different concentrations. Essential amino acids (histidine and threonine) were present in higher concentrations in SBMP than the values reported for SMP (0.92%, 1.61%) and WMP (0.66%, 1.16%) [102]. Germini et al. [103] reported that proteins partially hydrolyze into peptides and free amino acids during fermentation. Additionally, it is reported that free amino acids are produced during the first four hours of fermentation. After that, there is a decline of amino acid content because the bacterial use during the yoghurt's production generates aromatic substances. Acetaldehyde is the primary component of the aroma produced by *L. bulgaricus*, whereas

S. thermophilus mostly produces diketones in yoghurt. The uncontrolled fermentation of milk during curd making released free amino acids, and many of them were utilized by the lactic acid bacteria, as reported by Padgyan et al. [9].

Table 4. Amino acid profiling of sour buttermilk powder (SBMP).

Name	Peak Area mAU * min	Area Ratio (Std/IS)	Mg per g of Powder	mg per g of Protein
Argenine	2.2171	0.122	2.12	3.95
Serine	0.0127	0.274	2.68	4.99
Aspartic acid	0.008	0.361	5.41	10.08
Glutamic acid	3.4555	0.190	2.97	5.54
Threonine *	0.0002	0.153	2.07	3.85
Glycine	3.0864	0.169	1.24	2.31
Alanine	51.474	2.824	27.17	50.65
Proline	0.733	0.040	0.45	0.84
Methionine *	0.4738	0.026	0.40	0.75
Valine *	3.1443	0.172	1.97	3.68
Phenylalanine *	118.2526	6.487	120.18	224.04
Iso-Leucine *	30.1234	1.652	23.74	44.26
Leucine *	2.0047	0.110	1.50	2.80
Cystine	1.9833	0.109	16.04	29.91
Histidine *	5.677	0.311	2.62	4.88
Lysine *	0.3186	0.017	0.13	0.24
Nor-Leucine (IS)	18.23	1.000	131.74	245.58

* Essential amino acids.

3.2.8. Fourier-Transform Infrared Spectroscopy (FTIR) Spectra of SBMP

The fundamental concept behind infrared spectroscopy is that all molecules absorb frequencies due to their molecular makeup [104]. In order to characterize the organic components in a food system (solid, liquid, or gas), FTIR is a very helpful approach [105].

The primary constituents, fat, protein and lactose, each have distinctive peaks. IR peaks at 2920–2922, 2850–2852 and 1739–1741 cm^{-1} (Figure 6) emerge from the SBMP's fat. As the fat concentrations were reduced, the C=O bond's distinctive peak at 1739–1741 cm^{-1} intensified, and the vibrational frequency of C-O in fat, 1161 cm^{-1}, noticeably drops. Therefore, a lower concentration of fat in the developed SBMP was also revealed by the spectra. The protein's amide I and II vibrations of SBMP are represented by two broad peaks with middle intensities of 1620 and 1529 cm^{-1} (Figure 6), respectively, suggesting the higher concentration of milk protein in SBMP. The characteristic peaks of various C-O vibrations in carbohydrates are located between 800 and 1250 cm^{-1} (Figure 6), indicating the presence of milk lactose [106].

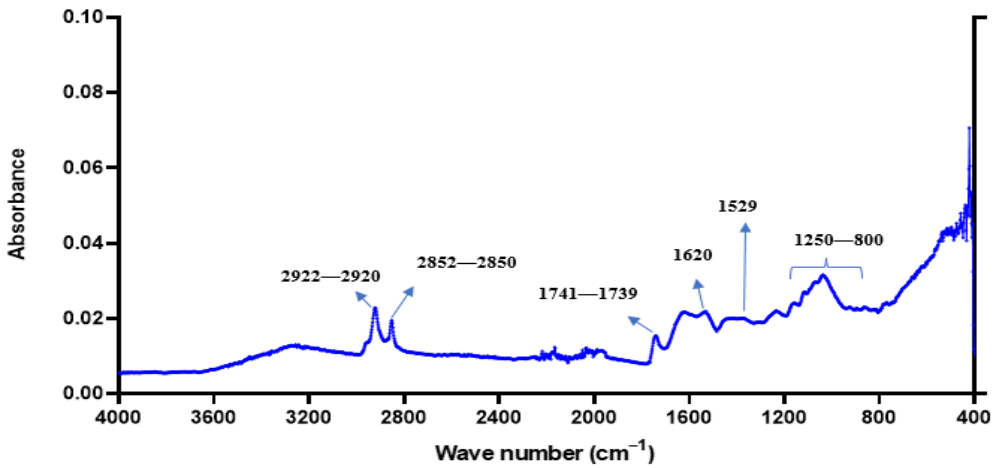

Figure 6. FTIR absorbance spectra of sour buttermilk powder (SBMP).

3.2.9. XRD Spectra of SBMP

X-ray diffraction (XRD) is a frequently used rapid analytical technique for identifying the phase of crystalline materials [107]. It can also detect all forms of lactose crystallization. The manufactured SBMP powder was analyzed to determine the crystallization pattern of its amorphous constituents. Jouppila et al. [108] reported that amorphous lactose crystallizes into different forms, such as α-lactose monohydrate, anhydrous β-lactose, anhydrous α-lactose and an anhydrous mixture of α and β-lactose in a molar ratio of 5:3, 3:2 and 4:1, respectively. According to Nijdam et al. [109], the amorphous lactose in dairy powders crystallized as α-lactose monohydrate and a 5:3 molar combination of α- lactose and β-lactose.

The presence of lactose crystals in SBMP was identified using the location (denoted by diffraction angle, 2θ) and intensity data of the peaks observed in XRD patterns, as depicted in Figure 7. The results were compared with the literature related to different crystalline forms of lactose in skim milk. The available scientific literature on XRD patterns showed peaks of α-lactose monohydrate, stable anhydrous α-lactose, and anhydrous α and β-lactose mixture (molar ratio of 5:3) at a diffraction angle of 19.1° [110], 20.0° [111] and 20.1° [112], respectively. The lactose crystallization peaks of the XRD pattern for SBMP were observed (Figure 7) at a 20° diffraction, with a subsequent intensity value of 156.67. Hence, 2θ value of SBMP sample directly advocating the presence of crystalline lactose in the sample, which eventually falls in the range (19.1° to 20.1°) reported in the scientific literature.

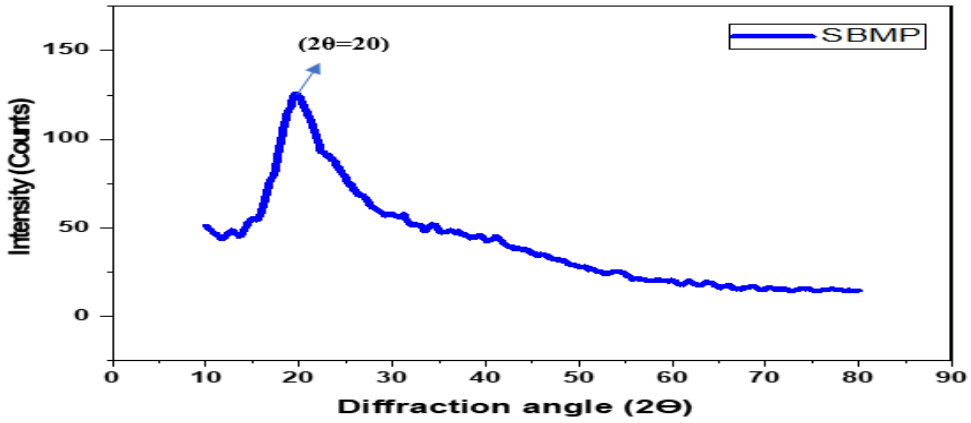

Figure 7. XRD spectra of sour buttermilk powder (SBMP).

3.2.10. Particle Morphology of SBMP

SEM micrographs of SBMP (Figure 5) showed the presence of spherical-shaped powder particles of variable size. The particle size distribution (Table 2) also advocated the variation in the size of SBMP particles. Moreover, it showed some particle infusion and clustering that could be the cause of poor dispersibility and solubility. Furthermore, powder particles were attached in a clustered manner that could be attributed to amorphous lactose and surface fat. Hence, the majority of smaller particles also has a lower bulk density and higher IAC values (Table 2). This could be attributed to the lower TS content of the CSBM (Table 1) that was spray-dried to obtain SBMP.

4. Conclusions

SBM is criticized for its low TS, high acidity and poor thermal stability. Its concentration by RO markedly enhanced total milk solids in retentate, which were converted into stable SBMP via spray drying. SBMP was an "instant powder" in wettability, and it exhibits excellent flowability (θ), good porosity, immediate dispersibility, lower solubility index, lower foam stability and capacity, higher emulsification capacity and emulsification stability. Additionally, it contains essential amino acids and fatty acids and shows good antioxidant properties. SBM, DSBM, CSBM (RO concentrate) and SBMP solution exhibited

typical shear-thinning behavior, and their apparent viscosity decreased with the rise in temperature. SBMP particles were smooth, variable in size and exhibited slight infusion and clustering. Overall, this study established that RO combined with spray drying can be used to valorize SBM into a novel dairy ingredient demonstrating excellent wettability, flowability, emulsification stability and antioxidant properties. The manufactured powder could be used to improve nutritional properties, viscosity, foaming capacity and stability of processed foods. However, its dispersibility, solubility index, oil-binding capacity and foam stability must be improved to further enhance its uses in different food formulations.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/membranes13090799/s1, Figure S1: Fatty acids profiling chromagraph of Sour buttermilk powder (SBMP); Figure S2: Amino acids profiling chromagraph of Sour buttermilk powder (SBMP);Table S1: Gradient program used for Quaternary RS Pump Compartment; Section S1: Methods.

Author Contributions: Conceptualization, G.S.M., A.K.S. and S.A.; Methodology, S.M., G.S.M. and A.K.S.; Validation, G.S.M.; Formal analysis, S.M.; Investigation, S.M.; Resources, A.K.S.; Data curation, S.M.; Writing—original draft, S.M.; Writing—review & editing, G.S.M. and Y.K.; Supervision, G.S.M., A.K.S., Y.K., R.S., S.A. and R.H.V.; Project administration, G.S.M. and A.K.S.; Funding acquisition, G.S.M. and A.K.S. All authors have read and agreed to the published version of the manuscript.

Funding: Haryana State Council for Science, Innovation and Technology (HSCSIT), Panchkula (Project number: HSCSIT/R&D/2021/539).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be provided upon request.

Acknowledgments: The authors would like to thank the Director of ICAR-NDRI, Karnal and Haryana State Council for Science, Innovation and Technology (HSCSIT), Panchkula, for providing funding and support.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

RO	reverse osmosis
SBM	sour buttermilk
TS	total solids
SBMP	sour buttermilk powder
SCBM	sweet cream buttermilk
DSBM	defatted sour buttermilk
CSBM	sour buttermilk concentrate
VRR	volume reduction ratio
CF	concentration factor
FM	flux mean
IF	initial flux
FF	final flux
IAC	interstitial air content
OAC	occluded air content
LBD	loose bulk density
PBD	packed or tapped bulk density
HR	Hausner ratio
CI	Carr index
SI	solubility index
WBC	water binding capacity
OBC	oil binding capacity
ηAppa	apparent viscosity

SSA	specific surface area
XRD	X-ray diffractometer
SMP	skim milk powder
WMP	whole milk powder
HMF	hydroxymethylfurfural
TBA	2-thiobarbituric acid
EC	emulsifying capability
ES	emulsion stability

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RESEARCH & DEVELOPMENT PROJECT

Development of ready to cook (RTC) food product through selective fermentation of biofortified and non-fortified Pearl Millet varieties for enhancing bioavailability of micronutrients

SUBMITTED BY

Dr. Prarabdh C. Badgujar

To

HARYANA STATE COUNCIL OF SCIENCE, INNOVATION AND TECHNOLOGY, PANCHKULA

HSCSIT Reference No. HSCSIT/R&D/2021/535 Dated: 05.03.2021

Project Duration: 2-years

Performa for submitting Annual Progress Report of the Project

Progress report: 2022-2024 (Final report covering remaining objective)

1) Title of the Project

Development of ready to cook (RTC) food product through selective fermentation of biofortified and non-fortified Pearl Millet varieties for enhancing bioavailability of micronutrients

2) **Date of start of Project:** 05 March 2021

3) Name of Principal Investigator and address

Dr Prarabdh C. Badgujar, Assistant Professor, Department of Food Science and Technology, National Institute of Food Technology Entrepreneurship and Management, Kundli, Sonepat, 131 028.

4) Name of Co-Investigators (if any)

1. Dr Tejpal Dhewa, Assistant Professor, Department of Nutrition Biology, Central University of Haryana, Jant-Pali, Mahendergarh, Haryana, 123031
2. Dr Gauri A. Chandratre, Assistant Professor, Department of Veterinary Pathology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar 125004 Haryana

5) **Place of work:** NIFTEM (Sonepat), CUH (Mahendergarh) and LUVAS (Hisar).

6) Objectives

1. Estimation of major micronutrients & quantification of anti-nutrients in locally grown Pearl Millet varieties (fortified & non-fortified ones)
2. Isolation of dominant probiotic strain from local fermented foods
3. Safety assessment of dominant probiotic strains isolated from locally made product in Swiss albino mice
4. Selective fermentation of both Pearl millet varieties & estimation of iron & zinc and antinutritional factors
5. Development of pearl millet based fermented ready to cook (RTC) product & its physicochemical characterization
6. *In vivo* assessment of iron and zinc bioavailability from developed RTC product in nutritionally induced anaemic rat model
7. Popularization of developed RTC product and human capacity building among population

7) Time schedule of Project indicating year-wise activities and financial requirements.

Activity	Year	
	1st year	**2nd Year**
Pearl Millet varieties estimation of micronutrient and antinutrients.	Yes	
Isolation of dominant probiotic strain from local fermented foods	Yes	
Safety assessment probiotic strains in Swiss albino mice	Yes	
Selective fermentation estimation of iron & zinc and antinutritional factors	Yes	
Development ready to cook (RTC) product & its physicochemical characterization:		Yes
In vivo assessment of iron and zinc bioavailability of RTC in anaemic rat model		Yes
Popularization of developed RTC product and human capacity building among population		Yes

Financial requirements. (Price in Lakhs)

Item	**1st Year**	**2nd Year**	**Total**
Salaries/wages **(1 JRF)**	401760	401760	803520
Consumables	400000	400000	800000
Travel	70000	89662	159660
Other Costs/ Contingencies	25000	30000	55000
Overheads (10%)	89676	92142	181818
Total	**986436**	**1013562**	**20,00,000**

8) (a) Summary of research work done so far viz-a-viz time schedule

A. Safety assessment of probiotic strains in Swiss albino mice (Annexure 1)

To evaluate safety of probiotic strain, male Swiss albino mice (4-6 weeks age) were orally administered with different doses (0, 10^7, 10^9 and 10^{11} CFU/d) of *Limosilactobacillus fermentum* in 28-day sub-acute toxicity study (Annexure 1 Fig 1). To assess immunomodulation, additional group of mice received 10^9 CFU/d lactobacilli. MS005 supplementation did not alter body weight, absolute and relative organ weight, hemato-biochemical end-points (Fig 1,2,3,4 Table 1,2), and hepatic GSH & SOD levels. In comparison with control, 1.6-fold reduction in liver lipid peroxidation and significant ($p<0.05$) increase in splenic lymphocyte proliferation (2.3-fold), NK cell activity (1.59-fold), and macrophage phagocytosis (1.3-fold) was observed in mice administered 10^9 CFU/d lactobacilli (Fig 8). Histopathological examination showed normal architecture of liver, spleen, ileum and colon in lactobacilli administered mice; while increased number of villi and dense population of lymphocytes in payer's patches of ileum was evident in 10^9 CFU/d dosed group as compared to control mice (Fig 5,6,7). Overall, results corroborated immunostimulatory effect of MS005**.** Hence, *Limosilactobacillus fermentum* (MS005) could be considered as a potential probiotic to enhance iron bioavailability of iron-rich foods as well as to formulate immunomodulatory functional foods.

B. In vivo assessment of iron bioavailability of RTC in anaemic rat model

An experimental study was conducted to determine the effect of Biofortified Pearl Millet Based Raabadi on Iron Bioavailability in Dietary Induced Anemia in Sprague Dawley Rats by Oral Route. All procedures followed for conduct of this study were as per the guidelines set by the Committee for Control and Supervision of Experiments on Animals (1723/PO/RcBiBt/S/13/CPCSEA), and with approval of Institutional Animal Ethics Committee (IAEC) is in place (IAEC-023-051). Total 24 female rats were acclimatized for a period of 03 days prior to dose administration. During this period, all animals were observed for mortality and morbidity twice daily. Rats were randomly divided into four groups G1, G2, G3 and G4, 6 animals per group.

C. Induction of Dietary Anemia

Anemia was induced in G2, G3 and G4 group animals by providing iron deficient feed (iron content 6-7 mg/kg feed) from day 1 to day 37. Approximately 25% decrease from initial hemoglobin concentration was considered as successful induction of anemia. All animals were provided with standard rodent diet *ad libitum* from Day 37 till terminal sacrifice. G1 served as control

D. Preparation of the Dose Formulation

6 to 8 gm skim milk powder was added in 100mL distilled water, to which 12-15 gm of Pearl Millet flour was added and stirred for 15 min at 95˚C. The mixture was allowed to cool (at 40-42˚C). Inoculm @2% L. fermentum MS005 was added and incubated at 37 ± 1-2˚C for 16 hr. Two separate Raabadi samples were prepared every alternate day; one with non-biofortified pearl millet flour and another with biofortified pearl millet flour. Raabdi was stored at in refrigerator (4˚C) and was used for 1-2 days for dose.

E. Experimental design

Groups	Treatment	Dose
G1	Normal control (Non-Anaemic)	Normal Diet
G2	Anaemic Control Group (AC)	Iron Deficient Diet
G3	Anaemic fortified Pearl millet raabadi group (AF)	Normal diet + Bio-fortified pearl millet based fermented Raabadi (3-5 gm/day)
G4	Anaemic non- fortified Pearl millet raabadi group (ANF)	Normal diet + Non-fortified pearl millet based fermented Raabadi (3-5 gm/day)

F. Dose Administration

From Day 38, Bio-fortified pearl millet based fermented Raabadi (3-5 gm/day) and non-fortified pearl millet based fermented Raabadi (3-5 gm/day) was administered to G3 and G4 group animals, respectively for subsequent 30 days. Various parameters evaluated included mortality, clinical signs, body weight, feed consumption, hematology, gross pathology organ weight to check the effect of Biofortified Pearl Millet Based Raabadi on Iron Bioavailability in Dietary Induced Anemia.

G. Results

There was 20% decrease in mean values of hemoglobin of animals fed with iron deficient diet. Similar findings were reported previously (Dhur *et al*., 1990). RBC morphologic evaluation revealed normocytic hypochromic anemia. The changes observed indicated successful development of anemia.

1. Mortality and Clinical Signs Observations

All animals survived throughout the experiment period and no mortality was observed. No abnormal clinical signs observed in comparison with control groups.

2. Body Weights

There was statistically significant reduction in body weight and body weight gain in anaemic control group. (Annexure 2 Table 1)

3. Feed Consumption

Feed consumption was significantly decreased in anaemic rats when compared to normal control group rats on day 42, 50, 56, 63 and 65. There was significant increase in feed consumption of rats fed with bio fortified Pearl millet raabadi (day 42, 50, 56, 63 and 65) and non- fortified Pearl millet raabadi (day 65). Biofortified PM-Rabadi showed more feed consumption (G3) than nonfortified PM Product (G4). (Annexure 2 Table 2)

4. Hematology

There was statistically significant increase in Hb and PCV on day 66 in rats treated with biofortified (G3) and nonfortified (G4) Pearl millet raabadi as compared to the anaemic rats suggesting reduction in anemia (Annexure 2 Table 3).

5. Serum iron and biochemistry

Serum iron concentration was significantly decreased in anaemic rats whereas significant increase in blood urea nitrogen (BUN) was observed in anaemic rats. Consumption of biofortified and non biofortified Pearl Millet Rabadi improved serum iron concentration in rats of G3 and G4 and reduced blood urea nitrogen indicating ameliorative effect on anemia.(Annexure 2 Table 4 Fig 2, 4)

6. Gross Pathology

Gross-pathological examination showed red discoloration of liver and kidney in anaemic rats and no lesion of pathological significance was observed in rats fed

biofortified and nonbiofortified pearl millet based Raabadi when compared with control group.

7. Organ weight

The relative spleen weight was increased significantly in anaemic control group as compared to normal control group. Relative spleen weight of rats fed biofortified and nonbiofortified PM Rabadi was comparable to control (Annexure 2 Table 5 Fig 1).

8. Histopathology (Annexure 3)

Histopathological examination of various vital organs revealed significant alterations in anaemic rats. Degeneration of hepatocytes, congestion of central vein and sinusoidal spaces was observed in liver (Fig 1). Kidney showed extensive hemorrhages, formation of hyaline casts in tubules and reduced glomeruli in renal parenchyma (Fig 2). Spleen showed hemorrhages in red pulp and decreased lymphocytes in white pulp (Fig 3). Ileum and colon showed increased mucosal thickness, distortion of mucosal epithelium and reduced number of goblet cells (Fig 4 &5). Bone marrow secriton revealed diffuse hemorrhages and reduced cellularity confirming anemia (Fig 6). All these alterations were significantly reduced in rats fed with biofortified PM based product and nonfortified PM based product. However biofortified PM rabadi showed better amelioration than nonbiofortified product.

9. Liver gene expression study

Feeding of biofortified and nonbiofortified PM based product upregulated the mRNA expression of hepcidin and FPN 1 gene which was down regulated in anaemic rats. Biofortified Pearl Millet rabadi showed significant upregulation in gene expression compared to non biofortified Pearl Millet rabadi. (Annexure 2 Fig 3)

H. CONCLUSION of Objective 6:

Feeding of Bio-fortified pearl millet based fermented Raabadi (3-5 gm/day) and Non-fortified pearl millet based fermented Raabadi daily to Sprague Dwaley rats for 30 days to anaemic rats resulted in the improvement (reduction from earlier levels) of anaemia.

However, feeding of bio-fortified pearl millet based fermented Raabadi caused significant improvement in health status of anaemic rats in terms of body weight, haemoglobin concentration, serum iron profile, feed consumption along with improvement in the functioning of vital organs such as liver kidney, bone marrow and spleen as compared to non-fortified pearl millet based fermented Raabadi.

Therefore, findings of the present study conclude that incorporation of biofortified pearl millet instead of routinely used (non-biofortified bajara varieties) should be utilized for the production of raabadi and should be fermented with our isolated and well characterized/studied probiotic strain, *Limosilactobacillus fermentum MS005*. This shall improve overall health status and iron availability and can reduce the problem of anaemia in humans, especially in girls and adults. Thus, detailed human clinical trials can help confirm the beneficial effect of use of biofortified pearl millet varieties/hybrids (especially, HHB-311) for the fermentation of Raabadi with the above mentioned strain. This shall go in long way to reduce anaemia burden in our state, Haryana as well as in the India.

9. Report of work done so far (please indicate time versus activity schedule)

Session 2023-2024 – FY 2023-24 and onwards, i.e., till August 2024

	April – June 2023	**July – Sept 2023**	**Oct-Dec 2023**	**Jan – Mar 2024**	**April -August 2024**
Storage study: Physicochemical parameter and microbiological analysis of fermented beverage. Sensory analysis of beverage					
Receiving confirmation for utilization of sanctioned funds in the extended duration of the project (i.e., March 2023 onwards)					
Safety determination of isolated *Limosilactobacillus fermentum MS005* in mouse model					
Procurement of biofortified pearl millet hybrids – HHB-311 – **Principal cause of delay;** caused delay as new crop was available only at the end October 2023 for in vivo anaemic rat bioavailability study					
Effect of feeding Raabadi (made from biofortified pearl millet, HHB-311) on iron bioavailability in dietary induced anaemic rat model					
Final Results and Report compilation					

9) Staff position: **One (JRF) – till Jan 2023**

10) Details of Head wise & year wise expenditure:

Full and final settlement of accounts and sanctioned amount.

Item/Head	Amount Carried Forward (A)	Amount Sanctioned/ Received (B)	Total Amount (C) (A+B)	Expenditure FY 22-23 (D)	Expenditure FY 23-24 (E)	Total Expenditure (F) (D+E)	Closing Balance (C-F)
Salaries/ Expenditure Wages (1 JRF)	15,624	4,01,760	4,17,384	3,30,336	38,880	3,69,216	48,168
Consumables	296	4,00,000	4,00,296	73,665	3,26,250	3,99,915	381
Travel	17,803*	89,662	1,07,465	7630	99798	1,07,428	37
Other Costs/ Expenditure Contingencies	1	30,000	30,001	23,868	6,132	30,000	1
Overheads (10%)	0	92,142	92,142	92,142	0	92,142	0
Total	**33,724/-**	**10,13,564 /-**	**10,47,288/-**	**5,27,641/-**	**4,71,060/-**	**9,98,701/-**	**48,587 /-**
Note*	**The amount of Rs. 17803 was wrongly mentioned as Rs. 17183 in the earlier submitted UC (2021-2022); however, the total mentioned (Rs. 33724) was correct; hence, overall the amount does not change and has no implications on the budget.**						

Bank Interest	Closing Balance	Total Amount
Rs.22,627/-	Rs.48,587/-	Rs.71,214/-

11) List of equipment purchased (if any): NIL

(a) Name of equipment

(b) Date of receipt

(c) Date of Installation

12) Difficulties/problems faced in implementation of project and suggestions for remedies.

Had mentioned in earlier reports.

13) List of publications indicating grant no. of HSCST along with reprints related to the project.

Three research articles published *so far; two more are in pipeline and shall be published within next 6 months.* ***All publications are in Sci-indexed journals.*** (copies of published articles attached after this report.

i. Samtiya, M., Chandratre, G. A., Dhewa, T., **Badgujar, P. C.**, Sirohi, R., Kumar, A., and Kumar, A. B. **2022.** A comparative study on comprehensive nutritional profiling of indigenous non-bio-fortified and bio-fortified varieties and bio-fortified hybrids of pearl millets. **Journal of Food Science and Technology**, 1-12. https://doi.org/10.1007/s13197-022-05452-x

(**Impact factor 2.6** as of 2023; indexed in Scopus; Publisher: Springer Nature)

ii. Samtiya, M., Bhushan, B., Sari, T. P., **Badgujar, P. C**., Chandratre, G. A., Singh, P., ... & Dhewa, T. **2024**. Characterization of indigenous lactobacilli from dairy fermented foods of Haryana as potential probiotics utilizing multiple attribute decision-making approach. **Food Production, Processing and Nutrition**, 6(1): 81. https://doi.org/10.1186/s43014-024-00259-z

(**Impact factor 4.0** as of 2023; Scopus indeed; Publisher: BMC, Springer Nature)

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(**Impact factor 6.5** as of 2023; indexed in Scopus; Publisher: Elsevier)

14) **Any other relevant information**. NIL

Signature of PI

Date: - 13.09.2024

Place: - Kundli, Sonipat

J Food Sci Technol
https://doi.org/10.1007/s13197-022-05452-x

ORIGINAL ARTICLE

A comparative study on comprehensive nutritional profiling of indigenous non-bio-fortified and bio-fortified varieties and bio-fortified hybrids of pearl millets

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Revised: 19 March 2022 / Accepted: 23 March 2022

Abstract Seven indigenous pearl millet varieties, including non-bio-fortified (HC-10 & HC-20) and bio-fortified (Dhanashakti) and bio-fortified hybrids, viz., AHB-1200, HHB-299, HHB-311, and RHB-233, were studied in the present work. There was not any significant difference observed in the crucial anti-nutrients content, i.e., phytate (24.88–32.56 mg/g), tannin (3.07–4.35 mg/g), and oxalate (0.33–0.43 mg/g). Phytochemical content and antioxidant activity showed significantly high ($p < 0.05$) TPC and FRAP, TFC, and DPPH radical scavenging activity in the HHB 299 and Dhanashakti, respectively. Quantitative analysis of polyphenols by HPLC (first report on these varieties) revealed that HHB-299 has the highest amount of gallic acid. Fatty acid profiling by GC-FID showed that Dhanashakti, AHB-1200, and HHB-299 have rich monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA). Mineral analysis by ICP-OES showed high iron (87.79 and 84.26 mg/kg) and zinc (55.05 and 52.43 mg/kg) content in the HHB-311 and Dhanashakti, respectively. Results of the present study would help facilitate the formulation of various processed functional food products (RTC/RTE) that are currently not reported/unavailable.

Keywords Bajra · Anti-nutritional factors · Linoleic acid · Polyphenols · Iron · HPLC

Introduction

Millets are one of the most essential crops consumed throughout the world, primarily in the semi-arid and arid regions of Asia (China and India) and Africa. These are the first cereals grains used domestically by humans, having a

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Published online: 16 April 2022

round shape, small seeds, and belong to the *Poaceae* family. Millets are ranked as the sixth essential crop that feeds 1/3rd of the world population (Samuel and Peerkhan 2020; Singh et al. 2017). Millets possess a rich quantity of essential fatty acids, dietary fiber, fats, protein, etc., although they contain a good quantity of major micronutrients such as iron, calcium, magnesium, zinc, and potassium (Saini et al. 2021). Recently, millets gained much researchers' interest due to the presence of essential nutrients (carbohydrates, proteins, major micronutrients, i.e., Fe and Zn, vitamin B) and bioactive ingredients, which have promising health-boosting attributes. Furthermore, millet is also explored for its therapeutic potential as a nutraceutical towards chronic ailments, including diabetes, cancer, cardiovascular diseases, and obesity (Majid and Priyadarshini 2020).

Pearl millet (*Pennisetum glaucum*) is one of the most important cereals, widely cultivated and well recognized as future food crops that confirm people's nutritional security. In India, PM production has improved constantly with high-yielding hybrid varieties. Due to the immense nutritional level of dietary fiber, micronutrients, and bioactive components, whole PM-based natural food products demand has also increased (Gong et al. 2018). PM is also reported to contain high lipids compared to other grains such as sorghum and maize. They also contain a rich amount of essential fatty acids like linoleic acid (39–45%) and oleic acid (21–27%). Literature evidence has concluded that PM has rich amount of phenolic components than other food sources such as rye, barley, wheat, sorghum, maize, and oats (Tomar et al. 2021). PM grains show a rich concentration of bioactive components (phenolics and flavonoids) with immense antioxidative attributes. Several methods, such as HFRSA, TAC, FRAP ABTS, and DPPH, have been used to evaluate the antioxidative potential of PM grains (Salar and Purewal 2017).

Cereals biofortification is an excellent approach to alleviate malnutrition conditions using genetic tools to improve the nutritional value of food crops. To overcome the concerns of micronutrient deficiency (mainly Fe and Zn), the All India Coordinated Research Project on Pearl Millet (AICRP-PM) started the biofortification trial to yield micronutrient-rich crops in 2014. Initially, ICRISAT, with the support of harvest plus, released a biofortified variety of pearl millet with large Fe and Zn. Dhanashakti (Zn-43 and Fe-81 ppm) is the first high-Fe pearl millet released in 2013. After this, high iron and zinc-containing biofortified pearl millet varieties/hybrids, i.e., AHB-1200, AHB 1269, HHB-299, RHB-234, RHB-233, and HHB-311, were released between 2018 and 2020. One meal of pearl millet high-Fe biofortified variety can fulfill up to 50–100% of the daily iron requirement and help mitigate the iron deficiency in the population (children, men, and women) (Satyavathi et al. 2021).

Despite its rich nutritional value in terms of minerals, it contains anti-nutritional factors such as phytate, which profoundly decreases their bioavailability. Phytate is one of the critical anti-nutrients that possesses chelating properties and chiefly reduces the bioavailability of micronutrients such as zinc, copper, and iron. Moreover, other anti-nutritional factors, such as oxalate, tannins, etc., have been reported to reduce the bioavailability of the minerals in the food (Kaushik et al. 2018; Samtiya et al. 2020). PM grains have rich nutritional value, but comprehensive data of the selected varieties of PM is still lacking.

Further, there is a dearth of literature about comprehensive nutritional profiling for these bio-fortified PM hybrids and bio-fortified PM variety, Dhanashakti. These bio-fortified hybrids and bio-fortified varieties have been developed for the rich iron and zinc content, but still, their other nutritional and anti-nutritional content has not been discussed in detail in the previously published studies. Thus, the present study was conducted to assess the proximate composition, anti-nutrients (Tannin, oxalate, and phytate), minerals (Fe, Zn, Ca, Mg, Mn, Na, and K), phytochemical content (Total flavonoids and total phenolic compound), antioxidant activity (DPPH and FRAP), Fatty acids profiling, and quantitative estimation of polyphenols present in the seven pearl millet varieties being majorly cultivated in India to help develop various functional food products from the bio-fortified hybrid/varieties having the better nutritional profiling and least possible anti-nutritional factors.

Material and methods

Chemicals/reagents

All the reagents and chemicals used in this research study were molecular/analytical grade. Phytic acid, Gallic acid, 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic Acid (Trolox), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), Quercetin, Diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma-aldrich Inc., USA. Methanol, Hydrochloric acid, Ferric chloride, Aluminium chloride, Sodium bicarbonate, Potassium acetate, Petroleum ether, Folin-Ciocalteu's reagent, Sulphuric acid, Sodium hydroxide, Acetone were procured from the Hi-Media Laboratories Pvt. Ltd., Mumbai. Supelco FAME Mixture (Supelco CRM No.47885), *n*-Hexane (HPLC Grade) (CAS No.110–54-3), Toluene (HPLC Grade) (CAS No. 108-88-3), Boron Trifluoride (7% in Methanol), Anhydrous sodium sulfate (AR Grade) (CAS No.7757-82-6), Grade-1 Reagent grade water as per IS 1070. Nitric acid, and Perchloric acid were

procured from Qualigens (ThermoFisher Scientific, Mumbai, India). Tannic acid was procured from the Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

Pearl millet varieties procurement and processing

Total seven varieties of Pearl millet (PM), i.e., Non-biofortified (HC-10 & HC-20) & biofortified (Dhanashakti), and bio-fortified hybrids, viz., AHB-1200, HHB-299, HHB-311, and RHB-233 were used in this study. Four PM varieties (HC-10, HC-20, HHB-311, HHB-299) were procured from the Bajra Section, CCS Haryana Agricultural University, Hisar, India. Other three PM Varieties (Dhanashakti, AHB-1200, RHB-233) were procured from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, Telangana, India. Each variety was collected in 2–2.5 kg of weight. All raw PM varieties were appropriately cleaned and dried in sunlight in 4–5 h up to 8–10 percent moisture content. Further, all seven samples were ground in a hammer mill into fine flour and stored in air-tight containers till further analysis.

Proximate analysis of raw PM varieties flour

Raw PM flour was analyzed for protein, moisture, ash, crude fiber content by following standard procedures of the Association of Official Analytical Chemists (AOAC 2005). Protein content (N × 6.25) was estimated using Kjeldahl apparatus (Gerhardt, Analytical Systems, Germany), and crude fat content was estimated by standard Soxhlet method using automated Soxhlet equipment (Pelican Equipments, Chennai, India). Moisture content was measured after drying the flour samples at 105 °C for 16 h and repeated till weight was constant. Ash content was calculated by ashing the flour samples for 4–5 h using a muffle furnace at 550 °C. The crude fiber of samples was estimated after digesting with 1.25% sulfuric acid and subsequently with 1.25% sodium hydroxide solution. Total carbohydrate was calculated according to formula: Total carbohydrate = [100 – (crude protein + crude fat + crude fiber + moisture + ash)].

Estimation of phytochemical content and antioxidant activity

Ultra-sonic assisted extraction (UAE) and conventional extraction (CE)

PM samples were extracted using Ge et al. (2021) method with some modifications, using 80% of methanol (methanol:water) as a solvent. Different concentrations of methanol (nil to absolute) were first experimented to finalize 80% methanol as a preferred solvent for the extraction of PM (data not shown). In the UAE method, 1 g of flour sample was mixed in 10 mL of methanol and ultrasonicated for 30 min using an ultrasonic water bath (MRC, Israel) operated at 40 kHz at 30 °C. The supernatant was collected, and the residue was subjected to ultrasonication in the same manner two more time. All the three supernatants were pooled and centrifuged at 8000 rpm for 15 min followed by filtering through a syringe filter 0.45 µm and stored in the amber falcon tubes at −20 °C till further analysis.

In the CE method, 1 g flour sample was mixed in 10 mL methanol and kept in a shaker incubator for 30 min at 150 rpm at 30 °C (Innova 42, New Brunswick Scientific, USA). The supernatant was collected and re-shaked in the same conditions twice, and afterward same steps were followed as discussed in the UAE method.

Phytochemical content

Total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC were estimated according to Singhal et al. (2021) with some modifications. Samples absorbance was measured at 765 nm using a multimode microplate reader with a cuvette port (Spectramax M2e system, Molecular Devices, USA). Gallic acid was used as standard with a range of 0–50 µg/mL to measure the TPC concentration, and results were expressed as mg of gallic acid equivalent (GAE)/g of flour.

For the estimation of TFC, sample extract (500 µL) was mixed with 1.5 mL methanol, followed by 100 µL of aluminum chloride (10% w/v), 100 µL 1 M potassium acetate, and 2.8 mL distilled water. Samples were incubated in dark conditions for 30 min at room temperature. The absorbance of the sample's solution was measured at 415 nm using a microplate reader with a cuvette port. Quercetin was used as standard with a range of 0 to 80 µg/mL, and results were calculated as mg of quercetin equivalent (QE)/g of flour.

Antioxidant activity

Ferric reducing antioxidant power (FRAP) activity

The FRAP assay was estimated according to the method described by Kumar et al. (2021) with minor modifications. The absorbance of the sample was measured at 593 nm using the cuvette port of the spectrophotometer. Trolox was used as a standard with a range (5–80 µM), and results were discussed in mM Trolox equivalent (TE)/g of flour.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (RSA)

Free radical scavenging activity of samples was measured by DPPH assay according to Kumar et al. (2020) and Verma et al. (2021) method with modifications. Sample absorbance was measured at 517 nm against a blank (methanol only). The same amount of DPPH and methanol mixture was used as a control. All the analyses were measured in triplicates. RSA ability was calculated by:

$$\text{Radical scavenging ability}(RSA\,\%) = [A_1 - (A_D - A_S)]/A_1 \times 100$$

Note: A_1 is control absorbance; A_D is DPPH solution absorbance with the sample; A_S is sample extract absorbance without DPPH.

Anti-nutritional factors

Phytic acid (PA)

The phytic acid content of the raw PM flour samples was estimated using the method described by Ahmed et al. (2020) with minor changes. The absorbance of the sample was calculated at 500 nm using a multimode microplate reader with a cuvette port. A standard curve of phytic acid (dissolved in 0.2 N HCl) was used to estimate PA content, and the results are discussed as PA in mg/gm of flour.

Tannin content

The tannin content of raw PM flour was estimated according to Owheruo et al. (2019) method with minor modifications. The absorbance of the sample was measured at 725 nm using a multimode microplate reader with a cuvette port. Tannic acid was used as a standard with a range (5–50 µg), and results were discussed as mg of tannic acid/gm of flour.

Oxalate content

The oxalate content of raw PM flour was estimated according to Ijarotimi et al. (2018) method. About 1 gm of flour sample was added into 100 ml of conical flask followed by 75 ml of H_2SO_4 (3 M). The sample solution was stirred for 1 h with a magnetic stirrer at 30 °C and filtered using filter paper (Whatman No 1). Exactly 25 ml of clear filtrate was taken and titrated hot (90 °C) against 0.1 M $KMnO_4$ solution till faint pink color appeared for at least 30 s. Oxalate was calculated using the below formula:

$$\text{Oxalate}\ (\text{mg/g}) = V_T \times 0.9004$$

Note: V_T = Titre volume (mL).

Polyphenol profiling

Extraction of polyphenols

For the quantification of polyphenols, 3 g of raw PM flour was extracted in 10 mL methanol and ultrasonicated for 30 min using an ultrasonic water bath (MRC, Israel) operated at 40 kHz at 30 °C. The supernatant was collected, and the residue was re-extracted in the same manner twice. All the three supernatants were pooled and centrifuged at 8000 rpm for 15 min. The supernatant thus obtained was then filtered through a nylon syringe filter 0.45 µm and was stored in the amber-colored falcon tubes and used for phenolics profiling analysis on the same day.

HPLC analysis of polyphenols

Polyphenols were quantified by high-performance liquid chromatography (HPLC) with photodiode-array (PDA) detector (Agilent 1260 Infinity II, Agilent Technologies, USA) as per the Seal (2016) method with some modifications. Polyphenolic compounds in samples and standards were estimated on a reverse-phase C18 column (Spherisorb, 250 × 4.6 mm, 5 µm). HPLC column was controlled at 40° C thermostatically. The total run time of the separation protocol was 32 min, 1.0 mL/min of flow rate, and 10 µL injection volume was used. After testing several gradient settings and acetic acid concentrations (1, 0.5, and 0.1%), the following mobile phase conditions were used for the separation: solvent A (0.1% aqueous acetic acid) and solvent B (acetonitrile). The gradient increased at 10 min from 20 to 80% solvent B and was maintained for 10 min. Solvent B was then decreased to 20% at 27 min and held for 5 min. Individual components were identified by injecting the standard solutions at 10 µg/mL based on their UV spectrum. Caffeic and quercetin were detected at 320 nm and Gallic at 300 nm. Individual polyphenols were quantified by the area comparison corresponding to their dilutions in mg/100 g.

Fatty acid profiling

Fatty acids of raw PM flour samples were measured by Gas chromatography (GC) having Supelco SP-2560 fused silica capillary column (Agilent technology, 7890B) as fatty acids methyl esters (FAMEs) (AOAC 2002, Official Method 996.06). Two–three mL of petroleum ether was added to the extracted fat and fat was dissolved completely. Reconstituted fat was transferred into a Pyrex culture tube with a teflon screw cap (16 mm × 120 mm). The solvent was evaporated entirely under a gentle stream of nitrogen at room temperature. After that, 2 mL of 7% Boron trifluoride was added in methanol followed by addition of

1 mL toluene. Then, the pyrex tube was incubated at 100 °C for 45 min in a hot air oven. At every 10 min, tube was shaken gently during incubation. Vial was allowed to cool down to room temperature (20–25 °C), and then 5 mL of distilled water, 2 mL hexane, and approximately 1.0 g sodium sulphate were added. The vial was capped and shaken for 5 min in a vortex mixer. Consequently, layers were allowed to separate, and then carefully upper hexane layer was pipetted. Finally, the hexane layer was filtered through a 0.22 µm PTFE filter into an amber coloured GC vial to be used for the further analysis. Data are reported as the percentage of fatty acids.

Instrument programming

Operating conditions: Injector temperature—250 °C; Detector temperature—280 °C; Initial temperature—100 °C (held for 4 min). The temperature was then increased by 4 °C/min up to 240 °C and held for 25 min.

Minerals composition determination

Micronutrients in the PM varieties were estimated according to Wheal et al. (2011) with modifications. Digestion of samples was carried out by using a diacid (Nitric (HNO_3) and perchloric ($HClO_4$) acid) mixture. After 3–4 h of digestion, samples were collected when HNO_3 white fumes ceased, and collected samples were diluted with the milli-Q type I ultrapure water up to 50 mL. Total minerals (Fe, Zn, Ca, Mg, Mn, K, and Na) were analysed using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Optima 7000DV, PerkinElmer, USA).

Statistics

All measurements were carried out in triplicates, and data were analysed using GraphPad Prism (GraphPad Software version 5.01, San Diego, CA, USA). The results were represented as mean ± SD (standard deviation). One-way and two-way analysis of variance (ANOVA) was used for statistical analysis of data followed by Tukey post hoc test to separate the mean ($p \leq 0.05$), which were considered statistically significant.

Results and discussion

Proximate analysis of raw PM varieties flour

The proximate composition of all the seven PM varieties is presented in Table 1. Our results showed that carbohydrate and moisture content of PM varieties was in the range of 66.98 ± 0.51 to 74.40 ± 0.28% and 6.13 ± 0.22 to 9.54 ± 0.15%, respectively. Dhanashakti variety had the highest carbohydrate content among all the seven varieties. The crude fiber and ash content of our samples was in the range 2.58 ± 0.02 to 1.606 ± 0.02% and 1.77 ± 0.21 to 3.08 ± 0.60%, respectively. Our results are in parallel to that of Kumari et al. (2018), who reported 70.77 to 67.90% carbohydrate in the 11different local, popular and new hybrid of PM varieties of Indian origin (such as PC 1201, HHB 203, Proagro 9444, HHB 67, Pioneer 86M86, etc.). Protein content was lowest in HHB-299 and was highest in the HC-10 variety. Fat content was highest in the AHB-1200 hybrid among all seven varieties. Siroha and co-workers have shown that different Indian pearl millet varieties have fiber, ash, protein, and fat content in the range 2.9–3.8%, 1.65–1.90%, 9.7–11.3%, and 5.1–7.2%, respectively, corroborating our results (Siroha et al. 2016). Findings suggested that PM is a rich source of protein and could be used to mitigate protein malnutrition by making different ready-to-cook/eat (RTC/RTE) products.

Phytochemical content

TPC and TFC

Figure 1a and b shows the comparative analysis of TPC and TFC for CE and UAE treatments. Results showed that TPC and TFC content in UAE was higher than that of CE in the studied PM varieties. TPC and TFC content of our samples for UAE treatment were in the range 2.58–3.25 mg GAE/g and 0.49–0.62 mg QE/g, respectively. Siroha et al. (2016) reported similar results for TPC; the study reported 3.13–2.39 mg of GAE/g of PM samples. Another study by El Hag et al. (2002) reported that raw flour of different PM cultivars had 3.04 mg/g and 4.44 mg/gm of total polyphenols content. There was a non-significant difference ($p > 0.05$) in the TPC between CE and UAE treatments for HHB-311 and AHB-1200 hybrids. Highest TPC and TFC content was found in the HHB-299 hybrid and Dhanashakti variety, respectively, for both the treatments (CE and UAE). The TFC results in the present study are in agreement with Owheruo and co-workers, who reported 0.91 mg/g in raw PM samples (Owheruo et al. 2019). Similar results were reported by Pushparaj and Urooj (2014) with 0.27 and 0.21 mg/g of TFC in two different varieties (Maharashtra Rabi Bajra and Kalukombu) of raw pearl millet.

Table 1 Proximate composition of non-bio-fortified and bio-fortified pearl millet cultivars

PM Varieties	Carbohydrate (%)	Protein %	Ash %	Fat %	Moisture %	Fiber %
HC-20	70.68 ± 0.42^{a}	9.60 ± 0.20^{ac}	2.28 ± 0.40^{ab}	6.76 ± 0.56^{ab}	8.46 ± 0.01^{af}	2.19 ± 0.005^{a}
HC-10	66.98 ± 0.51^{b}	10.46 ± 0.09^{b}	2.60 ± 0.69^{ab}	8.62 ± 0.32^{b}	9.54 ± 0.15^{b}	1.78 ± 0.03^{b}
HHB-299	73.37 ± 0.82^{c}	9.38 ± 0.08^{a}	2.05 ± 0.65^{ab}	5.09 ± 0.28^{a}	8.10 ± 0.22^{ae}	1.99 ± 0.03^{c}
HHB-311	68.46 ± 1.12^{ab}	10.20 ± 0.13^{bdc}	2.11 ± 0.20^{ab}	7.81 ± 1.29^{b}	9.19 ± 0.12^{bf}	2.21 ± 0.01^{a}
AHB-1200	67.21 ± 1.51^{db}	9.53 ± 0.17^{ac}	3.08 ± 0.59^{b}	10.67 ± 1.06^{c}	7.10 ± 0.17^{cde}	2.40 ± 0.01^{d}
RHB-233	69.88 ± 0.67^{a}	10.03 ± 0.13^{dc}	1.76 ± 0.20^{a}	9.81 ± 0.38^{bc}	6.12 ± 0.22^{d}	2.51 ± 0.02^{e}
Dhanashakti	74.40 ± 0.28^{c}	9.82 ± 0.087^{c}	1.78 ± 0.02^{a}	5.17 ± 0.52^{a}	7.21 ± 0.33^{e}	1.60 ± 0.01^{f}

Data is expressed as mean ± SD, $n = 3$. Mean values bearing different superscript letters (a, b, c, d) are significantly different at $p < 0.05$ in Tukey's multiple comparison post-hoc test

Values are in dry weight basis

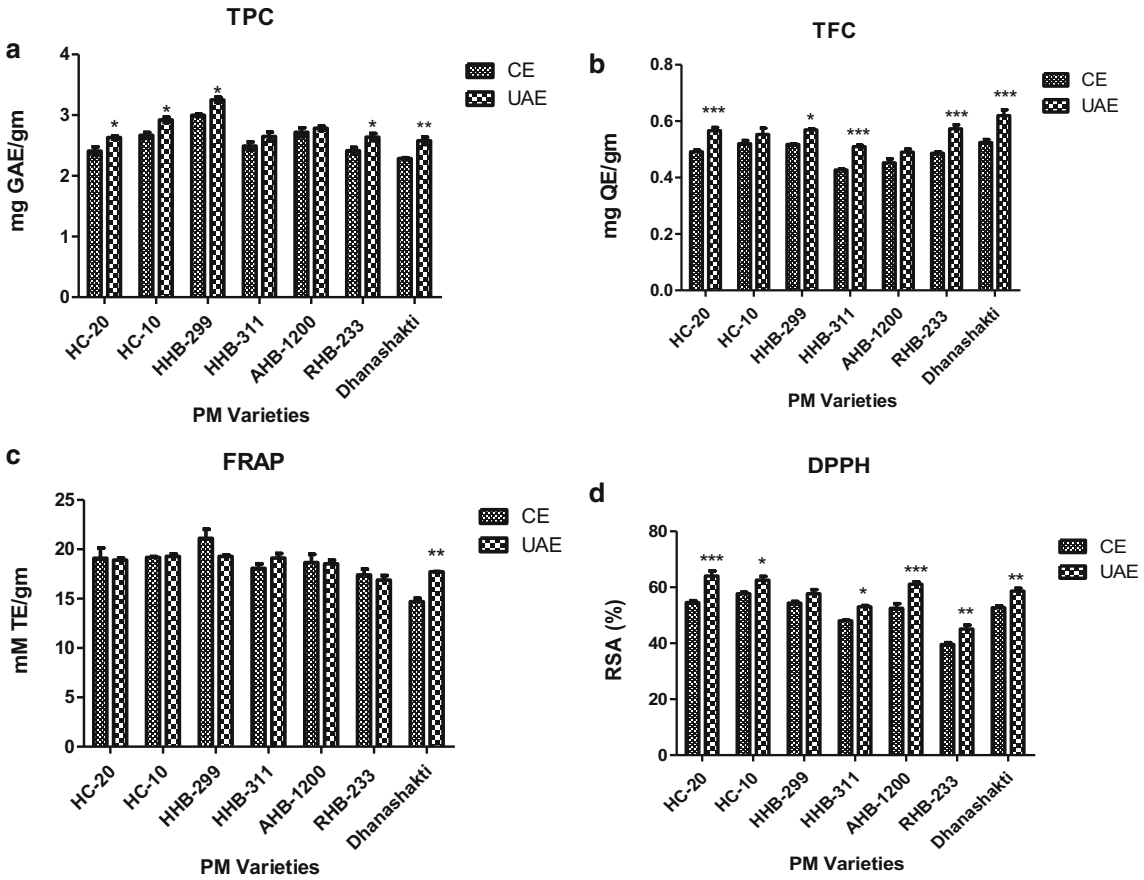

Fig. 1 Phytochemical content and antioxidant activity of methanolic extracts of non-bio-fortified and bio-fortified pearl millet cultivars using CE and UAE. Data is expressed as mean ± SD, $n = 3$. Mean values bearing *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$) are significantly different in Tukey's multiple comparison post-hoc test

Antioxidant activity

Ferric reducing antioxidant power (FRAP) activity

FRAP assay is an easy and fast method to evaluate the antioxidant potential of different food components. Results of the FRAP assay are presented in Fig. 1c. In this assay color (straw) of FRAP is reduced to blue color by electron donor bioactive component in acidic environments. Our results showed that the FRAP value of CE and UAE treated samples was 14.70–21.11 mM of TE/g and 17.72 to 19.28 mM of TE/g of flour, respectively. The highest

FRAP activity, i.e., 21.11- and 19.28-mM TE/g, was shown by the HHB-299 hybrid (CE treatment) and HC-10 variety (UAE treatment), respectively. Salar and Purewal (2017) reported FRAP activity ranging from 2.56 to 4.75 mM Fe^{2+}/g of sample in twelve indigenous varieties of pearl millet (such as HHB-226, HHB-197, HC-20, PUSA-605, etc). Moreover, they reported a non-significant difference in the FRAP potential of nine PM varieties. Researchers have reported that different processing methods (i.e., roasting, germination, etc.) did not significantly change the FRAP activity (Pushparaj and Urooj 2014).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging (RSA) ability

The results of the DPPH assay are presented in Fig. 1d. RSA of CE extracted PM samples was 39.55–57.71%; whereas, UAE samples showed 45.10–64.00% RSA. A decent quantity of RSA was shown by samples extracted with the UAE method, which may be due to the higher leaching of polyphenols by UAE treatment owing to effective breakage of the cell wall of the grain. Minimum RSA was found in the RHB-233 variety for CE and UAE extracts. Our results are well supported by the findings of Salar and Purewal (2017), who reported DPPH inhibition activity of the twelve indigenous varieties of pearl millet (Indian origin) (such as HHB-226, HHB-197, HC-20, PUSA-605, etc.) in between 22.71 and 54.90% RSA. Another study has reported 49.95% DPPH RSA in the raw pearl millet samples (Owheruo et al. 2019).

Anti-nutritional factors

Phytic acid (PA)

Phytic acid has the property to chelate cations like iron, zinc, calcium, and magnesium, ultimately reducing their bioavailability; it is considered as one of the most effective and critical anti-nutrients in foods, causing mineral deficiency in human and animal foods (Samtiya et al. 2020). Figure 2a shows the PA content of seven PM varieties used in this study. The PA content of the studied varieties was found to range from 24.88 ± 7.16 to 32.56 ± 1.29 mg/g of flour samples. Results clearly showed that there was not any significant difference ($p < 0.05$) between the PA content of all seven varieties. The highest PA content was observed in the HC-20 variety, whereas the lowest PA was found in the HC-10. The PA content of different pearl millet has been reported to be 17.72 mg/g (Owheruo et al. 2019), 9.9 mg/g (Pushparaj and Urooj 2014) and 10.76 mg/g (El Hag et al. 2002). Our results showed a high amount of PA content, possibly due to the different agro-climatic conditions, different primary processing methods, and quantification protocol.

Tannin content

Tannin is a type of anti-nutrient that mainly affects the digestibility of proteins and decreases essential amino acids (EAAs) by forming tannin–protein complexes between the carbonyl group of proteins and hydroxyl group tannins (Samtiya et al. 2020). Figure 2b represents the tannin content of PM varieties used in the present study. Our results showed that tannin content in the PM ranged from 3.07 ± 0.03 to 4.35 ± 0.64 mg TA/g of flour. Results showed no significant ($p < 0.05$) difference noticed in the tannin content of all seven varieties. The highest tannin content was found in the AHB-1200, whereas the lowest was measured in the HC-10 variety. Our results are in agreement with Singh and co-workers, who reported 4.59 and 3.01 mg/g of tannin in the pearl millet and finger millet varieties, respectively (Singh et al. 2017). Other studies have found tannin content to be between 2.3 and 2.1 mg/g in the indigenous pearl millet (Maharashtra Rabi Bajra and Kalukombu) and 1.64 mg/g in finger millet (Nigeria), respectively (Pushparaj and Urooj 2014; Owheruo et al. 2019). Again, the probable reason for high tannin content in our studies varieties and hybrids might be attributed to differences in agro-climatic conditions, different primary processing methods, and quantification protocols.

Oxalate content

Oxalate is one of the critical anti-nutrients that hinder calcium's bioavailability mainly by making complex. Figure 2c represents the oxalate content of PM varieties explored in the present study. PM flour contained oxalate ranging from 0.33 ± 0.01 to 0.43 ± 0.01 mg/g of flour. The highest oxalate content was found in the AHB-1200 biofortified hybrid, whereas the lowest was in the HC-20. Our results are in parallel with Suma and Urooj (2014) and Amalraj and Pius (2015), who reported 0.32 to 0.36 mg/g and 0.2 mg/g oxalate in their pearl millet samples, respectively. Among all varieties studied, oxalate content was significantly different ($p < 0.05$) only between AHB-1200 and HC-20.

Anti-nutrients mainly hinder the bioavailability of minerals (such as iron, zinc, calcium, etc.); our study shows a non-significant difference in the anti-nutrients content in almost all the varieties/hybrids studied. So, we will select varieties based on their minerals, fatty acids, and phenolic content to further make a suitable functional food.

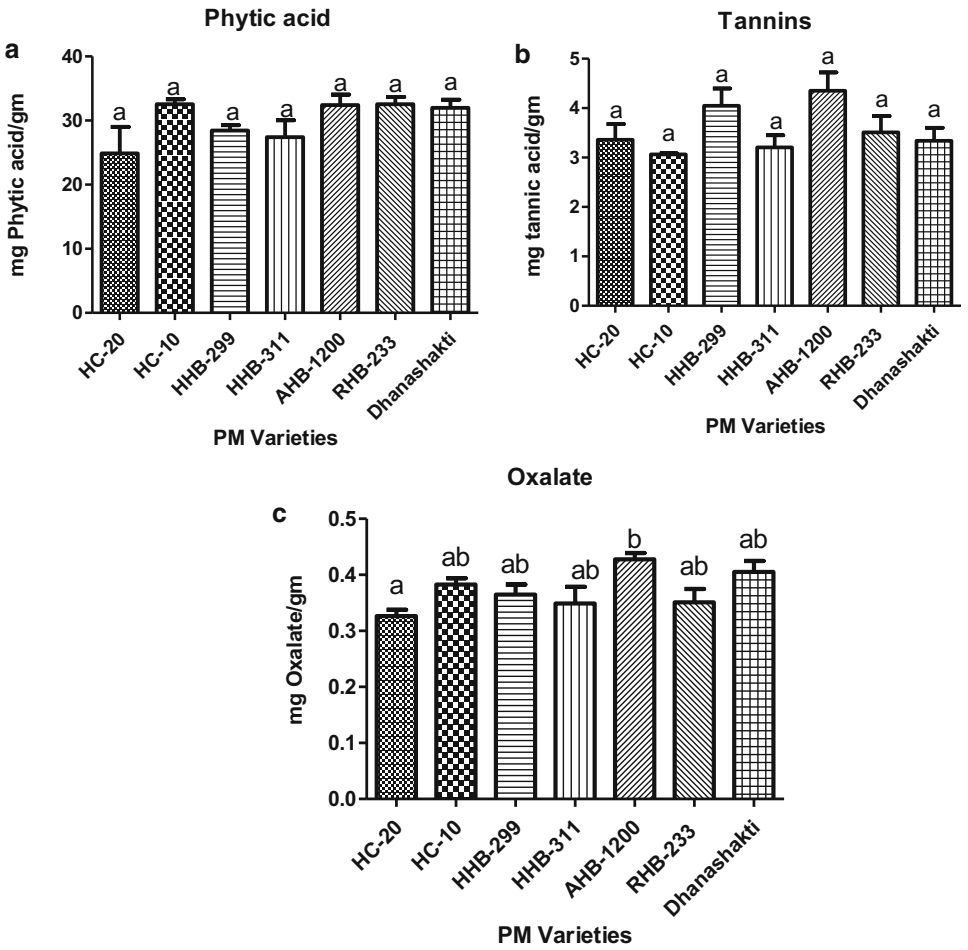

Fig. 2 Anti-nutritional factors in non-bio-fortified and bio-fortified pearl millet cultivars. Data is expressed as mean ± SD, $n = 3$. Mean values bearing different superscript letters (a, b, c, d) are significantly different at $p < 0.05$ in Tukey's multiple comparison post-hoc test

Quantification of phenolics

Pearl millet contains a diverse and ample quantity of phenolic compounds. Among some polyphenols quantified in this study, three phenolic compounds have been found to be present in the higher quantity in the seven PM varieties. Supplementary file S1 represents PM varieties' HPLC chromatograms of phenolics standard and one representative sample (HHB-299), and gallic acid, caffeic acid, and quercetin content of all varieties. The gallic acid, caffeic acid, and quercetin content ranged from 89.81 to 130.09 mg/100 g, 1.37 to 4.20 mg/100 g, and 41.85 to 57.39 mg/100 g, respectively. Biofortified hybrid, HHB-299 contained the highest amount of gallic acid, whereas the lowest was found in the RHB-233 hybrid. HC-10 had the lowest quantity of caffeic acid. AHB-1200 has the highest level of quercetin, while RHB-233 has the lowest quercetin content. Despite our best efforts with the literature search, we could not find a study to compare these results. This study is the first to quantify and report different types of polyphenols in the selected bio-fortified hybrids and bio-fortified variety, Dhanashakti. The results suggested that the HHB-299 could be an excellent candidate to formulate various RTE/RTC based functional food products, and the RHB-233 hybrid should not be the choice for the bio-fortified PM-based functional foods.

Fatty acid profiling

Our study results showed that PM varieties have suitable lipid concentrations. Saturated fatty acids like palmitic acid and stearic acid and polyunsaturated fatty acids like linoleic acid are the primary fatty acids creating the main portion of PM oil (Jukanti et al. 2016). In the present study, seven different fatty acids were identified in the PM lipid/fat: palmitic acid, oleic acid, stearic acid, linoleic acid, α-linolenic acid, arachidonic acid, and behenic acid. Table 2 represents the data of these seven fatty acids in percentage. Figure 3 shows the chromatograms of the three varieties (AHB-1200, RHB-233 and Dhanashakti) having rich essential fatty acid content. Supplementary file S1 represents the retention time (min) data for fatty acids of all varieties. The range of palmitic acid, oleic acid, stearic acid, linoleic acid, and α-linolenic acid were 18.75–

20.89%, 26.31–31.48%, 3.63–5.80%, 40.81–44.74%, and 1.88–2.22%, respectively. The other two fatty acids, arachidonic acid and behenic acid ranged from 0.89 to 1.18% and 0.25 to 0.34%, respectively. HHB-299 hybrid showed the highest oleic acid (31.48%), and HC-10 showed the highest palmitic acid (20.89%), whereas the highest linoleic acid (44.74%) and α-linolenic acid (2.22%) were found in AHB-1200 and RHB-233 varieties, respectively. The study suggested that Dhanashakti, AHB-1200, RHB-233, and HHB-311 have a rich amount of PUFAs (linoleic acid and α-linolenic acid), and HHB-299 has a rich source of oleic acid, making them suitable for consumption by growth retarded individuals, mainly children. Linoleic acid and α-linolenic acid are two essential fatty acids that cannot be synthesized by human de novo, so these must be taken from food. A recent study by Tomar et al. (2021) have also reported fatty acids present in the indigenous PM variety which is in concurrence with our results. The lipids of PM are the immense source of fat-soluble vitamins such as vitamins K, E, D, and A and a rich source of essential fatty acids. Tomar et al. (2021) reported that PM varieties mainly contains nearly 25% of saturated fatty acids (SFAs), almost 50% of PUFA, and about 25% monounsaturated fatty acids (MUFA). Furthermore, oleic acid alone has been reported to make up approximately 25% of MUFA and linoleic acid make up nearly 45% of the PUFA (Jukanti et al. 2016). Oleic acid can lower the hydrolysis of starches eventually playing a vital function of hypoglycaemic ability in the PM. Hence, the presence of rich oleic acid content in the PM lipids may play a significant role in the diet of people with hypoglycaemic concerns (Annor et al. 2015).

Our result is in concurrence with Slama and co-workers, who reported that pearl millet oil had a good amount of essential fatty acids, linoleic acid (47.5% ω-6 fatty acid18:2) and linolenic acid (2.15% ω-3 fatty acid18:3) (Slama et al. 2020). It has also been reported that a diet rich in polyunsaturated acids (PUFA) can provide ample benefits such as regulation of immune functions, maintaining blood cholesterol levels, and enhancing the high-density lipoproteins' fluidity. Consequently, rich PUFA fractions of the diet may be vital for the active transport molecules, enzymes functions, receptors, and structure and functions of several essential membrane proteins (Yaqoob 2002; Mehmood et al. 2008). Similarly, another study by Jellum and Powell (1971) has supported our fatty acids profiling results wherein different pearl millet varieties were shown to have 16.7–25.0% palmitic acid, 1.8–8.0% stearic acid, 40.3–51.7% linoleic acid.

Mineral composition

Pearl Millet has rich composition of minerals such as calcium, phosphorus, magnesium, manganese, potassium, zinc, and iron. Moreover, these minerals play several crucial functions, such as minerals acting as cofactors for many enzymes, modulating/controlling cell signaling, metabolic pathways, and immune functions in the human body (Krishnan and Meera 2018; Gharibzahedi and Jafari 2017). Our study results showed that PM varieties have favorable minerals composition. Seven micronutrients (i.e., Fe, Zn, Mg, Mn, Na, K, and Ca) were quantified in the present study. Table 3 represents the data of micronutrients in mg/kg of pearl millet. Results showed that in the selected varieties of PM, the Fe, Zn, Ca, Mg, Na, K and Mn ranged from 47.10 to 87.79, 38.37–55.05, 404–919.1, 1035–1716, 574.9–911.6, 2757–5205 and 6.69–12.45 mg/kg, respectively. The co-relation coefficient (r^2) value for the Fe and Zn standards were 0.998 and 0.999, respectively. Dhanashakti variety had the highest Mg, Ca, K, and

Table 2 Fatty acids profiling of non-bio-fortified and bio-fortified pearl millet cultivars by GC-FID

	Saturated fatty acids				MUFA	PUFA	
PM Varieties	Palmitic acid (%)	Stearic acid (%)	Arachidonic Acid (%)	Behenic acid (%)	Oleic acid (%)	Linoleic acid (%)	α-Linolenic acid (%)
HC-20	19.39	5.80	1.11	0.29	27.92	43.38	2.11
HC-10	20.89	5.39	1.18	0.34	29.44	40.81	1.95
HHB-299	18.75	5.42	1.13	0.32	31.48	41.03	1.88
HHB-311	19.66	5.02	1.10	0.31	28.79	43.09	2.04
AHB-1200	20.63	3.63	0.89	0.29	27.41	44.74	2.14
RHB-233	20.53	4.37	0.94	0.28	26.31	44.67	2.22
Dhanashakti	20.03	4.14	0.93	0.25	29.24	43.39	2.01

MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid

Values are presented as % of fatty acids in total % of lipids

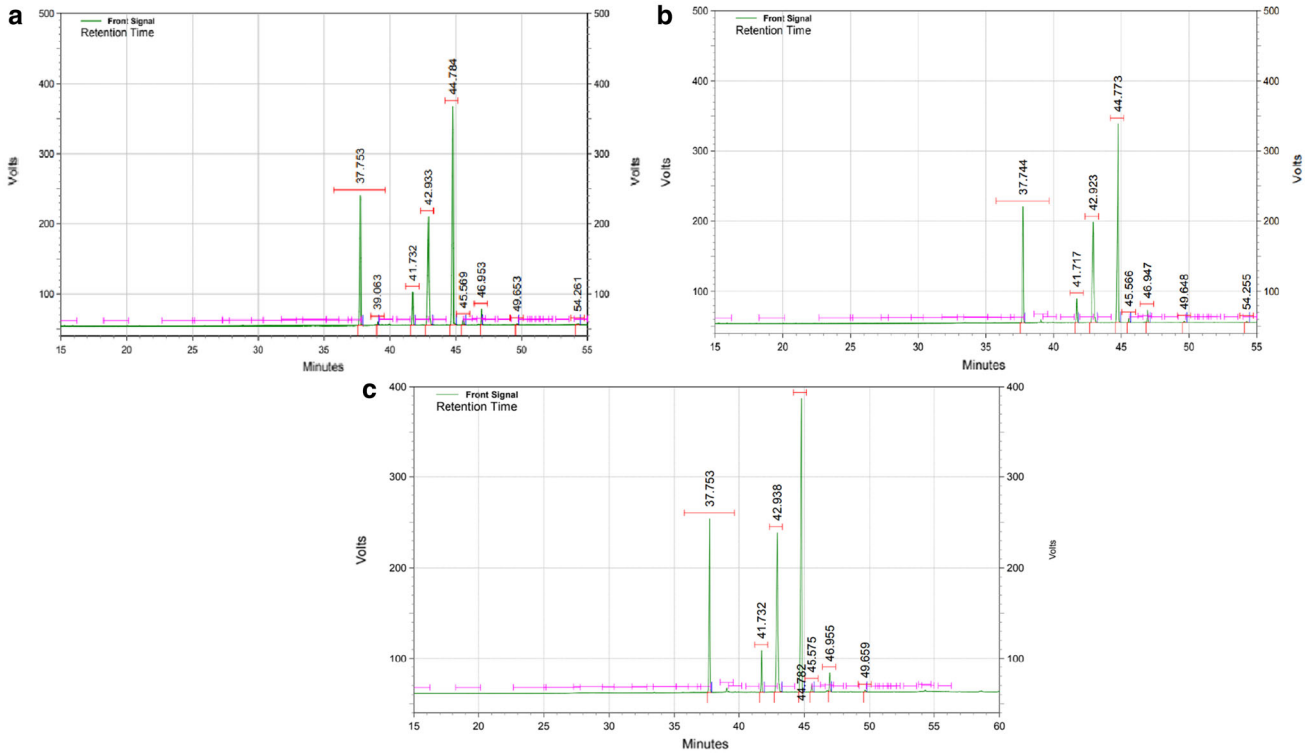

Fig. 3 Chromatograms of fatty acid analysis. **a** RHB-233, **b** AHB-1200 and **c** Dhanashakti

Table 3 Minerals composition of non-bio-fortified and bio-fortified pearl millet cultivars by ICP-OES

PM Varieties	Iron (mg/kg)	Zinc (mg/kg)	Calcium (mg/kg)	Magnesium (mg/kg)	Sodium (mg/kg)	Potassium (mg/kg)	Manganese (mg/kg)
HC-20	47.10	52.80	434.1	1330	749.9	3712	11.10
HC-10	54.88	38.37	464	1223	767.4	3183	6.69
HHB-299	77.71	50.76	436	1462	911.6	4210	8.02
HHB-311	87.79	55.05	564	1035	606	2757	9.78
AHB-1200	67.53	51.77	692.7	1419	574.9	4147	11.18
RHB-233	73.54	52.47	404	1704	874.7	3642	11.79
Dhanashakti	84.26	52.43	919.1	1716	793	5205	12.45

Mn; whereas HHB-299 contained the highest sodium (Na). Highest Fe and Zn content were found in the bio-fortified variety (Dhanashakti) and bio-fortified hybrids (AHB-1200, HHB-299, HHB-311, and RHB-233) compared to the indigenous varieties (HC-10 and HC-20). In the present study, high Fe and Zn content was recorded in the HHB-311 and Dhanashakti, i.e., (87.79 and 55.05 mg/kg) and (84.26 and 52.43 mg/kg), respectively. Our results are in agreement with the recent study by Tomar and co-workers, who have recorded iron (65.33–84.22 ppm), zinc (28.71–58.64 ppm), Ca (234.62–405.59 ppm), Mg (1635.23–2386.71 ppm), K (2495.57–4994.72 ppm), and Mn (8.21–12.57 ppm) in the different clusters of indigenous pearl millet germplasm (Tomar et al. 2021). Calcium and magnesium are vital minerals, and both have significant role in the bone formation, neuronal activities, and regulation of the internal cell environment. However, sodium controls the transmission of neural signalling, pH, contraction of muscle as well as regulation of the cellular fluid quantity. Our results evince that the bio-fortified hybrid, HHB-311, could be considered to formulate various iron-rich functional food products. These products could help tackle the crucial anaemia problem in the country by

simply supplementing/adopting this hybrid and taking care of its anti-nutritional factors, especially the phytate.

Conclusion

The present study showed that the Indian PM non-biofortified varieties, biofortified hybrids, and biofortified variety are rich in polyphenols, antioxidants, micronutrients (especially iron and zinc), and fatty acids (mainly essential); however, they also contain a significant amount of anti-nutritional factors (i.e., phytate, tannins, and oxalate). Biofortified hybrid, HHB-299, showed the highest TPC and FRAP, while biofortified variety, Dhanashakti, showed the highest TFC. Dhanashakti has a rich amount of carbohydrates and possesses a notably high concentration of omega-6 fatty acid, linoleic acid, and the omega-9 fatty acid, oleic acid. HHB-299 variety contained the highest amount of gallic acid and oleic acid among all the varieties. AHB-1200 has the highest amount of linoleic acid and quercetin among all the varieties tested. Dhanashakti variety had the highest Mg, Ca, K, and Mn; whereas, HHB-299 contained the highest sodium content. In the present study, high iron (87.79 and 84.26 mg/kg) and zinc (55.05 and 52.43 mg/kg) content were recorded in the HHB-311 and Dhanashakti, respectively. A non-significant difference was found in all the varieties' tannin and phytic acid content. Comparative data of our study suggested that the HHB-299, HHB-311, and Dhanashakti have rich nutritional profiles among the biofortified variety and bio-fortified hybrids of the pearl millet. Due to their rich fatty acid composition and nutritional attributes, these three varieties should be the choice to formulate various processed functional food products.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13197-022-05452-x.

Acknowledgements Authors are grateful to the Vice Chancellor, National Institute of Food Technology Entrepreneurship and Management, Kundli, Sonipat for providing necessary support and facilities to carry out the research project work.

Author's Contributions MS: Investigation, Formal analysis, Data curation, Validation, Writing—original draft. GAC: Methodology, Resources, Writing—review & editing. TD: Conceptualization, Resources, Methodology, Data curation, Writing—review & editing. RS: Resources, Formal analysis, Writing—review & editing. AK: Formal analysis, Validation, Writing—review & editing. AK: Data curation, Writing—review & editing. PCB: Conceptualization, Resources, Methodology, Data curation, Project administration, Supervision, Writing—review & editing.

Funding The author are thankful to the *Haryana State Council* for *Science Innovation and Technology* India, for funding this research work and the project (HSCSIT/R&D/2021/461).

Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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RESEARCH

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Characterization of indigenous lactobacilli from dairy fermented foods of Haryana as potential probiotics utilizing multiple attribute decision-making approach

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Abstract

The interest in region-specific ethnic fermented foods and their functional microbiota is rising. The demands for functional foods are continuously rising, so research is going on to develop nutritious food with many beneficial attributes and low safety concerns. The present study was designed to isolate and characterize lactobacilli probiotic candidates from locally resourced fermented foods (*dahi*, *lassi*, and *raabadi*) to make ready-to-eat fermented functional products later. Cultures were isolated from 82 fermented food samples collected from different villages. The initial experiments of gram staining, catalase test, and carbohydrate fermentation were assessed for the morphology, purity, and primary characterization on the genus level, which was verified through molecular characterization using PCR. Seven lactobacilli strains (no. MS001-MS007) were then assessed for safety, probiotic candidacy, phytase degradation, and biofilm forming abilities. All seven bacterial cultures showed no hemolytic activity and antibiotic sensitivity against more than 14 antibiotics out of 20. All seven lactobacilli isolates were able to tolerate pH 3.0, 0.3% bile 0.5% pancreatin, lysozyme (100 mg/L to 300 mg/L) and also shown possessed phytase degradation ability. All the cultures showed antioxidative potential and biofilm formation ability. Culture MS007 showed considerably higher bile salt hydrolase activity among all the isolates, whereas MS005 possessed excellent phytate degradation ability among others. Bacterial strains were identified using 16S rRNA gene sequencing. Moreover, the order of preference of isolates was calculated using the multidimensional Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) based on probiotic and other functional properties. The most promising attributes showing cultures were recognised as *Limosilactobacillus fermentum* MS005 and *Lactiplantibacillus plantarum* MS007, which could be further used for functional food product development.

Keywords Probiotics, *Lactobacillus*, Antioxidative attributes, Biofilm formation, TOPSIS, 16S rRNA sequencing

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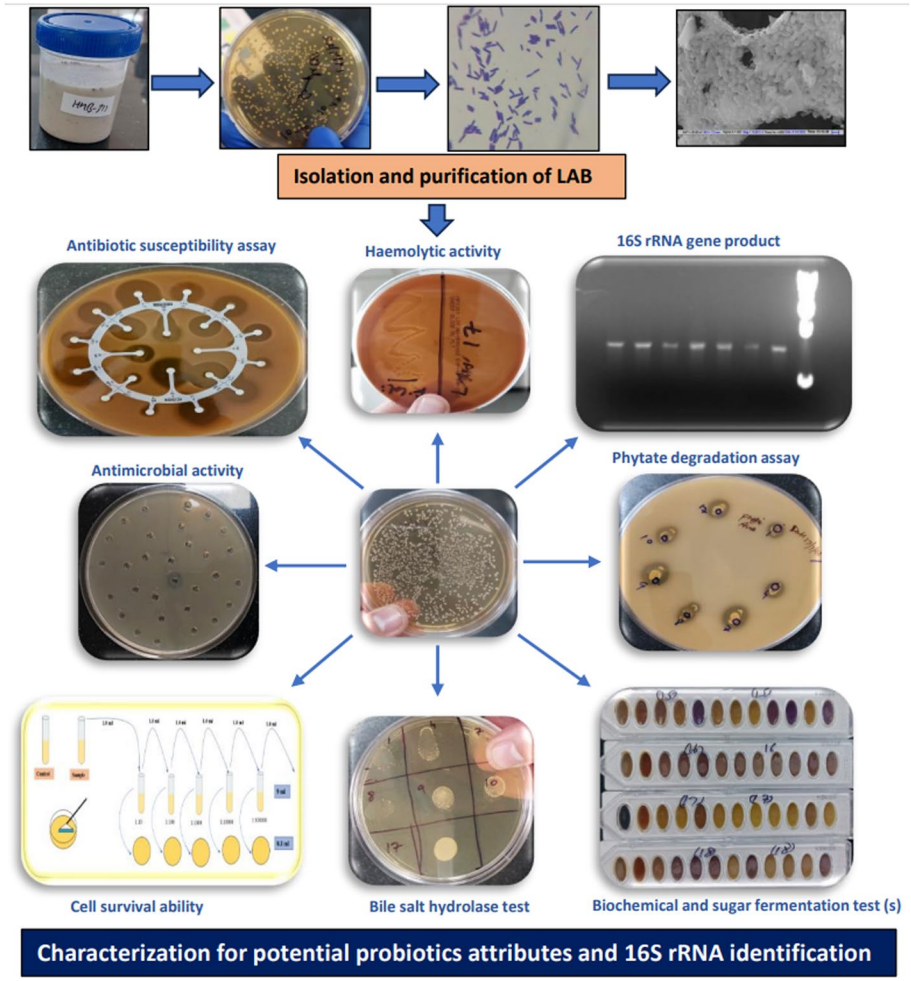
Graphical Abstract



Introduction

Probiotics are 'live microorganisms which, when administered in adequate amount confer health benefits on the host' (FAO/WHO 2002). Several sources, such as vegetables, rotten fruits, sausages, bovine and human faecal samples, mother's milk, fermented foods, etc., have been used to isolate and characterize probiotics cultures (Bhat et al. 2017). Among other lactic acid bacteria (LAB), lactobacilli are the prominent microorganisms used in dairy based fermented probiotic products as starters or adjunct cultures. Due to their non-pathogenic nature, most of them are classified as "generally recognised as safe" (GRAS) organisms (Kumari et al. 2022). Due to probiotics' diverse inherent health-enhancing properties, researchers' inquisitiveness has enhanced for isolating the novel strains having potential functional properties (Bhat & Bajaj 2019; Samtiya et al. 2022). The *Lactobacillus* genus is the most prominent type of LA bacteria used as a starter culture in food fermentation (Kumari et al. 2022). Presently, autochthonous *Lactobacillus* cultures having possible probiotics attributes are being progressively used in dairy foodstuffs as a potential starter culture to retain traditional foodstuffs typicality to enhance their health-promoting properties (Kumari et al. 2022; Saliba et al. 2021). Besides exploiting the conventional technological and probiotic capabilities, the recent research on characterizing the isolates with additional phytase activity is also in demand. In context of high popularity of cereal-based foods in India, such functionality might give an extra edge to the food starters of choice. Fermentation with such strains could enhance the

bioavailability of minerals (i.e., Fe, Zn, and Ca) through phytase production and decreasing phytic acid constituents in plant foods. Pearl millet-based fermented foods can be the food of choice to isolate phytase-producing lactobacilli (Samtiya et al. 2021). For the in vitro screening of potential strains of probiotics, several parameters are recommended by the Indian Council of Medical Research and Department of Biotechnology (ICMR-DBT) guidelines, such as resistivity against gastric acidity, tolerance to bile acid, antimicrobial activity, bile salt hydrolase activity, and for safety evaluation of hemolytic potential and antibiotic resistance patterns (Ganguly et al. 2011). Several other parameters, such as biofilm formation capacity, auto-aggregation ability, lysozyme tolerance, cell surface hydrophobicity, antioxidative capacity, phytase activity, ethanol tolerance, etc., could also be used for the evaluation of probiotic potentials (Bhushan et al. 2021; Pradhan & Tamang 2021; Shivangi et al. 2020). A scarcity has been seen in reports on isolating and characterizing safe probiotic lactobacilli starters with additional potential of reducing anti-nutrients. So, making a cereal-based fermented product with improved micronutrient bioavailability could be a promising approach that can help mitigate micronutrient deficiencies. The potential probiotic strains capable of reducing anti-nutrients and thereby enhancing micronutrients (Fe and Zn) are the need of the hour.

Considering the attention to the reports mentioned above, we designed a hypothesis and identified gaps in knowledge. The present research included the isolation of lactobacilli from fermented food sources (*dahi, lassi,* and *raabadi*) in rural Haryana and their *in-vitro* screening for safety (antibiotic sensitivity and haemolytic activity) and probiotic candidacy (cell survival capability, auto-aggregation, hydrophobicity, biofilm formation, antioxidative potentials, bile salt hydrolase activity, and, most notably, phytate degradation ability) along with the assessment of fermentation/technological superiority (NaCl and ethanol tolerance) of selected lactobacilli isolates. Furthermore, the current study underscored the importance of the multidimensional ranking technique (TOPSIS) in identifying phytate-degrading isolates with probiotic properties.

Materials and Methods

Chemicals/reagents/kits and microbial strains

Crystal violet, xylene, and ethanol were procured from Fisher Scientific International (Mumbai, India) 0.2,2-Diphenyl-1-picrylhydrazyl (DPPH), taurodeoxycholate, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and phytic acid, were procured from Sigma-Aldrich (Bangalore, India). Phosphate buffer saline, pancreatin, bile, lysozyme, NaCl, MRS broth, MRS agar, nutrient agar, nutrient broth, glycerol, gram's staining kit, negative staining kit, HiLacto test kit, HiPurA Genomic DNA Purification Kit, antibiotic disc ring (IC006, Icosa Universal-2), chloroform, ethyl acetate, n-hexadecane and MOPS buffer were procured from Hi-media (Mumbai, India). Only ACS/analytical grade chemicals and solvents were used in this investigation.

The indicator bacterial strains (*E. coli* ATCC 11229, *Listeria monocytogenes*, *Bacillus cereus* NCDC250, and *Staphylococcus aureus* NCDC109) were kindly gifted by Prof. Vijendra Mishra, Food Microbiology lab, NIFTEM, India.

Collection and processing of samples

The 82 fermented food samples (*dahi, lassi,* and *raabadi*) were collected from native households of rural Haryana in sterile sample containers (100 mL, Tarsons, Kolkata, India) and kept at 4 °C until delivered to the laboratory. The recording of sample pH was followed by serial dilution (0.85% saline), pour plating on MRS (de Mann Rogosa Sharpe) agar, and incubation for the next 24–48 h in aerobiosis. LAB colonies with diverse morphologies on the MRS agar plate were carefully chosen and further purified by repeated streaking to obtain a pure colony. The pure LAB cultures were preserved in glycerol stock (35% v/v) at − 80 °C for further use (Dhewa et al. 2010).

Morphological and biochemical characterisation of isolates

Isolate's primary identification was performed through culture characteristics, microscopic observations (gram's staining and negative staining), and colony morphology. Out of 120 isolated colonies, Gram's staining reduced the number of isolates to 20, which were further subjected to the biochemical analysis and carbohydrate fermentation pattern (Catalase, Esculin hydrolysis, Xylose, Celliobiose, Arabinose, Maltose, Galactose, Mannose, Mellibiose, Raffinose, Sucrose, and Trehalose). The test was carried out using the surface inoculation method to inoculate 50 µL of the test inoculum into each HiLacto test biochemical test kit well, followed by a 24-h incubation period at 37 °C.

Molecular identification of isolates for genus confirmation

Isolates showing gram's positive, catalase-negative, and rod-shaped nature were further considered for the PCR-based molecular identification for genus confirmation. Genomic DNA was collected for PCR using HiPurA genomic DNA purification Kit. DNA primers (amplicon length 250 bp) were used for the PCR (forward: CTCAAAACTAAACAAAGTTTC and reverse: CTTGTACACACCGCCCGTCA) to target the 16S–23S rRNA intergenic spacer region for *Lactobacillus*

genus identification (Dubernet et al. 2002). Amplification of DNA was carried out in a thermal cycler (CFX96™, Bio-Rad, California, United States); PCR program was as follows: denaturation at 95 ℃ for 5 min, followed by 35 cycles consisting of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, extension at 72 ℃ for 30 s, and a 7 min final extension step at 72 ℃. Gel electrophoresis was performed using aliquots (10 µL) of amplified products and a 50 bp marker on 1% agarose gel (Electrophoresis grade, Invitrogen, UK) in the Tris–Acetate EDTA (TAE) buffer. Gel was stained with ethidium bromide and examined using a gel-doc system (GelDoc Go, Bio-Rad, California, United States).

Strains identification of LAB isolates by 16S rRNA gene sequencing

The 16S rRNA sequencing technique was used for the strain's identification of LAB isolates. 16S rDNA gene PCR amplification was performed for all the seven LAB isolates using universal primers, the forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). Aliquots (10 µL) of the amplified products and 1000 bp marker were used for gel electrophoresis in 1% agarose gel (Electrophoresis grade, Invitrogen) in TAE buffer. Gel was stained with ethidium bromide and visualised under gel-doc system (GelDoc Go, Bio-Rad, California, United States). After confirming the correct amplification, the amplified PCR product was outsourced to Eurofins Genomics India Pvt., Ltd (Bengaluru, India) for purification and sequencing of 16S rRNA. Further, to identify the strains of LAB isolates, obtained sequences were evaluated by comparing with bacterial references, which are already available in GenBank database of NCBI (National Centre for Biotechnological Information) using BLAST search program with > 98% of DNA homology threshold.

Assessment of probiotic attributes (in vitro) of isolates

For the in vitro screening of potential strains of probiotics, parameters recommended by the Indian Council of Medical Research and Department of Biotechnology (ICMR-DBT) guidelines for safety evaluation of hemolytic potential and antibiotic resistance patterns and other potential probiotic attributes (Ganguly et al. 2011), were utilized for assessment in the current study.

Safety assessment

Haemolytic activity

Haemolytic activity of LAB isolates was determined by using blood agar plates (Himedia), following Bhushan et al. (2017). All the blood agar plates were streaked using active LAB isolates, followed by incubation at 37 ℃ for 48 h in a BOD incubator. After incubation, streaked plates were observed for haemolysis activity. Results were observed as γ-hemolysis (no zones or change), β-hemolysis (zones are lightened –yellow or transparent), and α-hemolysis (zone are greenish and dark).

Antibiotic susceptibility

All LAB isolates were evaluated using the Bauer-Kirby disk diffusion protocol with antibiotic disks. The antibiotic susceptibility was performed using methods of Bhushan et al. (2021) and Ahire et al. (2021), with some modifications. The soft MRS agar (0.8%, w/v) was inoculated with overnight-grown active LAB isolates, followed by pouring soft agar on preformed MRS agar (1.6% w/v) plates (200 mm, Himedia) and kept for 1 h in the laminar cabinet for proper drying. The soft agar surface was then covered with antibiotic discs, which were then incubated for 24 h at 37 °C. Results were analysed using breakpoint guidelines of the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek et al. 2014; CLSI 2023).

Cell survival in oral, gastric, and intestinal conditions in vitro

Tolerances to lysozyme, hydrochloric acid, bile and pancreatin

Tolerances of isolates to lysozyme (100–300 mg/L), HCl (pH 3.0), bile (0.3%, w/v), and pancreatin (0.5%, w/v) in MRS broth were tested as previously suggested protocols (Bhushan et al. 2017; Nath et al. 2021; Pradhan & Tamang 2021; Shivangi et al. 2020) with some modifications. For each of the tests, active LAB isolates (grown for 18 h in MRS broth) were harvested by centrifugation (8000 *g* for 5 min at 4℃), washed, and resuspended in phosphate buffer saline (PBS, pH 7.2). The control suspensions contained pH 6.8 to 7.0 and were not added to the test compounds. The incubation for all assays was done in shaking, after each incubation, the viability of the isolates was evaluated using MRS agar count plate method. The below-given formula was used to assess the survival percentages (%):

$$\text{Cells Survival \%} \frac{\text{Log value of cells survived in CFU/mL}}{\text{Log value of initial cells inoculated in CFU/mL}} \times 100$$

In-vitro tests for cell adhesion to intestinal walls

Auto-aggregation ability

Auto-aggregation ability of LAB isolates was determined following the method of Ahire et al. (2021) with some modifications. Overnight (16 h) grown active LAB cultures were harvested by centrifugation at 11,000 g for 10 min at 4 °C, followed by washing of cell pellet twice using PBS (pH 7.2), and OD_{600} was set as~ 0.8 by diluting. Three mL of culture suspensions were taken in test tubes, vortexed moderately for 30 s, and then incubated at 37 ℃. The upper aqueous phase of samples was taken carefully after 3 h, and after 24 h of incubation, OD_{600} readings were taken. The auto-aggregation ability of LAB cultures is presented in percentage using the below-given formula:

$$\text{Auto}-\text{aggregation } \% = \frac{OD_{600} \text{ of bacterial suspension} - OD_{600} \text{ of upper suspension}}{OD_{600} \text{ of bacterial suspension}} \times 100$$

Cell surface hydrophobicity

The isolates' ability to adhere to organic solvents was determined using the Microbial Adhesion to Hydrocarbon (MATH) assay using the method of Ahire et al. (2013) and Shangpliang et al. (2017) with some modifications. Xylene (non-polar solvent), ethyl acetate (polar solvent), n-Hexadecane (non-polar solvent), and chloroform (monopolar acidic solvent) were used as test solvents. Experiment was done as mentioned for auto-aggregation assay, except aqueous phase OD was taken instead of upper phase suspension. The percentage of hydrophobicity or microbial adhesion of LAB cultures was calculated using the below-given formula:

$$\text{Microbial adhesion } \% = \frac{OD_{600}\ (A) - OD_{600}\ (B)}{OD_{600}\ (A)} \times 100$$

Where, OD_{600}(A) and OD_{600}(B) are the aqueous phase absorbance (before and after addition with solvents).

Biofilm formation property

Biofilm formation ability of LAB isolates was determined according to the method of Bhardwaj et al. (2021) with some modifications. Overnight grown (18 h) active LAB isolates OD_{600} was adjusted to 0.1 (0.5 McFarland standard), and 100 µL cell suspension (MRS broth) was poured in 96-well of polystyrene plates (Greiner Bio-One, Kremsmünster, Austria). With a pipette, the planktonic cells were carefully removed from the wells after incubation, and non-adherent cells were eliminated by washing them with PBS (pH 7.2). After that, crystal violet (1% (w/v)) was added and incubated for 30 min at room temperature. The remaining color was removed from the wells following washing with PBS. At final step, 100 µL of pure ethanol was filled in all the wells and absorbance was measured on OD_{540} nm using a multimode microplate reader (Molecular Devices, USA) after 5 min. Moreover, all other plates were processed using the same protocol for the final absorbance. 100 µL of MRS broth was used as a control for this experiment.

Functional attributes

Tolerance to technological stresses

The tolerance of LAB isolates to ethanol (4, 6, 8, and 10%, v/v) and sodium chloride [(NaCl) 1, 2, 4, and 6%, w/v] concentrations were evaluated using the method of Gold et al. (1992) with slight modifications. The active MRS cell suspensions and controls were prepared as described previously. Each inoculated MRS test broth was incubated at 37 ℃ in a BOD incubator for 24 h, and then OD_{600} nm readings were recorded to assess the tolerance.

Enzymatic activity of isolates

Bile salt hydrolase (BSH) activity With a few adjustments, Ahire et al. (2021), methodology was used to determine the BSH capacity of isolated cultures. On MRS agar medium containing 0.37 g/L $CaCl_2$ and 0.5% (w/v) sodium taurodeoxycholate, overnight grown active cultures were spot inoculated (10 µL). The presence of hydrolysed salt precipitation surrounding the colonies directed BSH activity.

Phytate degradation ability The qualitative phytase activity test for LAB isolate's anti-nutrient degrading characteristics was assessed by plate screening assay according to Pradhan and Tamang (2021), with some modifications. Spot inoculations (10 µL) of the overnight-grown active cultures were made on modified MRS agar, and they were then incubated at 37 °C for 72 h. The appearance of clear zones around the spot-inoculated culture (caused by phytic acid breakdown) was recorded as a positive result.

Antioxidative properties

Preparation of intracellular cell-free extract (CFE) and intact cells (IC)

Overnight grown (18 h) active LAB isolates were harvested by centrifugation at 10000 *g* for 7 min at 4 ℃. For the Intracellular cell-free extract (CFE) preparation, cell pellets were washed with PBS (7.2) two times, and

cell absorbance values were adjusted at~ 0.8 at 600 nm. with the same PBS. Cell suspension was then subjected to ultrasonic extraction for 45 min using an ultrasonication unit (CPX3800H-E, Bransonic, USA). The CFE extracts were filtered using a sterile 0.22-µm syringe filter (Nupore Filtration Systems, India) and collected in sterile vials for further experiments. For intact cell (IC) preparation, cell pellets were washed with PBS (7.2) two times, cells OD_{600} was adjusted at~ 0.8 with the same PBS and used for further experiments.

DPPH and ABTS DPPH assay and ABTS assay were used to perform the antioxidative activity in cell-free extract (CFE) and intact cells (IC) of LAB isolates by following methods Samtiya et al. (2023) and Ayyash et al. (2018), respectively. IC samples were centrifuged (1 Min, 2000 g) before taking absorbance at 517 nm or 734 nm using a multimode plate reader (SpectraMax, M2e) with a cuvette port.

The DPPH free radical scavenging ability was estimated using the given formula:

$$\text{RSA } \% = \frac{OD_{517}\ (A) - OD_{517}\ (B)}{OD_{517}\ (A)} \times 100$$

Where, OD_{517}(A) and OD_{517}(B) are the control and sample absorbance after incubation, respectively.

The ABTS radical scavenging ability was estimated using the given formula:

$$\text{Scavenging } \% = \frac{OD_{734}\ (A) - OD_{734}\ (B)}{OD_{734}\ (A)} \times 100$$

Where, OD_{734}(A) and OD_{734}(B) are the control and sample absorbance after incubation, respectively.

Antimicrobial activity

Antibacterial activity of cell-free supernatant (CFS, pH 7.0) of isolates in LB was observed against the indicator using the agar-well diffusion method, according to Bhushan et al. (2021), with some modifications. Bacterial cells were centrifuged at 5000 *g* for 10 min at 4 ℃. Cell-free supernatants (CFSs) were separated, pH was corrected to 7.0 with 5 M NaOH, and filtered through a sterile 0.22-µm syringe filter (Nupore Filtration Systems, India). All the target pathogen OD_{600} was adjusted to 0.1 (0.5 McFarland standard) and used for inoculating Mueller Hinton (MH) Agar. Through sterile borer (6 mm), well were formed in the MH agar containing different targeted bacterial cultures. Following the addition of 100 µL of CFSs (pH 7.0) to the wells, the plates were incubated at 37 °C in a BOD incubator for 12 to 16 h. Acetic acid (5%) and MRS broth (filter sterilised with 0.45-µm syringe filter) were used for controls.

Morphological visualisation of selected cultures using scanning *electron* microscopy

To visualise the *L. fermentum* MS005 and *L. plantarum* MS007 morphology, Scanning Electron Microscopy (Model: EVO 18, Zeiss Pvt. Ltd., UK) was performed. Cultures were freeze-dried and outsourced (Central Research Facility, Indian Institute of Technology, Delhi) for scanning electron microscopy.

TOPSIS: Multiple attribute decision making (MADM) analysis

Multiple Attribute Decision Making (MADM) approach called as Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) was employed in the study to rank the cultures in order of probable best probiotic candidate. TOPSIS is a multi-attribute decision-making technique that delivers a ranking based on the characteristics in terms of perceived weight and satisfaction. The ranking also determines the distances to the positive ideal solution and the distances to the negative ideal solution. The best cultures will be those that are closest to the positive-ideal solution and the furthest away from the negative-ideal solution (Zavadskas et al. 2016). TOPSIS is used in various fields, such as engineering, business, biotechnology, and environmental management. It allows decision-makers to estimate the ranks of alternatives based on multiple criteria or attributes. TOPSIS uses a geometric method that associates each alternative with an ideal solution. By evaluating the closeness of each alternative to the ideal solution, TOPSIS yields a comprehensive ranking that allows decision-makers to make informed choices that balance conflicting objectives. This method provides a structured and systematic way to handle the intricacies of multi-criteria decision-making, facilitating the selection of the most suitable alternative in a transparent and quantitative manner. The method is consisting of the following steps:

Step 1: Make a decision matrix of order $m \times n$ where m is the number of alternatives and n is the number of attributes (say $A = e_{ij}$ of size $m \times n$).
Step 2: Calculate the normalized decision matrix n_{ij} by normalize the column wise data of the decision matrix by using the following equation:

$$n_{ij} = \frac{e_{ij}}{\sum_{i=1}^{m} e_{ij}} \qquad i = 1, 2, 3, \ldots, m; \quad j = 1, 2, 3, \ldots, n$$

Step 3: Calculate the information entropy h_j by using the following equation:

$$h_j = \sum_{i=1}^{m} n_{ij} \times ln(1/n_{ij})$$

Step 4; Calculate the degree of divergence as $d_j = 1 - h_j, \quad j = 1, 2, 3, \ldots, n$, and the degree of importance by using the equation

$$w_j = \frac{d_j}{\sum_{j=1}^{n} d_j} \qquad j = 1, 2, 3, \ldots, n$$

Step 5: Calculate the comprehensive weight as

$$w_j{}' = \frac{b_j \times w_j}{\sum_{j=1}^{n} b_j \times w_j}$$

where b_j represents the value of weight associated with an attribute.

Step 6: Calculate the weighted normalize matrix V_{ij} as

$$V_{ij} = n_{ij} w_j{}' \qquad i = 1, 2, 3, \ldots, m; \quad j = 1, 2, 3, \ldots, n$$

Step 7: Evaluate the positive (V^+) and negative (V^-) ideal solutions from V_{ij} matrix obtained from Step 7.

$$V^+ = \left\{V_1^+, V_2^+, \ldots, V_n^+\right\} = (\max\{V_{11}, V_{21}, \ldots, V_{m1}\}, \max\{V_{11}, V_{21}, \ldots, V_{m2}\}, \ldots, \max\{V_{11}, V_{21}, \ldots, V_{mn}\})$$

$$V^- = \left\{V_1^-, V_2^-, \ldots, V_n^-\right\} = (\min\{V_{11}, V_{21}, \ldots, V_{m1}\}, \min\{V_{11}, V_{21}, \ldots, V_{m2}\}, \ldots, \min\{V_{11}, V_{21}, \ldots, V_{mn}\})$$

Step 8: Evaluate the distance between each alternative from positive and negative ideal solution as:

$$d_i^+ = \sqrt{\sum_{j=1}^{n} (V_{ij} - V_i^+)^2}, i = 1, 2, 3, \ldots, m; \quad j = 1, 2, 3, \ldots, n$$

$$d_i^- = \sqrt{\sum_{j=1}^{n} (V_{ij} - V_i^-)^2}, i = 1, 2, 3, \ldots, m; \quad j = 1, 2, 3, \ldots, n$$

Calculate the closeness coefficient (R_i) between the alternative and ideal solution by using the following equation:

$$R_i = d_i^- / (d_i^+ + d_i^-)$$

Statistical analysis

Microsoft Office (version 2019) was used for raw data tabulation. GraphPad Prism (version 5.01) was used for grouped and column statistics, and one-way analysis of variance (ANOVA) was used for statistical analysis of data, followed by Tukey post-hoc test to separate the mean ($p \leq 0.05$), which was considered statistically significant. The p-values < 0.05 were considered to be statistically significant. The data are expressed as mean ± standard deviation of replicates.

Results and discussion

The current work employed a subtractive screening strategy to find potential probiotic strains from traditional fermented food. Before functional and technological evaluation, crucial probiotic factors such as safety and tolerance to gastrointestinal conditions were taken into account.

Origin, colour appearance, and pH of collected samples

Eighty two dairy-fermented foods (*lassi, dahi,* and *raabadi*) were collected from the different districts of Haryana. Sample source, pH range, and appearance of color are presented in Table S1. Fig. S1 represents the districts of Haryana from where dairy-fermented samples were collected in this study.

Morphological, biochemical test (s) and molecular identification

In the present study, LAB colonies were selected based on their morphology (irregular and round shape), those devoid of pigmentation, white and creamy from MRS agar plates, and have shown rod-shaped appearance in gram-positive staining and negative staining. Results of the biochemical and carbohydrate fermentation test for the 20 selected isolates (based on the preliminary character) are represented in Table 1. Fifteen LAB isolates were found catalase-negative, which were further selected

Table 1 Biochemical test (s) of the selected lactic cultures

Lactic cultures	Biochemical test												*Lactobacillus* confirmation through PCR, along with their assigned ID
	Esculin hydrolysis	Catalase	Xylose	Celliobiose	Arabinose	Maltose	Galactose	Mannose	Mellibiose	Raffinose	Sucrose	Trehalose	
1	-	-	V	V	V	V	+	+	+	+	+	V	MS001
2	+	-	+	V	+	+	+	V	+	V	-	-	
3	+	-	+	V	+	+	+	-	+	-	-	-	
4	-	-	V	-	-	-	+	V	+	+	+	V	MS002
5	-	+	-	-	-	+	V	+	-	-	+	-	
6	+	-	+	V	+	+	+	V	+	V	V	+	
7	-	-	V	-	-	-	+	V	+	+	+	**V**	MS003
8	-	-	+	V	+	+	+	V	+	+	+	+	MS004
9	-	-	+	-	+	+	+	-	+	+	+	V	MS005
10	-	-	V	V	-	-	+	V	+	+	+	V	MS006
11	-	-	-	-	-	+	+	+	V	V	+	-	
12	+	-	+	+	V	+	-	+	+	+	-	+	
13	+	+	-	V	-	V	-	V	-	-	-	-	
14	-	+	-	V	-	+	+	+	+	-	-	-	
15	-	+	V	+	-	+	+	+	V	-	+	-	
16	-	-	V	V	-	V	V	V	V	V	V	V	
17	+	-	+	+	V	+	+	+	+	+	+	+	MS007
18	-	-	+	V	V	V	+	V	+	+	+	V	
19	+	+	-	-	-	-	-	-	-	-	-	-	
20	-	-	V	-	-	+	V	+	V	-	+	V	

'-'=Negative, '+'=Positive, V=Variable'

for molecular identification at the lactobacilli genus level. PCR confirmed that seven out of 15 LAB isolates were lactobacilli. The gel electrophoretic separation of genus-specific PCR products for confirmed lactobacilli isolates (Fig. S2). In this study, the morphological and biochemical characteristics of isolated cultures resembled other reported *Lactobacillus* cultures isolated from the fermented food samples (Nath et al. 2020; Yadav et al. 2016). Selected lactobacilli isolates were given specific code numbers, i.e., MS001, MS002, MS003, MS004, MS005, MS006, and MS007 (Table 1). Further, selected lactobacilli were evaluated for their potential probiotic's attributes. All the strains were molecularly identified by 16S rRNA gene similarities (> 99%) with the existing sequence of NCBI GenBank, as presented in Table S2. A gel electrophoresis photograph of the 16S rRNA gene product of lactobacilli isolates with a marker (Fig. S3). Six isolates were identified as *Limosilactobacillus fermentum* (MS001, MS002, MS003, MS004, MS005 and MS006) and one isolate as *Lactiplantibacillus plantarum* (MS007).

Safety assessment of isolates

The safety of lactobacilli isolates was determined using a blood haemolysis assay and the antibiotic susceptibility test. Probiotic bacteria (lactobacilli) are commonly considered safe for consumption, so these strains should be non-hemolytic in nature (Peres et al. 2014). So, only those cultures should be selected for further research, which cannot lyse erythrocytes of hosts and must be confirmed for γ-haemolytic activity on a blood agar plate. Our isolates were assessed for haemolytic activity, and results confirmed that all isolates (MS001, MS002, MS003, MS004, MS005, MS006, and MS007) are safe, as they do not show any kind of haemolysis activity (γ-haemolytic) and growth on sheep blood agar plate, hence can be used for further food development. Similar observations (γ-haemolytic activity) for *Lactobacillus* strains were reported by several studies such as *L. plantarum* CS (Nwachukwu et al. 2019), *L. plantarum* BIF43, BBC32B, BBC32A, and BBC33 (Bhushan et al. 2021), LAB cultures (Wu et al. 2021), *L. plantarum* UBLP40 (isolated from fermented food) (Ahire et al. 2021), *Lactobacillus* isolates (Yadav et al. 2016), *Lactobacillus* strains (isolated from goat milk) (Saliba et al. 2021), *L. fermentum* (NMCC-27, NMCC-17, NMCC-14, and NMCC-2) (Abid et al. 2022). Antibiotic tests are primarily used to evaluate probiotic bacterial cultures; cultures must be antibiotic sensitive to avoid the spread of undesired antibiotic resistance. According to the breakpoint scale (15–19 = ZOI ≥ 20), all tested isolates demonstrated sensitivity (intermediate to highly sensitive) to the majority

Table 2 Antibiotics susceptibility pattern/assay of lactic cultures

Antibiotic class	Antibiotics	LAB isolate						
		MS001	MS002	MS003	MS004	MS005	MS006	MS007
β-lactams	Ampicillin (10 µg)	S	S	S	S	S	S	S
	Amoxicillin (10 µg)	S	S	S	S	S	S	S
	Penicillin (10 Unit)	S	S	S	S	S	S	S
	Cloxacillin (1 µg)	R	R	R	R	R	R	R
Cephalosporins	Ceftazidime (30 µg)	S	S	S	S	S	S	S
	Cefoperazone (75 µg)	S	S	S	S	S	S	S
	Cefadroxil (30 µg)	S	S	I	S	S	S	S
	Ceftriaxone (30 µg)	S	S	S	S	S	S	S
Quinolones	Norfloxacin (10 µg)	R	R	R	R	R	R	R
	Ciprofloxacin (5 µg)	S	S	S	I	S	I	R
	Nalidixic acid (10 µg)	R	R	R	R	R	R	R
Azolidiones	Nitrofurantoin (300 µg)	S	S	S	S	S	S	S
Glycopeptides	Vancomycin (30 µg)	R	I	I	I	R	I	R
Aminoglycosides	Tobramycin (10 µg)	R	I	I	I	I	R	R
	Gentamicin (10 µg)	S	S	S	S	S	S	S
	Netillin (10 µg)	S	S	S	S	S	S	S
	Amikacin (30 µg)	I	I	S	S	S	S	R
Macrolides	Erythromycin (15 µg)	S	S	S	S	S	S	S
Sulfa drug Others	Co-trimoxazole (25 µg)	R	R	R	R	R	R	R
	Chloramphenicol (30 µg)	S	S	S	S	S	S	S

R resistant (ZOI ≤ 14), *I* intermediate (ZOI 15–19), *S* sensitive (ZOI ≥ 20), Clinical and Laboratory Standards Institute (CLSI), 2023

of the tested antibiotics (Table 2). If probiotic culture contains resistance genes, then it is more possible to transfer these genes to other pathogenic strains (in the intestine) and make them resistant to antibiotics. Therefore, it is necessary to evaluate new probiotic isolates for antibiotic susceptibility before using them in foodstuffs. Results of our study exhibited that all isolates showed sensitivity (ZOI ≥ 20) or intermediate sensitivity (ZOI 15–19) for more than 14 antibiotics (β-lactams, Cephalosporins, Quinolones, Azolidiones, Glycopeptides, Aminoglycosides, Macrolides antibiotics classes), and could be considered safe for further use. Our results are supported by the findings of several previous research studies that evaluated *Lactobacillus* strains (Ahire et al. 2021; Bhushan et al. 2021; Wu et al. 2021). In agreement with the previous findings (Yadav et al. 2016; Zhou et al. 2005), our LAB isolates showed natural resistance against nalidixic acid (antibiotic). Moreover, our isolates confirmed natural resistivity against Cloxacillin, Norfloxacin, and Co-trimoxazole antibiotics, so the resistivity may benefit their insistent establishment in the gut over antibiotic treatment. Although resistivity to antibiotics (Aminoglycopeptides and Quinolones) is an inherent characteristic (Ammor et al. 2007; Hummel et al. 2007), though, we didn't notice any such resistance, hence using our strains as probiotics is safer in terms of antibiotic resistance transmission and emergence. Henceforward, there may not be any safety concerns for the consumption of lactobacilli isolated in the present study.

Cell survival in oral, gastric, and intestinal conditions in vitro

The lysozyme tolerance ability of probiotics depicts its stability in the oral cavity as lysozyme is present in human saliva. Before reaching the intestinal cavity, they must face a stressful environment of mouth (Singhal et al. 2021). In the current evaluation, isolated lactobacilli showed high survivability rates (98 to 99%, 91 to 98%, and 85 to 92%) at different lysozyme concentrations (100 mg/L, 200 mg/L, and 300 mg/L), respectively, even after 3 h of exposure (Table 3). The lowest viability was observed for MS004 isolate, i.e., 91.02% and 85.71% at 200 mg/L and 300 mg/L lysozyme, respectively. Further, results depicted that MS005 LAB isolate was shown the highest tolerance, i.e., 92.44% at 300 mg/L of lysozyme. In agreement with our observations, lysozyme tolerance at a different concentration by other lactobacilli cultures was quantified by previous studies (Bhushan et al. 2017; Bosch et al. 2012). Our strains are showing better stability at oral conditions, compared to the previous isolated lactobacilli strains from cereal-based fermented food (Yadav et al. 2016). As per the current characterisations, potential probiotics must persist in the stomach's acidic environment if they come to the small intestine and colonise the host GI tract, where they can perform their activity (Hsiung et al. 2021). Our results confirmed that the majority of lactobacilli isolates showed high acid tolerance (between 85 to 97%) at pH 3.0; out of seven lactobacilli, MS005 and MS007 showed 97% of survivability, which denotes that these strains have good tolerance against acidic conditions (Table 4). Results of acid tolerance found that MS001 isolate showed a significant ($p < 0.001$) reduction to acidic conditions compared to the other six isolates. Our results corroborate the previous study, which reported that 13 out of 15 *Lactobacillus* strains showed survivability (84% to 100%) at acidic pH, isolated from fermented foods (Simões et al. 2022). Another study by Zielińska et al. (2015) and Nath et al. (2020) reported *Lactobacillus* strain's survivability at pH 3 in the range of 30 to 100% and 90%, respectively, when incubated for 3 h. Compared to previous findings some of our strains are showing better sustainable capacity at acidic pH. Besides gastric acid tolerability, tolerance to bile salt is considered a requirement to utilise beneficial

Table 3 Survival capability (%) of lactobacilli cultures in various concentrations of lysozyme

LAB isolate	100 mg/L (%)	200 mg/L (%)	300 mg/L (%)
MS001	98.31 ± 0.73^{a}	96.30 ± 0.56^{ae}	91.45 ± 0.46^{ad}
MS002	99.03 ± 0.81^{a}	92.15 ± 0.21^{b}	85.74 ± 0.82^{b}
MS003	99.71 ± 0.16^{a}	97.46 ± 0.59^{acd}	90.15 ± 0.09^{ad}
MS004	99.10 ± 0.44^{a}	91.02 ± 0.33^{b}	85.71 ± 0.84^{cb}
MS005	99.02 ± 0.07^{a}	98.43 ± 0.039^{cd}	92.44 ± 0.66^{a}
MS006	98.79 ± 0.74^{a}	98.39 ± 0.57^{d}	91.72 ± 1.05^{ad}
MS007	98.45 ± 0.75^{a}	95.06 ± 0.34^{e}	89.59 ± 0.61^{d}

Data expressed as mean ± SD

a–e: different superscript lowercase letters (a, b, c…..) in the same column indicate significant difference ($P < 0.05$)

Table 4 Survival capability (%) of lactobacilli cultures in acid (pH 3.0), bile (0.3%) and pancreatin (0.5%)

LAB isolate	pH 3.0	0.3% Bile	0.5% Pancreatin
MS001	85.03 ± 0.19^{a}	86.03 ± 0.011^{a}	92.99 ± 0.69^{a}
MS002	94.63 ± 0.09^{b}	96.55 ± 0.15^{b}	84.07 ± 1.02^{bc}
MS003	95.12 ± 0.42^{bc}	77.77 ± 0.19^{c}	83.05 ± 1.03^{b}
MS004	91.73 ± 0.55^{d}	79.98 ± 0.18^{d}	86.42 ± 0.12^{c}
MS005	97.16 ± 0.95^{b}	77.70 ± 0.44^{c}	96.33 ± 0.17^{d}
MS006	94.94 ± 1.20^{b}	88.59 ± 0.53^{e}	91.61 ± 0.46^{a}
MS007	97.09 ± 0.54^{b}	86.56 ± 0.51^{a}	82.55 ± 0.25^{b}

Data expressed as mean ± SD

a–e: different superscript lowercase letters (a, b, c…..) in the same column indicate significant difference ($P < 0.05$)

health effects (Daoudou et al. 2011). Commonly, the amount of bile salts was in the small intestine's range of 0.2 to 0.3% (Terpou et al. 2019). The potential of isolated lactobacilli culture to bypass the bile has also been evaluated in this study; results confirmed that most cultures showed more survivability than 77% (Table 4); MS002 isolate showed the highest survival percentage (more than 96%). In the current study, a few LAB isolates showed a low survival rate (77%); it has been reported in the earlier study that food-originating lactobacilli culture has less resistivity against intestinal conditions compared to gastric juice (Tokatlı et al. 2015). Pancreatic enzymes, particularly protease, lipase, and amylase, are crucial for regular digestion of proteins, fats, and carbohydrates. Hence, the ability to tolerate these enzymes is vital for the potential probiotic selection (Rayavarapu & Tallapragada 2019). In the present investigation, all the selected lactobacilli recorded more than 82% survival rate even at 0.5% pancreatin incubation for up to 48 h (Table 4). Our results showed that MS005 LAB isolates exhibited the highest cell survival capacity (< 96%) even after incubation of 48 h, which is in agreement with the previous finding, reported excellent growth ability of *Lactobacillus* strains at 0.5% pancreatin when incubated for up to 48 h (Khagwal et al. 2014). Overall result of the cell survival ability of LAB isolates in oral, gastric, and intestinal conditions suggested that MS005 isolate showed good potential to tolerate gastrointestinal environment.

In-vitro tests for cell adhesion to intestinal walls

Auto-aggregation is important parameter to evaluate the probiotic culture's ability to colonise and maintain itself in the intestinal tract. Different ranges of auto-aggregation potential from low (16–35%), medium (35–50%), and high (< 50%) can be exhibited by bacterial strains (Montoro et al. 2016). All the LAB isolates showed aggregation ability (Fig. 1) from 10.33 to 19.82% and 52.70 to 92.94% at incubation for 3 h and 24 h, respectively. A recent study reported that lactobacilli strains isolated from fermented food samples showed auto-aggregation ability (upto 82%) (Meena et al. 2022), which is lower than our isolate. MS007 isolates showed significantly ($p < 0.05$) the highest cell aggregation potential among all LAB isolates at 3 h and 24 h of incubation.

Bacterial adhesion to different solvents/hydrocarbons is another measure to estimate the isolate's adherence ability to intestinal tract cells (Shangpliang et al. 2017). Bacterial adhesion ability % for the different solvents (hexadecane, xylene, chloroform, and ethyl acetate) were in the range (1.37 to 55.04, 3.22 to 50.05, 50.09 to 85.59 and 36.72 to 84.82), respectively (Fig. 2). However, significantly ($p < 0.001$) highest adhesion property was observed for MS007 LAB isolate for hexadecane, xylene, and chloroform, while MS005 isolate showed the maximum adhesion (84.82%) for ethyl acetate. Our results are in agreement with the study of Bhushan et al. (2017), who reported adhesion ability in the range of 17 to 26% and 19 to 27% for hexadecane and xylene, respectively. Another study reported a very low ability of adhesion for *L. plantarum* to chloroform (13%) and ethyl acetate (2%) (Ahire et al. 2021), compared to our isolates. Comparatively, for chloroform, and ethyl acetate, isolates in present study were shown excellent cell adhesion capacity. A number of

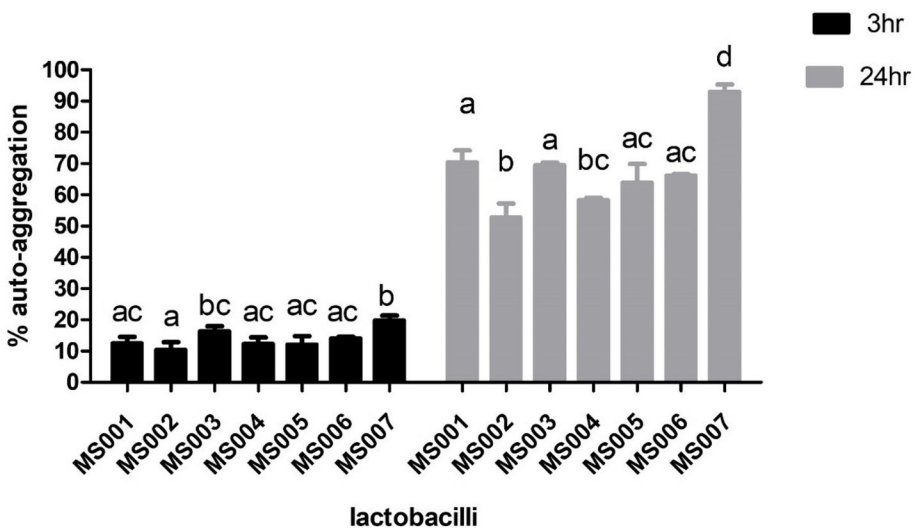


Fig. 1 Auto-aggregation potential of lactobacilli cultures at 3 h and 24 h incubation. Data expressed as mean ± SD, Different lowercase letters (a, b, c…..) above the bars denote statistically significant differences ($P < 0.05$)

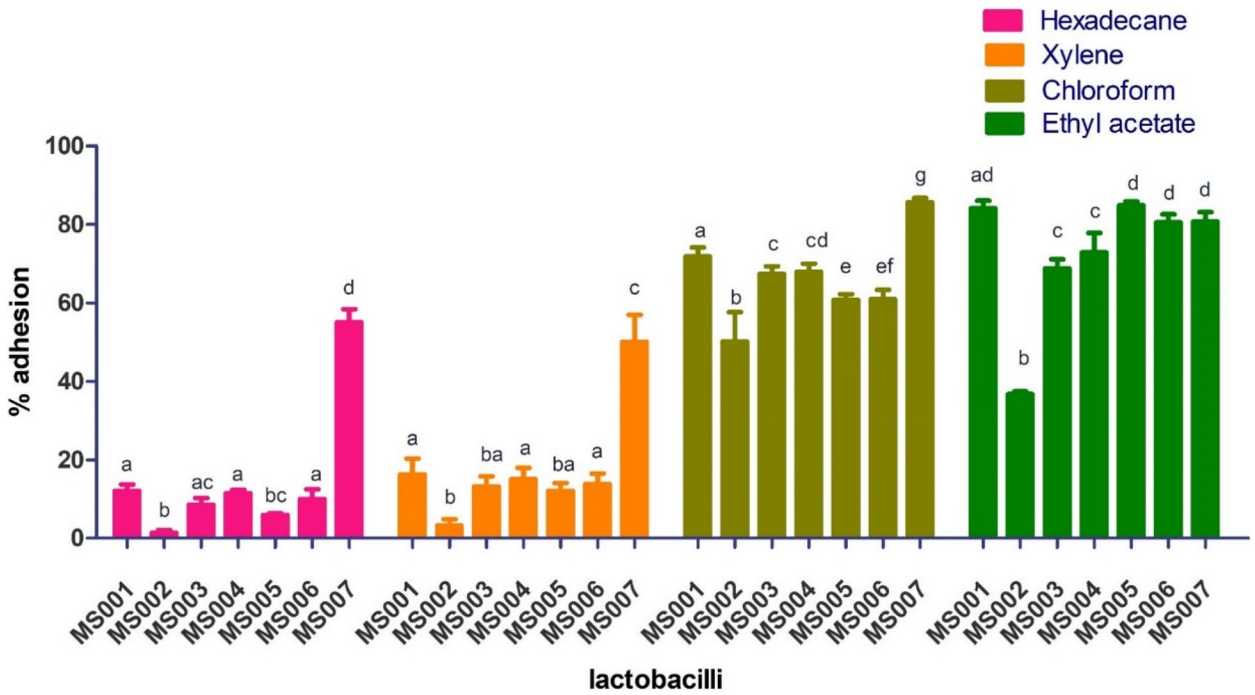


Fig. 2 Adhesion capability of lactobacilli cultures. Data expressed as mean ± SD, Different lowercase letters (a, b, c…..) above the bars denote statistically significant differences ($P < 0.05$). Data expressed as mean ± SD, Different lowercase letters (a, b, c…..) above the bars denote statistically significant differences ($P < 0.05$)

Table 5 Biofilm formation ability of lactobacilli cultures

LAB isolate	24 h	48 h	72 h	96 h
MS001	2.08 ± 0.23^{a}	2.27 ± 0.12^{a}	2.41 ± 0.20^{a}	2.25 ± 0.25^{ab}
MS002	0.47 ± 0.04^{b}	2.29 ± 0.50^{ac}	1.41 ± 0.23^{b}	1.29 ± 0.29^{a}
MS003	1.03 ± 0.14^{c}	2.75 ± 0.14^{ac}	2.25 ± 0.24^{a}	1.52 ± 0.58^{ac}
MS004	1.53 ± 0.17^{d}	2.79 ± 0.03^{bc}	2.59 ± 0.27^{a}	1.55 ± 0.20^{abc}
MS005	2.08 ± 0.23^{a}	2.23 ± 0.24^{ac}	2.50 ± 0.23^{a}	2.42 ± 0.26^{bc}
MS006	2.05 ± 0.10^{a}	2.29 ± 0.29^{ac}	2.47 ± 0.30^{a}	2.42 ± 0.18^{b}
MS007	1.35 ± 0.21^{cd}	2.79 ± 0.04^{c}	2.83 ± 0.07^{a}	2.45 ± 0.51^{bc}

Data expressed as mean ± SD

a–d: different superscript lowercase letters (a, b, c…..) in the same column indicate significant difference ($P < 0.05$)

variables, including the bacterial growth stage, the content of the surrounding medium, and the structure of the components of the cell surface, affect the hydrophobicity and auto-aggregating properties of bacteria, which are essential for the formation of biofilms and adhesion (Chaffanel et al. 2018). Different strains or genera of bacteria showed very different adhesion abilities against the same solvent, even though adhesion potential will be used to estimate the adhesiveness of LAB cultures. So, to further confirm the adhesion property, in vitro study (such as the Caco-2 cells model) should be used to assess these properties.

The capacity to form biofilm by beneficial bacteria (such as probiotics) could be useful to protect the host from diseases. This attribute permits the bacteria to maintain itself in the intestinal conditions, oppose pathogens for surface colonisation, and act as a defense purpose improving the microorganism survival rate (Probert & Gibson 2002). Table 5 shows the biofilm-forming potential of all LAB isolates for different incubation times. Significantly ($p < 0.05$), higher biofilm was formed by MS001, MS005, and MS006 isolates, at 24 h of incubation, while the lowest was formed by MS002 isolate at 24 h as well as 72 h of incubation. And as the time of incubation was increased, biofilm formation considerably increased, and maximum biofilm formation was achieved at 72 h of incubation for the MS007 isolate. The findings of our study are corroborated by the recent study of Rezaei et al. (2021), who also obtained similar results as incubation time was increased, and after 72 h of incubation, biofilm formation was decreased. Another recent study reported identical observations when LAB culture was incubated for 72 h on an abiotic polystyrene surface (Parolin et al. 2021).

Tolerance to technological stresses

The ability of probiotic cultures to withstand the intestinal environment with high salt concentration is one of the crucial characteristics for their selection. Results of

our study revealed that all the isolated lactobacilli showed maximum tolerance up to 4% of NaCl concentrations, which was supported by the previous finding, where lactobacilli isolated from curd tolerated 1–6% NaCl concentration (Prabhurajeshwar & Chandrakanth 2017). Since several fermented food's end products are alcohol, we here screened LAB isolates for ethanol tolerance ability. The present study showed that all the isolated LAB cultures recorded a maximum tolerance of up to 6% ethanol concentration, which is insisted by a recently reported study (Pradhan & Tamang 2021). Another study confirmed that the *Lactobacillus* strain could tolerate ethanol concentrations up to 16% (Gold et al. 1992), which is higher than our isolates. Tolerance to different concentrations of ethanol or NaCl mainly depends on the substrate and the strain of the cultures.

Enzymatic activity of isolates

Deconjugation of bile salts by the probiotic is the vital attribute of cultures assessed through bile salt hydrolase activity (Adebola et al. 2020; Horackova et al. 2020). In the present investigation, the qualitative assay for BSH activity indicated that all the strains, except MS005, MS006, and MS007, showed only slight growth and no ability to deconjugate TDC (bile salt) (Table 6). Furthermore, MS007 isolate were observed for slight precipitation zones also, which means MS007 strain possessed excellent BSH activity among all seven isolates. Our results are supported by the previous study, which reported diverse bile salt hydrolase activities for the LAB cultures isolated from *Raabadi* (a cereal-based fermented food) (Yadav et al. 2016). Previous studies supported our results of bile salt hydrolase activity for the probiotic bacteria isolated from fermented foods, such as *Lactobacillus* strains from fermented food samples of Rajasthan (Meena et al. 2022), *L. plantarum* subsp. *plantarum* NMB7 (Pradhan & Tamang 2021), *L. plantarum* UBLP40 isolated from traditional fermented food idli batter (Ahire et al. 2021).

Table 6 Bile salt hydrolase (BSH) activity and Phytate degradation potential of the lactic cultures

Lactic culture	BSH (Taurodeoxycholate)	Phytase activity
MS001	+	+ ve
MS002	+	+ ve
MS003	+	+ + ve
MS004	+	+ ve
MS005	+	+ + + ve
MS006	+	+ ve
MS007	+ +	+ + ve

For BSH: + growth only, + + slight precipitation

For Phytate degradation ability: + ve (weak); + + ve (good); + + + ve (excellent)

Phytic acid is one of the key anti-nutrients that crucially affect micronutrient bioavailability by forming complexes with essential micronutrients such as iron and zinc (Jatuwong et al. 2020; Samtiya et al. 2020). The present study confirmed that all LAB cultures showed positive results for phytic acid degradation (Table 6). Moreover, MS001, MS002, MS004, and MS006 LAB isolates documented weak positive results, whereas the MS005 isolate was noted for excellent results for phytase degradation compared to all isolates. Previous several studies confirmed that LAB strains isolated from fermented foods produce phytase enzyme and phytic acid degradation properties such as LAB strains isolated from Ethnic Indian Fermented Foods (Sharma et al. 2019), *L. plantarum* (Uslu et al. 2016), and *Lactobacillus* strains (Saraniya & Jeevaratnam 2015). Culture possessing phytase production ability could be a subtle approach in selecting probiotic cultures, and this culture may further be used to develop micronutrient-rich plant-based foods.

Antioxidative properties

Probiotic bacteria comprise antioxidants; when they are lysed in the intestinal cavity by the action of bile, their antioxidant components are released in the lumen. The antioxidative ability of intact cell and cell-free extract samples of all the LAB isolates is shown in Fig. 3 (a to d). Results showed a non-significant ($p > 0.05$) difference in the antioxidative potential of all LAB isolates cell-free extract using DPPH assay (Fig. 3a). MS002 and MS007 intact cell samples showed significantly ($p < 0.05$) lowest antioxidative % inhibition (Fig. 3b), i.e., 14% and 15%, respectively, for DPPH assay, whereas MS003 shows highest (34%) among all. For ABTS assay, % scavenging activity was found to be in the range 19.65 ± 0.34 to 21.53 ± 0.27% (Fig. 3c) and 18.52 ± 1.66 to 26.05 ± 1.44% (Fig. 3d) for cell-free extract and intact cell sample, respectively. The cell-free extract and intact cell suspension of isolate MS006 showed higher ABTS radical scavenging capacity of 26% and 21%, respectively. A study by Shokryazdan et al. (2017) showed similar result trends for ABTS antioxidative potential; intact cell samples of *L. acidipiscis* ITA44 and *L. pentosus* ITA23 were recorded for much higher antioxidant activity than cell-free extracts samples. Our results corroborated previous study findings that intact cell samples of 12 LAB strains (showed higher DPPH scavenging activity 6.69 to 37.74%) than cell-free extracts samples (Chen et al. 2014). Still, the percentage of inhibition was quite minimal, which could be attributed to lower concentrations of antioxidant chemicals such as antioxidant enzymes, iron chelators, and others, which will need to be validated using

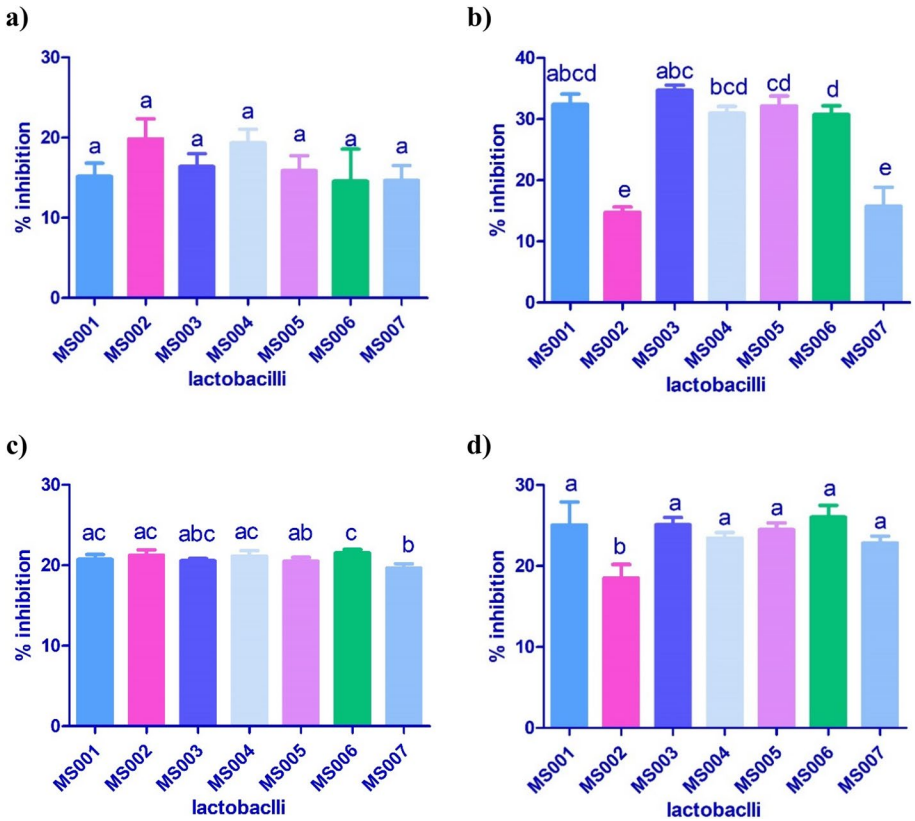


Fig. 3 Antioxidative potential of lactobacilli cultures. **a**. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) cell free extract (CFE). **b**. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Intact cell (IC). **c**. 2,2′-azino-bis 3-ethylbenzo-thiazoline-6-sulphonic acid (ABTS) cell free extract (CFE). **d**. 2,2′-azino-bis 3-ethylbenzo-thiazolin e-6-sulphonic acid Intact cell (IC). Data expressed as mean ± SD, Different lowercase letters (a, b, c…..) above the bars denote statistically significant differences ($P < 0.05$)

sophisticated methods such as chromatography. Overall results suggested that the intact cell sample of LAB isolates recorded considerably high antioxidative potential compared to cell-free extract for both ABTS and DPPH scavenging assays.

Antimicrobial activity

Antimicrobial compounds generally inhibit pathogens' growth by competitive exclusion in the intestinal lumen. Compounds like bacteriocins, surfactants, hydrogen peroxide, organic acids, and bacteriocin-like inhibitory substances are the components produced by lactic acid cultures to hinder the pathogen's growth (Ghanbari et al. 2018; Jung et al. 2019; Silva et al. 2020). The present study used CFSs of isolates to assess the antimicrobial potential of bacterial metabolites. None of the isolates CFSs showed any antimicrobial activity at adjusted pH of 7.0 (data not shown). Similar results for LAB cell-free supernatants (adjusted at 7 pH) were reported by several previous studies (De Keersmaecker et al. 2006; Gunyakti & Asan-Ozusaglam 2019; Layus et al. 2020; Tsai et al. 2005), our results were in agreements with these findings. Previous study results are with the agreements of our outcomes; all the LAB strains except strain C16 lost their antimicrobial potential supernatants were adjusted to pH 7 (Reuben et al. 2020). These findings recommended that inhibition of pathogenic strains at low pH supernatants is mainly due to the production of acidic substances (such as acetic acid, propionic, and lactic acid). Furthermore, more study on different pH needs to be evaluated to confirm isolated cultures' antimicrobial potential.

Scanning electron microscopy micrographs

Two strains (*Limosilactobacillus fermentum* MS005 and *Lactiplantibacillus plantarum* MS007) that were isolated for this study's objectives were observed by scanning electron microscopy technique. Figure 4 represents the scanning electron micrographs (Magnification: 15.00 K X) of *L. fermentum* MS005 (Fig. 4a) and *L. plantarum* MS007 (Fig. 4b).

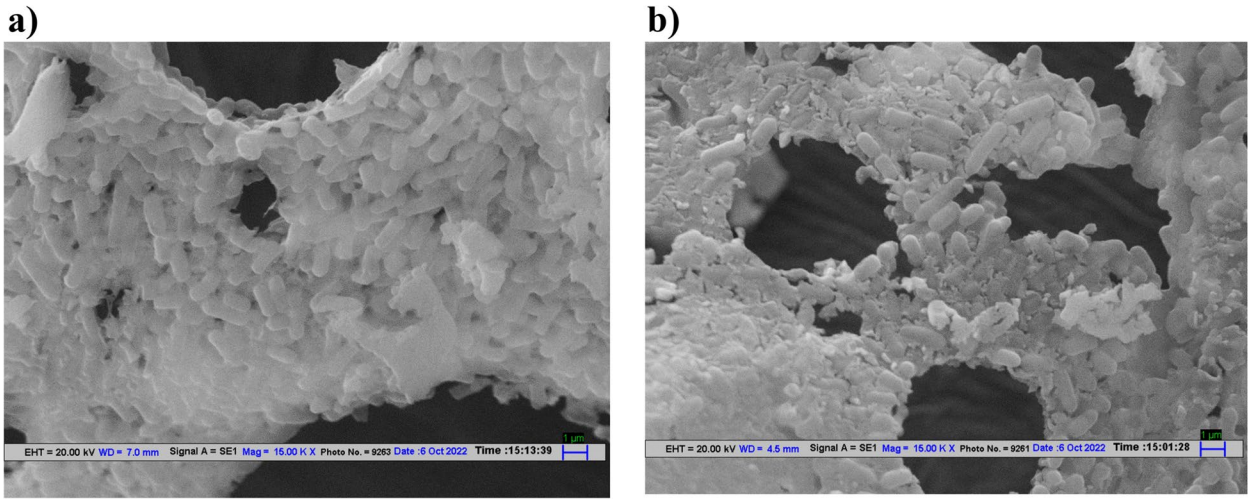


Fig. 4 Scanning electron micrographs (15.00 K X) of lactic cultures. **a** *Limosilactobacillus fermentum* MS005. **b** *Lactiplantibacillus plantarum* MS007

TOPSIS: Multiple attribute decision making (MADM) analysis

Multiple Attribute Decision Making (MADM) analysis, especially the Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS), is a prevailing method for embark upon complex decision-making problems (Jaglan et al. 2023; Tzeng & Huang 2011). Herein, we work on seven alternatives possessing twenty-four attributes. All weights b_j associated to each attribute are considered as one except the phytate degradation, which is taken as 5. The normalized decision matrix, weighted normalize matrix, and closeness coefficients are calculated. Finally, corresponding to seven alternatives MS001 to MS007, we obtain the 4, 7,3,6,1,5,2 ranks, respectively. In a recent study by Jaglan et al. (2023), similar modelling was utilized to identify the potential gluten degrading probiotic candidate based on all screening criteria.

Phytate degradation assay is an important attribute in this study. We varied the weight associated with phytate degradation ability from 1 to 10. From Table 7, we can easily observe that MS007, which is initially at rank one changes to rank 2, whereas MS005 that is at rank 2 initially changes to rank 1 as we increase the weight of phytate degradation in this study. This is also established in the Fig. 5.

Conclusions

The present research was carried out to isolate and characterise the potential cultures from the traditional fermented foods (*Dahi, Raabadi,* and *Lassi*) of Haryana state, India. Seven LAB cultures were selected after being identified as lactobacilli through PCR using a genus-specific primer of *Lactobacillus*. For BSH activity, MS007 LAB isolate was detected for the slight precipitation zone. All the LAB isolates showed no haemolysis (γ-haemolytic activity) and were positive for the phytate degradation test, whereas MS005 recorded for an excellent degradation zone surrounding the colonies. The best isolate was chosen based on its attributes and degree of proximity to the chosen parameters, and this was explained logically by a straightforward mathematical equation generated with TOPSIS. Higher levels of flexibility in TOPSIS

Table 7 Effect of weight of phytate degradation potential on the ranking of the samples

Sample	Weight of phytate degradation test									
	1	2	3	4	5	6	7	8	9	10
MS001	3	4	4	4	4	4	4	4	4	4
MS002	7	7	7	7	7	7	7	7	7	7
MS003	6	3	3	3	3	3	3	3	3	3
MS004	5	6	6	6	6	6	6	6	6	6
MS005	2	2	2	1	1	1	1	1	1	1
MS006	4	5	5	5	5	5	5	5	5	5
MS007	1	1	1	2	2	2	2	2	2	2

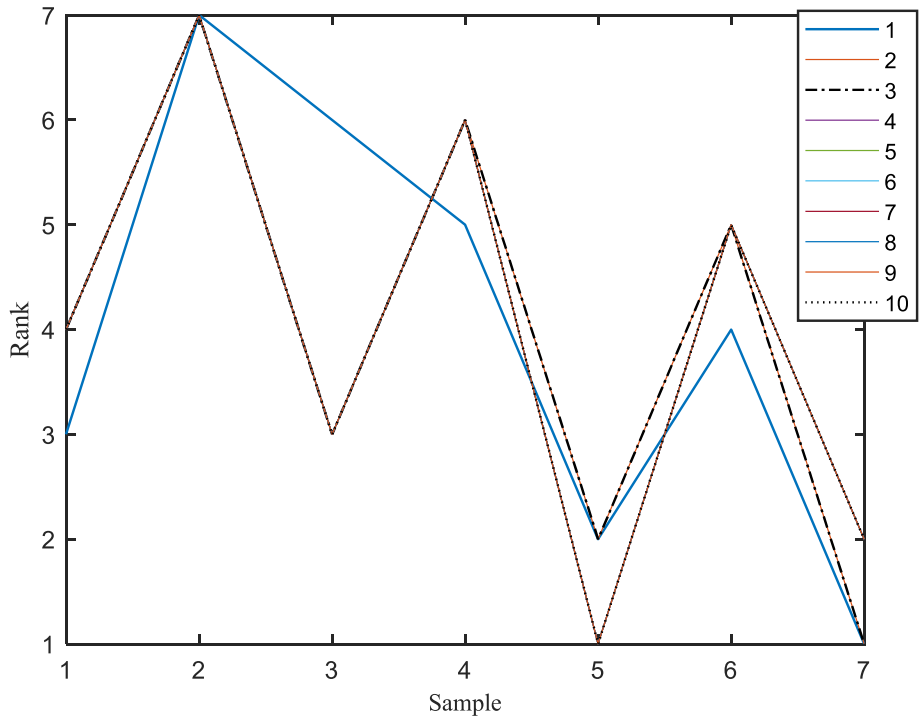


Fig. 5 Rank versus sample for different values of weight of the phytate degradation

application can aid in decision-making for investigations involving several analytic dimensions. Based on their potential probiotic qualities, strains *Limosilactobacillus fermentum* MS005 and *Lactiplantibacillus plantarum* MS007 were identified by the TOPSIS method as promising candidates for further use in the development of foods and other industrial applications. However, further in vivo trials are needed to validate their health-promoting attributes.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-024-00259-z.

Supplementary Material 1.

Acknowledgements
Authors are grateful to the Director, National Institute of Food Technology Entrepreneurship and Management, Kundli (NIFTEM-K), Sonipat and Vice Chancellor, Central University of Haryana, Jant-Pali, Mahendergarh for providing necessary support and research facilities. The NIFTEM-K internal manuscript reference number is NIFTEM-P- 2024-74.

Author's contributions
MS: Investigation, Formal analysis, Data curation, Validation, Methodology, Writing—original draft, Writing—review & editing. BB: Methodology, Writing—review & editing. STP: Investigation, Methodology. PCB: Conceptualization, Resources, Methodology, Data curation, Writing—review & editing. GAC: Resources, Writing—review & editing. PS: Methodology, Formal analysis, Validation, Writing—Reviewing and Editing. AK: Data curation, Writing—review & editing. TD: Conceptualization, Resources, Methodology, Data curation, Project administration, Supervision, Writing—review & editing.

Funding
The authors are thankful to the Haryana State Council for Science Innovation and Technology, India (Project No.: HSCSIT/R&D/2021/461) for funding this research work.

Availability of data and materials
The datasets used during the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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Received: 1 December 2023 Accepted: 6 May 2024
Published online: 10 September 2024

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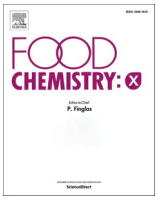
Publisher's Note

Food Chemistry: X 22 (2024) 101483

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Effect of selective fermentation on nutritional parameters and techno-functional characteristics of fermented millet-based probiotic dairy product

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ARTICLE INFO

Keywords:
Probiotics
Fermented food
Nutritional attributes
Biofortified pearl millet
Micronutrients
Bioavailability
Lactic acid bacteria

ABSTRACT

The primary goal of this study was to assess the effect of selective fermentation on the nutritional and techno-functional characteristics of fermented millet-skim milk-based product. The product was made with HHB-311 biofortified pearl millet (PM) flour, skim milk powder, and isolated cultures (either alone or in combination) of *Limosilactobacillus fermentum* MS005 (LF) and *Lactobacillus rhamnosus* GG 347 (LGG). To optimize fermentation time, time intervals 8, 16, and 24 h were explored, while the temperature was kept 37 °C. Results of protein digestibility showed that LF (16 h) and LGG (24 h) fermented samples had significantly higher ($P < 0.05$) protein digestibility of 90.75 ± 1.6% and 93.76 ± 3.4%, respectively, than that of control (62.60 ± 2.6%). Further, 16 h fermentation with LF showed enhanced iron (39%) and zinc (14%) bioavailability. The results suggested that LF with 16 h fermentation is most suitable for making millet-based fermented products with superior techno-functional attributes and micronutrient bioavailability.

1. Introduction

Fermentation is a type of metabolic processing that oxidizes carbohydrates to release energy when no external electron acceptors are present (Rollán, Gerez, & LeBlanc, 2019). As per the expert panel report of The International Scientific Association for Probiotics and Prebiotics (ISAPP), fermented food is "*food made through desired microbial growth and enzymatic conversions of food components*" (Marco et al., 2021). Traditional/indigenous fermented foods can contribute to enhanced nutritional state of the consumers and are connected to cultural relationships across other civilizations. Fermentation has grabbed the interest of academics seeking to optimize the process to improve specific nutritional qualities and health benefits of fermented products (El Sheikha & Hu, 2020). Microorganisms employed in the process of fermentation can create a variety of enzymes (i.e., amylase, lipase, protease, phytase, etc.) that hydrolyze carbohydrates, proteins, and lipids into easily digested elements with good texture and flavor (Dhull et al., 2020). During food fermentation, endogenous enzymes are activated due to lower pH, which contributes to the reduction of phytic acid. Fermentation improves mineral (i.e., Ca, Zn, and Fe) bioavailability by producing phytases that reduce phytic acid constituents in plant-based foods (García-Mantrana, Yebra, Haros, & Monedero, 2016; Gupta, Gangoliya, & Singh, 2015). Studies have shown that natural fermentation and strain-specific fermentation provide better mineral bioavailability through a more effective degradation of the plant food's phytic acid content (Nkhata, Ayua, Kamau, & Shingiro, 2018). Nutri-cereals, such as millet, are nutritionally equivalent to cereals because they are abundant sources of nutrients like dietary fiber and phytochemicals that benefit all (Kumar, Tomer, Kaur, Kumar, & Gupta, 2018; Rasane, Jha, Kumar, & Sharma, 2015) and could be utilized to make functional

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https://doi.org/10.1016/j.fochx.2024.101483
Received 26 February 2024; Received in revised form 14 May 2024; Accepted 15 May 2024
Available online 17 May 2024

products. Fermented milk cereal flour products like *Raabadi*, Boza, Mahewu, and Togwa are widely consumed throughout Africa and the Indian subcontinent (Sudha, Devi, Sangeetha, & Sangeetha, 2016). Combining cereals and milk can improve nutrition and create a more functional product. Incorporating cereal into dairy products might compensate for the lack of fiber in milk and the low digestibility and amino acid deficiencies in cereal (Ganguly, Sabikhi, & Singh, 2022). Most probiotic food items available in global markets are milk-based, with very few developed employing alternative substrates. Cereals are considered the most affordable sources of nutrition and protein for a considerable portion of the human population, especially in underdeveloped countries (Di Stefano et al., 2017). The combination of cereal and milk will result in a synergistic impact, providing improved nutrition and eventually leading to a value-added functional food (Ganguly, Kumar, Singh, & Sabikhi, 2014). Research is being done to prepare millet and milk-based fermented products/substrates to improve nutritional health. Several such kinds of products were prepared previously, such as a composite dairy-cereal substrate (Ganguly & Sabikhi, 2012), *Rabadi*-like fermented milk beverage using pearl millet (Modha & Pal, 2011), fermented millet milk based curd (Sheela, Moorthy UmaMaheswari, Kanchana, Kamalasundari, & Hemalatha, 2018), pearl millet based fermented skim milk product (Basu & Tomar, 2016), whey skim milk-cereal based probiotic beverage (Ganguly, Sabikhi, & Singh, 2021), fermented finger millet-based yogurt-like beverage (Vila-Real et al., 2022), milk-cereal (millet) based composite substrate (Ganguly et al., 2022) and probiotics enriched rabadi beverage (PERB) (Yadav, Shukla, Kumari, Dhewa, & Kumar, 2024). Selective fermentation of millet and skim milk-based blend with desired culture (phytate degrading bacteria) could be a potential strategy to make micronutrient-enriched products with improved bioavailability. There are minimal studies on millet (biofortified) and milk-based composite food items made using desired culture (phytate degrading bacteria) specifically for improving micronutrient bioavailability. In the current study, we have used two lactobacilli, i.e., *Lactobacillus rhamnosus* LGG 347 (LGG) and *Limosilactobacillus fermentum* MS005 (LF). LGG is a well-known probiotic culture that has several health-beneficial properties. LF is one of the best phytate degrading cultures out of seven indigenous cultures isolated from dairy-fermented foods and characterized in our lab. Previous studies showed that fermentation improves the bioavailability of cereal and milk-based fermented products (Basu & Tomar, 2016; Ganguly et al., 2021; Ganguly et al., 2022). However, they did not perform selective fermentation with desired cultures at different durations.

Several indigenous biofortified pearl millet (PM) varieties have been released with high iron and zinc contents, e.g., the HHB-311. However, there is a dearth of micronutrient bioavailability data on these biofortified millet varieties. Therefore, in the present study a millet-based skim milk fermented food developed using the HHB-311 variety in combination with LGG and LF bacteria. The main reason for selecting LF in the present study was its phytate degrading potential, and LGG was chosen due to its well-established health-beneficial attributes. The study also investigated the effects of fermentation on nutritional attributes and techno-functional properties of the millet-based fermented substrate.

2. Materials and methods

2.1. Procurement and processing of raw materials/cultures for product development

HHB-311 PM variety was procured from the Bajra Section of Chaudhary Charan Singh Haryana Agricultural University (CCS HAU), Hisar, Haryana. Skim milk was procured from a grocery store in New Delhi, India. *Lactobacillus rhamnosus* GG (LGG) probiotic culture was purchased from the National Centre for Dairy Cultures (NCDC) at ICAR-National Dairy Research Institute-Karnal, Haryana, India. Indigenous LF probiotic culture was selected from the LAB cultures isolated in our lab. Both cultures were used in the present investigation for product development purposes. MRS broth media was used to maintain both lactobacilli. PM seeds were cleaned and washed with double distilled water, then dried at 40 °C for 16 h. PM seeds were ground into flour using a hammermill and then sieved using a 200 μm mesh. After sieving, the flour was placed in a ziplock pouch and stored in a cool and dry place for further use.

2.2. Development of fermented millet-skim milk product (FMSMP)

Fermented products based on PM flour and skim milk powder were developed in the Animal Product Technology Lab, Department of Food Science and Technology, National Institute of Food Technology Entrepreneurship and Management, Kundli, Sonepat, Haryana, India. Fig. 1 describes treatments used for the selective fermentation to prepare FMSMP. In the first step, 12 g of skim milk powder was dissolved in 100 mL distilled water, followed by the addition of 10 g of PM flour (HHB-311), and then the blended sample was continuously stirred and heated at 95 °C for 10–15 min until the mixture was properly gelatinized (Basu & Tomar, 2016). Then, the samples were cooled to 40–42 °C and inoculated with 2% each of LF and LGG, or a combination of both (LF + LGG, 1% each). The inoculation was performed with desired probiotic bacterial cultures at their log phase (16 h of activation). At the time of inoculation, CFU/mL level of probiotic bacteria was~10^{10}. All three experimental samples were incubated in a biochemical oxygen demand (BOD) incubator at 37 °C temperature for different periods, i.e., 8, 16, and 24 h. After each incubation, the fermented samples were removed and stored in the refrigerator (4–6 °C) for further analysis. The nutritional analysis was conducted within 24 h. A sample without culture/fermentation was prepared and used as a control.

2.3. Physico-chemical evaluation of FMSMP

For the estimation of physico-chemical quality of the product, parameters such as pH, total titratable acidity, and total soluble solids were measured for the control (unfermented), and samples (8 h, 16 h, and 24 h) fermented with different cultures.

2.3.1. pH value

The pH of fermented and control samples was estimated using a calibrated pH meter (Eutech Instruments) with a single junction pH electrode (ThermoFisher, Scientific).

2.3.2. Total titratable acidity (TTA)

The total titratable acidity of the fermented and control samples was estimated by standard titration, and results were expressed as percent lactic acid (LA). Calculation of lactic acid (%) was done by following formula:

$$TTA\ as\ LA\ (\%) = (V \times 0.1\ N\ \ NaOH\ \ \times\ \ 9)/M$$

where, V = Volume of sodium hydroxide used for titration; 0.1 N NaOH = Normality of NaOH used for titration; 9 = Factor for lactic acid; M = Mass of the sample used for the titration.

2.3.3. Total soluble solids (°Brix)

Total soluble solids (TSS) of the control (unfermented) and fermented samples were measured using a high-accuracy touchscreen digital refractometer (RX-7000i, ATAGO, Japan). For the assessment of the TSS content of samples, nearly 250 mg of sample was taken and put on the sample stage of the refractometer, and results were recorded as the percentage of Brix.

2.4. Proximate composition of FMSMP

For the estimation of moisture, protein, ash, and carbohydrate content of the control and fermented samples, the standard method of AOAC

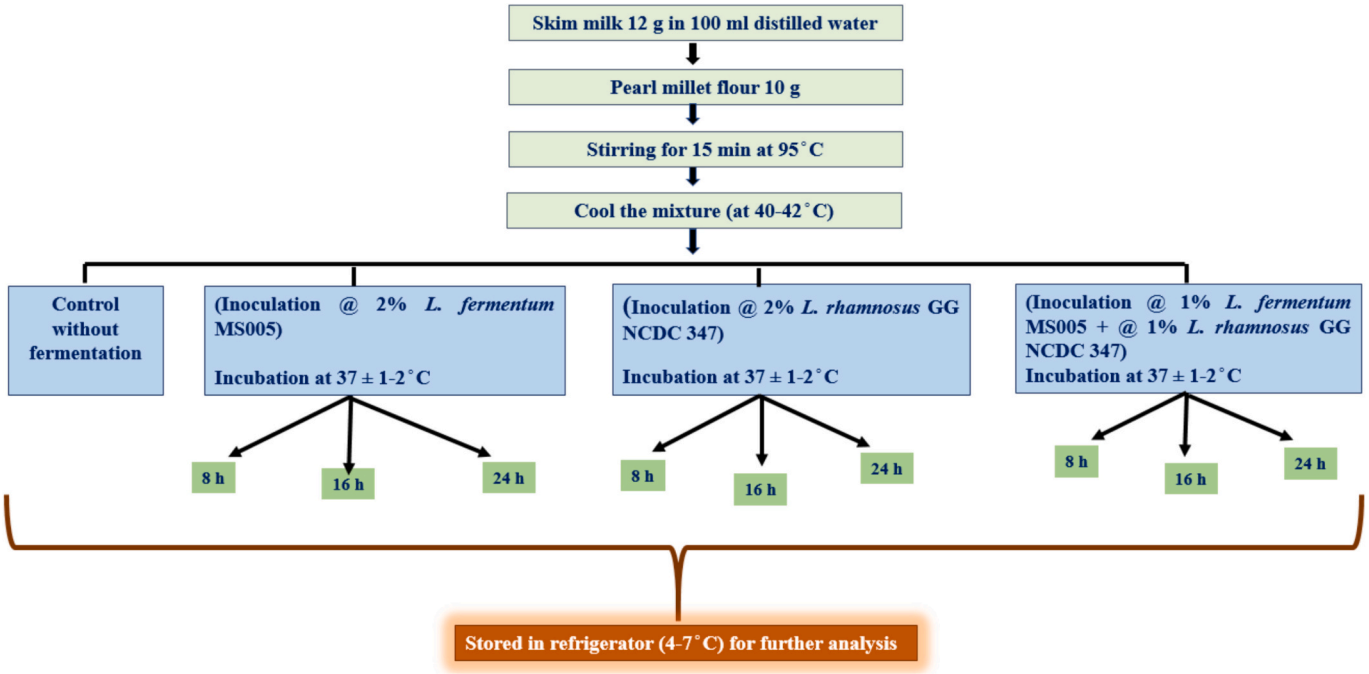

Fig. 1. Schematic illustration of fermentation treatments for selective fermentation.

(2005) was followed.

2.4.1. Estimation of fat

Estimation of fat in control and fermented samples was performed using the Mojonnier method (AOAC, Official Method 989.05, 2012). Fat content was measured using the given below formula:

$$Fat\ (\%) = [(WS - WB)/WP] \times 100$$

where WS = Weight (g) of beaker with fat content (after drying); WB = Weight (g) of empty beaker; WP = Weight (g) of sample.

2.5. Estimation of phytochemical content and antioxidative activity of FMSMP

2.5.1. Extraction

The control and fermented samples were extracted to evaluate phytochemical content and antioxidant activity with slight changes to the technique of Öztürk et al. (2018). For the extraction, 3 g samples were dissolved in 15 mL of 80% methanol and vigorously homogenized for 5 min. The samples were kept undisturbed for 1 h and were then homogenized for 5 min. After centrifugation (~7000 *g* for 10 min at 4 °C), the supernatant was passed through a 0.45 μm syringe filter, and the filtrate was collected in amber-colored falcon tubes (15 mL) and stored at 4 °C.

2.5.2. Phytochemicals

2.5.2.1. Total phenolic content (TPC). The total phenolic content of the samples was estimated according to the method of Kennas, Amellal-Chibane, Kessal, and Halladj (2020) with minor changes. Absorbance of the samples was read at 765 nm (Spectramax M2e system, Molecular Devices, USA). Findings were represented as mg of gallic acid equivalent (GAE)/100 g of sample.

2.5.2.2. Total flavonoid content (TFC). The total flavonoid content in the samples was estimated according to the colorimetric assay Ge et al. (2022) with slight modifications. After incubation, the mixture's absorbance was read at 510 nm, and results were recorded as mg quercetin equivalent (QE)/100 g of sample.

2.5.3. Antioxidative potential

2.5.3.1. Ferric reducing antioxidant power (FRAP) activity. With slight modifications, the FRAP activity was evaluated using a technique reported by Wu et al. (2020). The absorbance was read at 593 nm, and the results were expressed in mM Trolox equivalent (TE)/100 g sample.

2.5.3.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA) assay. The 2,2-diphenyl-1-picrylhydrazyl RSA of samples was determined using the procedure of Singhal, Kumar, and Badgujar (2021), with slight changes. The absorbance was taken at 517 nm, and the control/blank of the assay was prepared by using DPPH and extraction reagent (methanol) only. The results were recorded as the DPPH RSA (%) and the following formula used for calculation:

$$RSA\ (\%) = [A_1 - (A_D - A_S]/A_1 \times 100$$

where A_1 = Control absorbance; A_D = DPPH solution absorbance with the sample; A_S = Sample extract absorbance without DPPH.

2.6. Anti-nutritional factors (ANFs) of FMSMP

2.6.1. Phytic acid

The phytic acid content of the samples was assessed using a phytate estimation kit (Megazyme, K-PHYT, Ireland). Sample (1 g) was taken into a 50 mL conical flask, and 20 mL 0.66 M HCl was added. The conical flask was covered with foil paper and incubated in an incubator shaker (Innova 42, New Brunswick Scientific, USA) at 250 rpm for 3 h at room temperature. A 1 mL aliquot of the extracted solution was transferred into a 1.5 mL tube and centrifuged for 10 min at 18,000 *g*. A portion (0.5 mL) of the resultant extracted supernatant was placed into a new 1.5 mL tube immediately, and subsequently, 0.5 mL of 0.75 M NaOH solution was added to neutralize it. Other components were added following the instructions provided in the kit. Phosphorus standard was prepared at different concentrations (0, 0.5, 2.5, 5, and 7.5 μg), and 0.5 mL of color reagent was added to each tube, following the same procedure as for the sample.

Calculation:

To determine the phytic acid content, the absorbance of "Free Phosphorus" sample was subtracted from the absorbance of "Total Phosphorus" sample, thereby obtaining $\Delta A_{phosphorus}$.

For the estimation of phytic acid, the below-given formula was used:

$$Phosphorousg/100\ g) = \text{mean M} \times 0.1112 \times \Delta A_{phosphorus} \quad (a)$$

where Mean M = Mean obtained from the standard curve of phosphorus; 0.1112 = Multiplication factor given in the kit; $\Delta A_{phosphorus}$ = Absorbance obtained after subtraction of absorbance of the "Free Phosphorus" sample from the absorbance of "Total Phosphorus" sample

$$Phytic\ acid\ (g/100\ g) = \frac{\text{Phosphorous (g/100 g)}}{0.282}$$

where Phosphorous (g/100 g) = Value taken from (a); 0.282 = Factor given in kit to calculate phytic acid.

2.6.2. Tannin

Tannin content of the samples was determined using Anaemene and Fadupin (2022) technique with few modifications. After color development of the standard and sample solutions, absorbance was taken at 760 nm, and results were discussed as mg of tannic acid equivalent (TAE)/100 g of sample.

2.7. In vitro protein digestibility (IVPD) assessment

IVPD of the samples was assessed by following the method of Verma et al. (2021) and Gong et al. (2022) with modifications. The digestion model included three phases: oral/mouth, gastric, and intestinal. The final mixture was kept undisturbed at 37 °C for 1.5 h; subsequently, the reaction was stopped by heating the mixture for 10 min in a 95 °C water bath. The supernatant was collected and kept at −20 °C for subsequent analysis after centrifugation at 5000 *g* for 15 min at 4 °C. Nitrogen content of the 1 mL supernatant was estimated using Kjeldahl apparatus (Gerhardt, Analytical Systems, Germany). The IVPD (%) was calculated using the below-given formula:

$$IVPD\ (\%) = \left(N_{supernatant} - N_{control}\right)/N_{sample} \times 100$$

where $N_{supernatant}$ = Nitrogen content in the sample supernatant (after digestion); $N_{control}$ = Nitrogen of control (following the same process, but without sample); N_{sample} = Nitrogen content in the original sample (without digestion).

2.8. In vitro bioavailability of iron (Fe) and Zinc (Zn)

Fe and Zn bioavailability of the control and fermented samples was estimated according to the approach of Kumar et al. (2017) with slight variations. The sample (5 g) was transferred to a 100 mL conical flask, and 30 mL of distilled water was added. The flask was then shaken in an incubator at room temperature for 16 h at 150 rpm. Then, 2 mL α-amylase solution (6.25 mg/mL) was added and placed in a water bath to incubate for 45 min at 37 °C. The mixture was adjusted to pH 4.0 using HCl solution, followed by addition of 8 mL pepsin (1.25 mg/mL) solution. A water bath was subsequently utilized for incubating samples (1 h at 37 °C). A NaOH solution was added to bring the mixture to pH 6.0, and 10 mL pancreatin solution (20 mg/mL) was added. The resulting mixture was centrifuged at 13,000*g* for 12 min at 4 °C after being left undisturbed at 37 °C for 1 h. The sample supernatant was passed through a 0.45 μm syringe filter and analyzed for the Fe and Zn content using ICP-OES (Optima 7000DV, PerkinElmer, USA) against a water blank (processed similarly). The undigested sample was first digested with acid, and micronutrients were estimated using ICP-OES. The micronutrient bioavailability was measured using the formula:

$$Bioavailability\ (\%) = (M_S - M_B)/M_U \times 100$$

where M_S = Micronutrient in the sample supernatant (after digestion); M_B = Micronutrient in water blank (processed similarly); M_U = Micronutrient in the sample (without enzymatic digestion/undigested).

2.9. Molar ratio of phytic acid/minerals

The molar ratio of minerals to anti-nutrients was determined using the method of Norhaizan, Ain, and A. W. (2009). The mole of phytic acid/minerals was evaluated by dividing the weight of phytic acid (PA) and minerals present in 100 g of sample with the atomic weight of PA (660 g/mol) and Fe (56 g/mol) or Zn (65 g/mol). The molar ratio between PA/mineral was determined after dividing the mole of phytic acid by the mole of minerals.

2.10. Techno-functional properties of FMSMP

2.10.1. Functional groups (FTIR) analysis

Functional groups of unfermented and fermented samples were determined using a Fourier-transform infrared spectrophotometer (Cary 630, Agilent Technologies, USA). The sample was placed on Attenuated Total Reflectance (ATR), which presented the peaks between the wavelength range of 4000–600 cm^{-1}, and the scan was completed in 1–2 min.

2.10.2. Texture profile analysis (TPA)

To analyze the texture profile of unfermented and fermented samples, the TA.HDplusC Texture Analyser (Stable Micro Systems, England, UK) was used. The TPA method was performed as it automatically calculates a range of food texture properties. Program conditions were: 1 mm s^{-1} of heat speed, 25-mm stainless steel cylinder probe with 5 mm s^{-1} moving speed, and 1 mm s^{-1} of test speed.

2.10.3. Apparent viscosity (rheological property)

The apparent viscosity of the samples was determined based on the approach given by Ge et al. (2022) using a rotating rheometer (MCR 52, Anton Paar Co. Ltd., Australia). The apparent viscosity was measured using operating parameters: measuring the temperature of 25 °C, PP50 detector, the gap between the flat and detector was set to 0.5 mm, shear rate ranged from 0.01 to 100 s^{-1} in 300 s, and 30 points were collected for a single sample.

2.10.4. Color profile analysis

Unfermented and fermented samples were assessed for their color properties in terms of L*, a*, and b* using a digital colorimeter (KONICA MINOLTA, INC., Japan).

2.11. Statistical analysis

Microsoft Office (version 2019) was used for raw data tabulation and descriptive statistical calculations. GraphPad Prism (version 5.01) was used for grouped and column statistics, and one-way analysis of variance (ANOVA) was used for the statistical analysis of data, followed by the Tukey post-hoc test to separate the mean ($P \leq 0.05$), which was considered statistically significant at 95% confidence level. The results were represented as mean ± SD (standard deviation).

3. Results and discussion

3.1. Effect of fermentation on physico-chemical properties and proximate composition of FMSMP

A fermented product's pH, acidity, and total soluble solids (TSS) affect its acceptability. As a result, TSS, pH, and acidity were used to optimize the probiotic bacterial culture and incubation duration in this investigation (Fig. S1A–C). The initial TSS value for the control

(unfermented, 0 h) was 15.51 ^{0}Brix; the pH was 6.3; and the acidity was 0.18 (% LA). LF and LF + LGG fermented products recorded a significant decrease ($P < 0.05$) in the TSS (11.35 and 10.27) and pH (4.68 and 3.94), respectively after 24 h of fermentation. Our findings are consistent with recent research by Vila-Real et al. (2022), who reported lower pH values in the finger millet slurries fermented with co-cultures than in single-culture fermentation. The microflora generates organic acid with an increased duration of fermentation, subsequently causing the pH level to drop (Ahmed, Xua, Sulieman, Mahdi, & Na, 2019). Furthermore, the lowest decrease in the TSS and pH was observed for the LGG substrate after 24 h of fermentation. Compared to the LF and LGG, the LF + LGG fermented product revealed a faster decline in TSS, pH, and a rise in acidity after 8, 16, and 24 h of fermentation. The results of our study corroborated those of Di Stefano et al. (2017), wherein the authors reported the strain-dependent fermentation capability of probiotic strains in terms of pH and lactic acid for PM-based probiotic products. Similarly, a reduction in the total solids content has been reported in the fermented skim milk/cereal combinations compared to their unfermented equivalents due to fermentation, corroborating the present investigation findings (Ganguly et al., 2022). Titratable acidity negatively correlated with the pH value, which was expected. For LF, LGG, and LF + LGG, respectively, there was a considerable increase in acidity during fermentation. The utilization of carbohydrates by lactic acid bacteria throughout the metabolic processes leads to a rise in the organic acids, especially lactic acid (Kuria, Matofari, & Nduko, 2021), which increases TTA, as seen in the present investigation.

Table 1 lists the fermented products' proximate composition (protein, fat, ash, carbohydrates, moisture). There were statistically substantial variations in the moisture content readings between the samples fermented with different cultures for varied fermentation times, with values ranging from 79.71 ± 0.18 to 81.67 ± 0.13%. Values for the ash content ranged from 0.90 ± 0.01 to 1.09 ± 0.07%. The control sample had the lowest value for ash content; however, the 8 h fermented sample (LF + LGG) recorded significantly ($P < 0.05$) higher ash content than that of control (unfermented). The fat content ranged between 0.28 ± 0.07 to 0.75 ± 0.10%, with the 8 h fermented sample (LGG) showing the highest fat. Recent research by Divisekera et al. (2021) supported our findings, who found that the moisture, ash, and fat contents of the control and fermented finger millet-based products ranged from 74.00 to 82.33%, 0.57 to 0.76%, and 0.08 to 0.14%, respectively. The present evaluation recorded varying protein content (4.27 to 5.59%) and carbohydrates (11.82 to 14.44%). Carbohydrate content was slightly decreased in most of the fermented substrates compared to the control. According to observations, the carbohydrate content decreased gradually during fermentation at different times in the process. These findings align with earlier results claiming that carbohydrates are a primary carbon source for bacteria to ferment sugars (Adams, 1990; Jan et al., 2022). The maximum protein level was found in the 8 h fermented product (LF), with values ranging from 4.01 ± 0.28 to 5.59 ± 0.22%. Our findings are consistent with those of Basu and Tomar (2016), who found that the PM-based fermented skim milk product contained protein (5.01%), ash (0.82%), fat (0.73%), and moisture (82.18%). The range of carbohydrate content values was 11.82 ± 0.02 to 14.44 ± 0.01%. Carbohydrate reduction could be related to the elevated efficiency of amylolytic enzymes, which results in complex carbohydrates being broken down into simpler forms of sugar. The same pattern of reduced carbohydrate content was observed resulting from the fermentation of maize (Gernah, Ariahu, & Ingbian, 2011), blends of maize-soybeans for weaning (Amankwah, Barimah, Acheampong, Addai, & Nnaji, 2009), 'Fura' (Inyang & Zakari, 2008), which are in consonance with data from present study. The biological activities of microorganisms could cause a decrease in dry matter during the fermentation process, which consumed a certain amount of the substrate nutrients, leading to an overall dry matter reduction (Wedad, El Tinay, Mustafa, & Babiker, 2008).

Table 1
Effect of selective fermentation on proximate composition of fermented millet-skim milk products.

Samples	Moisture (%)	Fat (%)	Ash (%)	Protein (%)	Carbohydrates (%)
Control	79.71 ± 0.18^{a}	0.45 ± 0.08^{ab}	0.90 ± 0.01^{a}	5.29 ± 0.16^{ac}	13.64 ± 0.25^{ade}
LF (8 h)	80.74 ± 0.14^{b}	0.28 ± 0.07^{a}	0.92 ± 0.01^{ab}	5.59 ± 0.22^{a}	12.48 ± 0.15^{bc}
LF (16 h)	81.51 ± 0.16^{cd}	0.49 ± 0.03^{ab}	1.01 ± 0.04^{ab}	4.39 ± 0.05^{bde}	12.60 ± 0.03^{abc}
LF (24 h)	81.18 ± 0.13^{c}	0.55 ± 0.06^{ab}	0.99 ± 0.11^{ab}	4.49 ± 0.39^{bde}	12.80 ± 0.35^{abc}
LGG (8 h)	79.87 ± 0.25^{a}	0.75 ± 0.10^{b}	0.97 ± 0.04^{ab}	4.00 ± 0.28^{be}	14.41 ± 0.67^{de}
LGG (16 h)	80.82 ± 0.12^{bc}	0.50 ± 0.05^{ab}	1.00 ± 0.01^{ab}	4.33 ± 0.01^{b}	13.35 ± 0.06^{abd}
LGG (24 h)	80.96 ± 0.17^{bc}	0.70 ± 0.12^{b}	0.10 ± 0.01^{ab}	4.50 ± 0.10^{bde}	12.83 ± 0.18^{abc}
LF + LGG (8 h)	80.59 ± 0.13^{bc}	0.52 ± 0.07^{ab}	1.09 ± 0.07^{b}	5.02 ± 0.22^{ade}	12.77 ± 0.21^{abc}
LF + LGG (16 h)	79.81 ± 0.11^{a}	0.45 ± 0.11^{ab}	1.03 ± 0.02^{ab}	4.27 ± 0.01^{bde}	14.44 ± 0.01^{e}
LF + LGG (24 h)	81.67 ± 0.13^{d}	0.74 ± 0.05^{b}	0.96 ± 0.04^{ab}	4.80 ± 0.13^{ce}	11.82 ± 0.02^{c}

Data expressed as mean ± SD.
At $p < 0.05$, mean values in the same column with distinct superscript letters (a, b, c …..) are statistically different.
Abbreviations: LF, *Limosilactobacillus fermentum* MS005; LGG, *Lactobacillus rhamnosus* GG 347.

3.2. Effect of fermentation on phytochemical and antioxidant of FMSMP

Phenolic substances are considered to provide several health advantages, primarily serving as potent antioxidants. Total polyphenol content (TPC) was higher in the control (unfermented) substrate compared to all fermented products except the 16 h and 24 h LGG fermentation (Fig. 2A). TPC ranged from 12.58 ± 0.53 to 53.91 ± 2.57 mg GAE/100 g sample. The 16 h and 24 h LGG fermentation products had TPC values of 39.68 ± 2.28 and 53.91 ± 2.57 mg GAE/100 g, respectively. Study results indicate that milk protein and phenolics from PM might interact, and phenolic releases during LGG fermentation were relevant. Sample fermented for 8 h, 16 h, and 24 h with LF or LF + LGG were found to have considerably ($p < 0.05$) lower TPC concentrations than the control sample, which may be due to the LF culture. LAB strain's hydrolytic enzymes degrade complex phenolic substances into simpler compounds, which results in a rise in TPC after LAB fermentation (Li et al., 2020). The results obtained in this study may be due to using LF culture for fermentation because the products from LF, singly or in combination, showed reductions in TPC. Polyphenol levels decreased during the fermentation of treated grains, indicating that the microflora can ferment phenolics (Bravo, 1998). Additionally, phenolic molecules might exhibit a prebiotic impact and promote the abundance of probiotics along with other beneficial bacteria, demonstrating a reciprocal link among phenolic substances and probiotics (de Llano et al., 2017; Gibson et al., 2017; Ozdal et al., 2016; Succi et al., 2017). Consequently, probiotics and starter cultures used in the present study might contribute to decreasing these bioactive substances by promoting the biotransformation of phenolic molecules during fermentation. The TFC overall trend in all samples differed from the TPC, as seen in Fig. 2B. TFC levels in fermented samples were generally higher in most categories than in control samples. However, only the 16 h and 24 h LGG fermented products demonstrated significant increases ($p < 0.05$) in TFC. TFC content ranged from 12.20 ± 1.24 to 20.70 ± 0.57 mg QE/100 g sample. Additionally, although the percentage of PM remained the same, TFC varied dramatically between samples fermented with different probiotic strains. This revealed that the growth of different cultures could considerably impact the TFC. The basis for this increase has been attributed to the LAB, which form compounds as metabolic byproducts

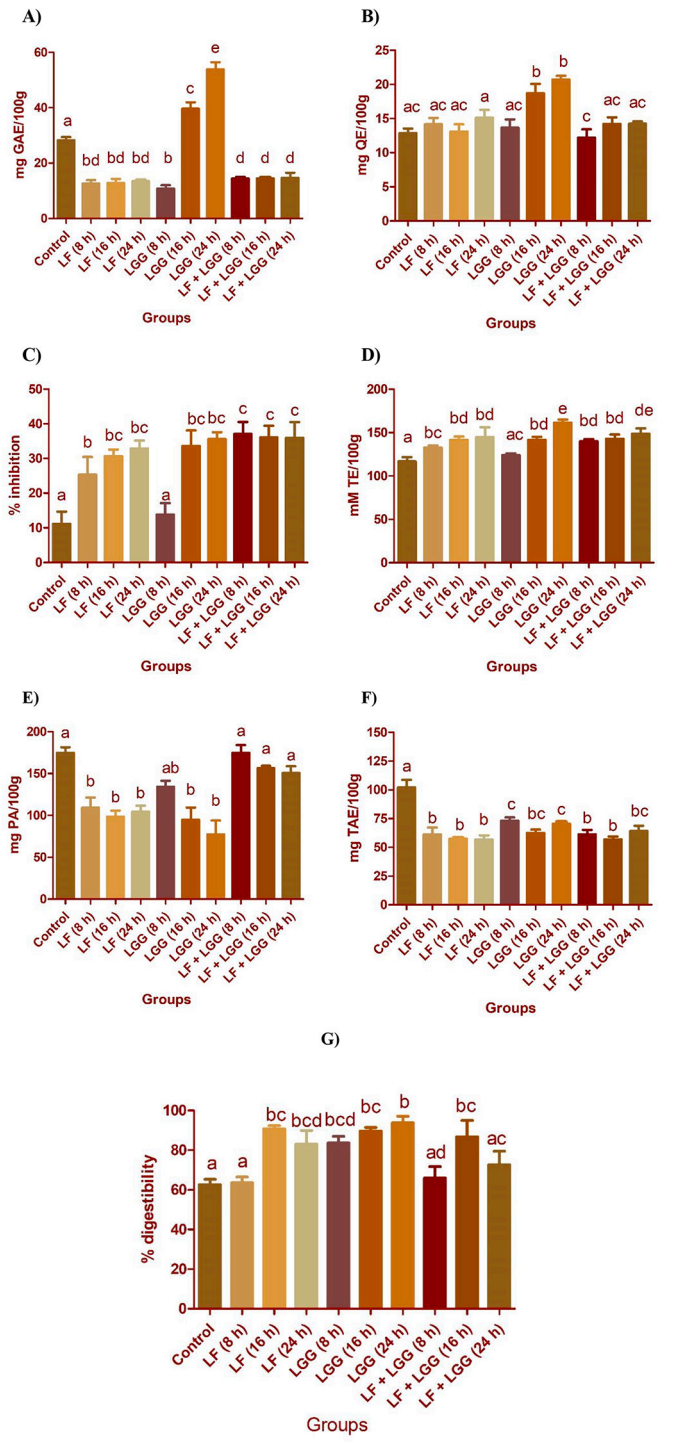

Fig. 2. Effect of selective fermentation on phytochemical content, antioxidative potential, anti-nutritional factors and protein digestibility (%) of fermented millet-skim milk products. Total Polyphenol Content (A); Total Flavonoids Content (B); 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (C); Ferric reducing antioxidant power (FRAP) (D); Phytic acid (PA) (E); Tannin Content (F); In vitro protein digestibility (IVPD %) (G).

Data expressed as mean ± SD,

At P< 0.05, Mean values bearing different letters (a, b, c, d) are significantly different.

Abbreviations: GAE, Gallic acid equivalent; QE, Quercetin equivalent; PA, Phytic acid; TE, Trolox equivalent; TAE, Tannic acid equivalent; LF, *Limosilactobacillus fermentum* MS005; LGG, *Lactobacillus rhamnosus* GG 347.

to increase the concentration of accessible bioactive molecules (Malik, Krishnaswamy, & Mustapha, 2022). The results are most likely related to the LAB's metabolic processes and may result in the breakdown of highly complex phenolic substances or the release of phenolic substances (such as flavonoids), which are linked to the structure of food materials (Dabbagh Moghaddam, Garavand, Razavi, & Dini Talatappe, 2018). According to a related work by Banwo, Asogwa, Ogunremi, Adesulu-Dahunsi, and Sanni (2021), the fermentation of millet and sorghum enriched with L. *fermentum* KL4, *L. plantarum* MOBL1, and *Candida tropicalis* OBY6 and MKY improved total flavonoids in the products.

The DPPH radical scavenging activity (RSA) and FRAP activity systems were used to test the antioxidant's effectiveness. Fig. 2C showed the results of DPPH activity (ranging from 11.14 ± 3.56% to 37.05 ± 3.51%) of all the samples fermented by different LAB cultures at varying fermentation times. Our findings are corroborated by Byresh et al. (2022), who observed an increase in antioxidative capability (DPPH) in a probiotic beverage (pineapple peel powder and white finger millet) fermented with L. *rhamnosus* GG NCDC 347. These results can be linked to a rise in TFC levels. In a fermented sample containing 10% PM flour and fermented with LF + LGG (24 h), the DPPH scavenging activity peaked at 37.05%, demonstrating that the millet has a high potential for free radical inhibition, which is enhanced by fermentation. Although it is worth noting that samples fermented by different strains of LAB had varying DPPH scavenging behavior even when the PM content was the same, this may be attributed to strain-specific effects and fermentation duration. As a result, fermentation with lactic culture influences the antioxidant potential of the products, which must be considered. However, observations also revealed that samples fermented with numerous LAB cultures exhibited substantial variations in DPPH potential even though the amount of sea buckthorn was still equal (Ge et al., 2022). According to the FRAP technique, an antioxidant might convert the Fe^{3+}-TPTZ complex into the violet-colored Fe^{2+}-TPTZ form. Fig. 2D showed the results of FRAP activity (ranging from 116.8 ± 4.95 to 161.4 ± 3.73 mM TE/100 g) of all the samples fermented with different culture conditions at various durations. The FRAP nearly followed the same pattern as the DPPH results showed. The FRAP peaked at 161.4 ± 3.73 mM TE/100 g in a sample fermented for 24 h with LGG, exhibiting that PM has a significant potential for free radical scavenging activity, which is improved by fermentation. All of the fermented products, except that of 8 h LGG, had considerably ($p < 0.05$) elevated reducing antioxidant power compared to the control sample. Therefore, it is essential to consider that fermentation influences the antioxidant activity. Several studies have demonstrated the breakdown of proteins into small peptides during LAB fermentation to enhance the effectiveness of antioxidants (Aloğlu & Öner, 2011; Xiao et al., 2015). Furthermore, an increase in the activity of antioxidants has been shown to be positively linked to the level of proteolysis. Moreover, lactic acid, free amino acids, and isoflavones generated by the fermentation of legumes can enhance the antioxidant capacity of the products (Gan, Shah, Wang, Lui, & Corke, 2017).

3.3. *Effect of fermentation on anti-nutritional factors and mineral molar ratio*

Anti-nutritional substances prevent proteins and minerals from being digested and assimilated by chelating metals and preventing their hydrolysis. Phytate and tannin contents of fermented substrate ranged from 77.47 to 174.80 mg/100 g and 56.91 mg to 102.10 mg/100 g, respectively. Fig. 2E represents the results of phytic acid in fermented and control (unfermented) products. The tannin content in all the fermented samples was much reduced after fermentation treatments when compared to the control (Fig. 2F). Samples fermented with LF for 8 h, 16 h, and 24 h showed tannin contents of 61.13 ± 2.43 mg TAE/100 g, 57.40 ± 0.24 mg TAE/100 g, and 56.65 ± 3.82 mg TAE/100 g, respectively. The primary mechanism of action involves microbial

breakdown by enzymes like tannin acyl hydrolases, and phytases. Under the present investigation, results showed that all the singly fermented treatments of LF and LGG led to considerable ($p < 0.05$) reductions in phytic acid quantity as compared to the control (unfermented). However, all the co-culture (LF + LGG) fermented substrates showed a slight decrease in phytic content but were not significant ($p > 0.05$) when compared to the control. Our findings for phytic acid decrease owing to fermentation align with those of Chaudhary and Mudgal (2020), who observed phytic acid and tannin content reductions in a milk-finger millet composite probiotic fermented product. Present observations are supported by Jan et al. (2022), who observed a substantial ($P < 0.05$) reduction in the anti-nutrient compounds in finger millet flour after diverse fermentation procedures. Similarly, a most recent investigation validated the influence of fermentation on phytic acid level reduction in fermented skim milk-cereal (PM) mixtures compared to unfermented substrates. Previous work also showed that fermentation produced a substantial ($p < 0.01$) 78% drop in phytic acid concentration (Ganguly et al., 2022). Under the present investigation, tannin content was significantly reduced in the fermented substrates (28.30 to 44.5%) when compared to the control (unfermented). Results suggest that the highest tannin reduction was observed in the product obtained from LF culture fermentation for 24 h, while the lowest tannin reduction was recorded for the LGG 8 h fermented product. Our findings are supported by a recent study that confirmed the fermentation of both proso and kodo millets, with initial tannin concentrations of 17.50 g/L and 16.45 g/L, which reduced to 3.01 g/L and 5.07 g/L, respectively (Malik et al., 2022). Likewise, Asres, Nana, and Nega (2018) demonstrated that spontaneous fermentation reduced the anti-nutritional components present in cereal grain products, subsequently improving the mineral's extraction level. Study outcomes confirmed that the FMSMP obtained from co-culture (LF + LGG) showed the lowest decrease in the phytic acid content, which suggests that combined fermentation is not as efficient as single-culture fermentation in enhancing the bioavailability of iron and zinc in millet-based functional beverages.

PA is recognized for its well-known negative impact on the availability of minerals (Frontela, Ros, & Martínez, 2011). Under the present investigation, PA/mineral molar ratios were determined for the iron and zinc content of control (unfermented) and fermented products (Table S1). The mineral molar ratio results correlated with findings of in vitro micronutrient bioavailability; thus, both demonstrate that co-culture fermented substrates have reduced micronutrient bioavailability and maximum PA/minerals for iron and zinc. In the present scenario, some crucial parameters of the phytate/mineral molar ratio have been interpreted as a marker of the possible bioavailability of minerals (Marin, Siqueira, & Arruda, 2009). Comparable prior investigations suggested the following anticipated critical values: (PA/Zn) > 15 for Zn, and (PA/Fe) > 1 for Fe (Hallberg, Brune, & Rossander, 1989; Turnlund, King, Keyes, Gong, & Michel, 1984), which were followed in the current study. The phytate content and its molar ratios are assumed to measure the product's dietary minerals bioavailability, and the quantities have been estimated to compare with the ratios recommended as crucial values. While the molar ratio of all the fermented products made from the PM-skim milk composite was considerably less ($p < 0.05$) than the unfermented control, the molar ratio of all the samples was still above the critical value of iron, i.e., >1 Fe. The outcomes are consistent with the recent finding by Ayub, Castro-Alba, and Lazarte (2021), who also reported a significant reduction in the PA/Fe molar ratio of quinoa fermented instant-mix probiotic beverage. However, the PA/Fe molar ratio was still above the critical value of iron, i.e., >1. Similarly, other studies reported mineral molar ratios of fermented groundnut flour samples above the critical values of iron (>1) and zinc (> 15) (Ijarotimi, Ogunmola, & Oluwajuyitan, 2022). Similarly, a recent investigation found that extending the fermentation period lowered the median molar ratio of PA/Fe of all kisra bread considerably ($p < 0.05$), but it was above the crucial value (>1) for iron (Ahmed, Xu, Sulieman, Na, & Mahdi, 2020), which is also in line with present investigation findings. In the present study, the molar ratio of PA/Zn for the 16 h and 24 h LF or LF + LGG fermented products were below or near 15, which suggests that these samples have high zinc bioavailability. Mainly, the molar ratios of PA/Fe and PA/Zn reduced considerably over the fermentation duration for all fermented substrates in the present investigation, even though the ratios of iron for all samples and ratios of zinc for most samples were above the proposed critical values.

3.4. *Effect of fermentation on in vitro protein digestibility and micronutrient bioavailability*

The nutrient's digestibility, in addition to the nutritional and bioactive constituents of food, is essential. As shown in Fig. 2G, the IVPD of most of the fermented PM-containing samples in the current investigation was significantly higher than the control (unfermented). Results confirmed that the digestibility of protein was significantly increased in all the fermented samples compared to control (unfermented), except for the LF (8 h) and LF + LGG (8 h and 24 h) fermented samples. The IVPD data showed that LF (16 h) and LGG (24 h) fermented samples had excellent digestibility, i.e., 90.75% and 93.76%, respectively, compared to 62.60% for the unfermented sample. Our results are supported by a recent study, which reported that a fermented finger millet yogurt-like beverage had a higher IVPD of 64% than 25% for unfermented flour (Vila-Real et al., 2022). Similarly, Sharma, Sharma, and Singh (2022) confirmed that the IVPD of fermented proso millet was significantly enhanced compared to untreated proso millet due to the fermentation process. Relative changes in dry matter loss occur during the fermentation process due to the activity of microbes that metabolize and hydrolyze lipids and carbohydrates as energy sources. Plant protein digestibility, on the other hand, increases with fermentation (Nkhata et al., 2018). Similarly, according to our findings, De Pasquale, Pontonio, Gobbetti, and Rizzello (2020) reported that fermentation with *L. brevis* and L. *plantarum* improved the nutritional characteristics of treated legume flour by increasing protein digestibility. Reasons for this possibly beneficial impact include the fact that a decrease in pH coupled with increased activity of bacteria's proteolytic enzymes causes the protein to be broken down into smaller peptides with elevated nutritional value as an outcome of fermentation (Sripriya, Antony, & Chandra, 1997). Data on IVPD also support the present investigation's results on anti-nutrients, as a recent study reported reduced amounts of tannins, which leads to increased protein digestibility (Joye, 2019). In the same way, fermentation improved protein digestibility in PM, which might be explained by the microflora's potential production of proteolytic enzymes. Anti-nutritional elements have been shown to negatively impact the digestibility of proteins because they can bind to proteases, restricting their actions and preventing them from being hydrolyzed (Cirkovic Velickovic & Stanic-Vucinic, 2018).

Fermentation also improves mineral bioavailability by producing a phytase enzyme that degrades phytic acid in plant-based foods. For instance, phytic acid reduction may enhance calcium, iron, and zinc levels several-fold (Samtiya, Aluko, Puniya, & Dhewa, 2021). The data show that the Fe level in control and fermented samples was in the 5.01 to 7.23 mg/kg range (Fig. 3A). The iron bioavailability findings for control and fermented samples are presented in Fig. 3A. As a result of fermentation compared to the control (unfermented), Fe bioavailability significantly improved in most of the fermented samples. The range of the Zn level in control and fermented samples was 5.72 to 7.21 mg/kg (Fig. 3B). The results of zinc bioavailability for control and fermented samples are presented in Fig. 3B. Blue column showing the micronutrient present in the unfermented (control) and fermented samples in mg/Kg, however, brown column presents micronutrient bioavailability (%) in unfermented (control) and fermented samples after in vitro micronutrient bioavailability assay. Results confirmed that iron bioavailability was considerably increased in all the fermented products using LF or LGG when compared to the control (unfermented). However, a considerable reduction in vitro micronutrient (Fe and Zn)

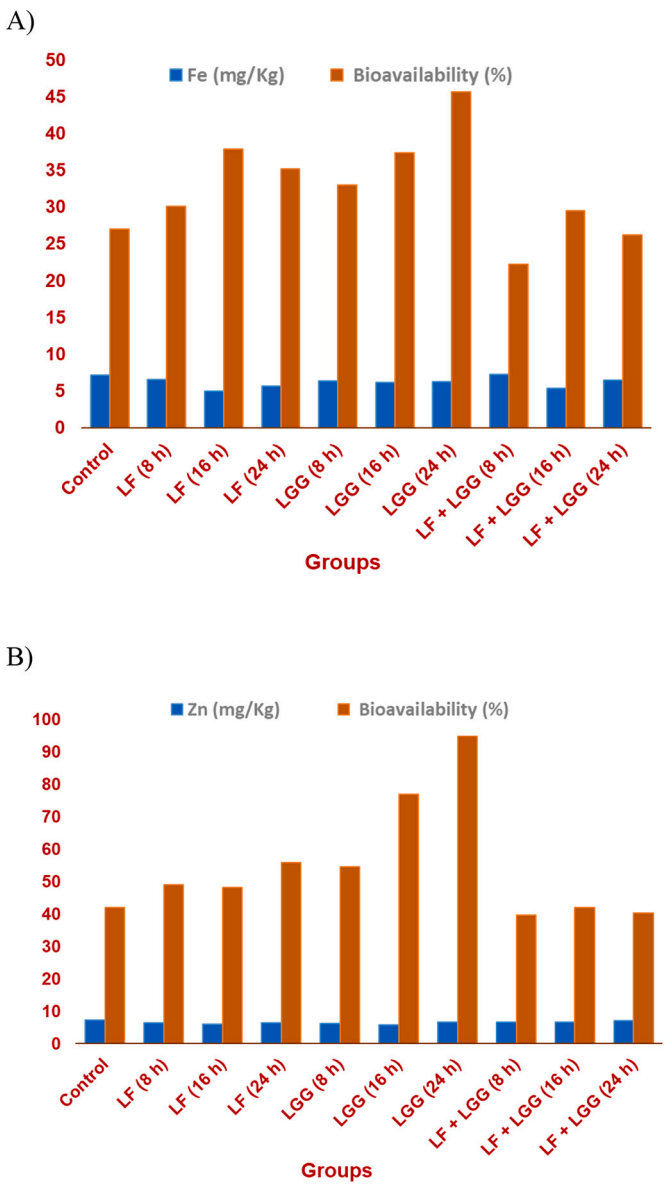

Fig. 3. Effect of selective fermentation on in vitro micronutrients bioavailability of fermented millet-skim milk products. Iron (mg/kg) and bioavailability (%) (A); Zinc (mg/kg) and bioavailability (%) (B).
Abbreviations: Fe, Iron; Zn, Zinc; LF, *Limosilactobacillus fermentum* MS005; LGG, *Lactobacillus rhamnosus* GG 347.

bioavailability was observed for the substrates fermented by co-culture (LF + LGG) treatment. These results are supported by the phytic acid content, where no considerable reduction was observed for all the samples fermented by co-culture (LF + LGG) treatment. The micronutrient bioavailability results of the present investigation agree with a previous study by Basu and Tomar (2016), who assessed the micronutrient bioavailability of PM-based fermented skim milk products. Results found that fermented products' micronutrient bioavailability (Ca, Fe, Zn, Mn, and Cu) was much improved compared to unfermented products. Similar to this, earlier research found that the bioavailability of iron in skim milk cereal-based fermented substrates was improved (Ganguly et al., 2022). The data concluded that LGG (24 h) fermented samples measured for the highest iron and zinc bioavailability compared to other fermented samples. However, LGG could not develop an organoleptically acceptable sour flavor during sample fermentation on its own and did not adequately ferment the sample even after 24 h. Conversely, LF (16 h) fermented samples were recorded for the highest iron bioavailability (37.82%) compared to all other fermented samples. Consequently, LF ferments samples properly and produces excellent organoleptic flavor, so LF (16 h) could be used to make millet skim milk based fermented products. The increase in bioavailability in the LF (16 h) sample compared to the control group was 39.8% and 14.5% for iron and zinc, respectively. Our findings are reinforced by previous studies, which reported that fermentation by lactobacilli strains decreased phytate levels and enhanced the availability of micronutrients (mainly Fe and Zn). Recently, a study reported that fermentation (4 or 10 h at 30 °C) of milled quinoa seeds using L. *plantarum* 299v reduced the phytic acid content significantly and enhanced the availability of micronutrients including zinc, iron, and calcium (Castro-Alba et al., 2019; De Castro, Cunha, Barreto, Amboni, & Prudencio, 2009). Consequently, the resultant reduction of the phytate level turned fermentation into a possible technique to enhance mineral bioavailability. Therefore, as an outcome, the ultimate nutritional value of the fermented item may be improved.

3.5. *Effect of fermentation on texture, functional groups, rheology, and color profile*

The phrase “food texture” refers to a broad range of textural features or attributes that consumers consider when assessing the quality and acceptability of food products (Paredes, Cortizo-Lacalle, Imaz, Aldazabal, & Vila, 2022). The parameters of hardness, chewiness, gumminess, cohesiveness, and springiness (Table 2) were evaluated for control and fermented samples; a non-significant ($P > 0.05$) difference was detected between both in terms of cohesiveness or springiness. Results of the study confirmed that non-significant ($P > 0.05$) variations were observed for the chewiness, gumminess, cohesiveness, and springiness of all the fermented samples compared to the control. However, all the co-culture (LF + LGG) samples fermented for 24 h showed considerable ($P < 0.05$) changes in chewiness in comparison to the unfermented sample. Adhesiveness represents the force needed to remove the material that attaches to the teeth while consuming food, which is an opposing force (Delikanli & Ozcan, 2014). Likewise, another study by Park et al. (2005) described the texture parameters (hardness, springiness, gumminess, and cohesiveness) in the yogurt-like products (prepared by skim milk and soymilk containing saccharified rice), which supported the results of the present study. Similarly, our results align with the previous investigation, which reported oat-based yogurt texture properties (Raikos, Juskaite, Vas, & Hayes, 2020). Our springiness, gumminess, and cohesiveness observations agree with the data reported by Mudgil, Barak, and Khatkar (2017), which recorded the same range of these texture parameters. Under the present study, fermented products' springiness was higher than the control sample, suggesting that fermented samples containing PM regained their initial form more quickly once the force deformed it was removed.

The FTIR technique identifies functional groups and structural variations at the molecular scale of several food samples. The chemical interactions that exist in the molecule's structure in the form of peaks contributed to identifying the functional groups in samples based on natural frequencies of vibration (Fanelli, Zimmermann, Totoli, & Salgado, 2018). Fig. 4 shows the FTIR spectra of the fermented and control samples. All the fermented and control (unfermented) samples showed a broad peak between 3300 and 3200 cm^{-1} and a sharp peak near 2930 cm^{-1}, which signifies that the stretching of O—H groups (H-bonds) and C—H were present in all the samples. Our findings were corroborated by the prior investigation, which revealed that the test and control samples displayed identical peaks around 3450.65 cm^{-1}. This would indicate that stretching the hydroxyl group (O—H) bond in alcohols could be responsible (Byresh et al., 2022). Similarly, in a fermented beverage, Zhao et al. (2021) indicated a distinctive peak at 2937 cm^{-1}, denoting the aromatic ester's existence. The present study recorded all the samples for other peaks near 2350, 2120, 2000, 1650 to 1550, and 1450 to 1000 cm^{-1}. Several absorption zones are associated with stretching the

Table 2
Effect of selective fermentation on texture parameters of fermented millet-skim milk products.

Samples	Hardness	Chewiness	Gumminess	Cohesiveness	Springiness
Control	19.15 ± 0.99^{ae}	2.51 ± 0.20^{ac}	7.78 ± 0.35^{ac}	0.41 ± 0.04^{a}	0.32 ± 0.012^{a}
LF (8 h)	24.16 ± 2.42^{ae}	3.01 ± 0.09^{ac}	8.39 ± 0.18^{abc}	0.35 ± 0.04^{a}	0.36 ± 0.003^{a}
LF (16 h)	25.63 ± 2.28^{ad}	2.46 ± 0.13^{ac}	7.35 ± 0.05^{ac}	0.29 ± 0.03^{a}	0.34 ± 0.015^{a}
LF (24 h)	34.85 ± 1.16^{bdf}	3.45 ± 0.00^{ad}	9.96 ± 0.17^{ab}	0.29 ± 0.01^{a}	0.35 ± 0.006^{a}
LGG (8 h)	19.07 ± 0.98^{ae}	2.49 ± 0.00^{ac}	7.84 ± 0.11^{ac}	0.34 ± 0.01^{a}	0.33 ± 0.010^{a}
LGG (16 h)	20.35 ± 4.39^{aed}	2.09 ± 0.56^{c}	6.39 ± 1.95^{ac}	0.31 ± 0.03^{a}	0.33 ± 0.013^{a}
LGG (24 h)	16.55 ± 0.12^{e}	2.44 ± 0.07^{ac}	6.18 ± 1.63^{c}	0.37 ± 0.10^{a}	0.41 ± 0.095^{a}
LF + LGG (8 h)	29.07 ± 3.64^{cd}	3.03 ± 0.31^{ac}	8.39 ± 0.68^{acd}	0.29 ± 0.01^{a}	0.36 ± 0.008^{a}
LF + LGG (16 h)	36.35 ± 0.82^{f}	3.38 ± 0.05^{ad}	9.93 ± 0.19^{ad}	0.27 ± 0.001^{a}	0.34 ± 0.002^{a}
LF + LGG (24 h)	39.33 ± 0.99^{cf}	4.44 ± 0.74^{bd}	11.73 ± 1.17^{bd}	0.30 ± 0.04^{a}	0.38 ± 0.025^{a}

Data expressed as mean ± SD.
At $p < 0.05$, mean values in the same column with distinct superscript letters (a, b, c …..) are statistically different.
Abbreviations: LF, *Limosilactobacillus fermentum* MS005; LGG, *Lactobacillus rhamnosus* GG 347.

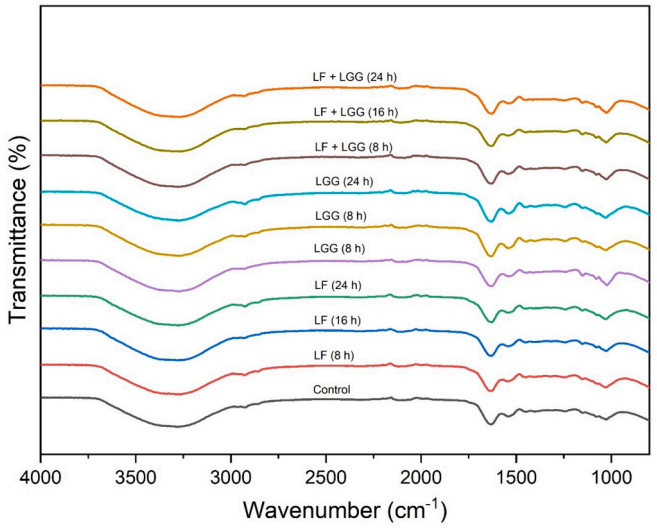

Fig. 4. Fourier transform infrared spectroscopy (FTIR) spectrum obtained from fermented millet-skim milk products.

CH and OH groups of molecules like organic acids and sugars, including glucose and fructose, that may be observed between 1500 and 960 cm^{-1} (Cozzolino, Cynkar, Shah, & Smith, 2011; Wu et al., 2015). Nevertheless, FTIR spectrum bands with certain variations within the wavenumber 1000–1500 cm^{-1} may be attributed to the binding to specific biologically active elements that exist (such as phenolic substances) and milk proteins' carboxyl groups (Mehanna et al., 2014). Similarly, another study's results are in line with our findings, reporting that the band between 1542 cm^{-1} and 965 cm^{-1} is assigned to a vibration of the C—O, C—N, and C—N, suggesting the presence of organic acids, sugars, and ethanol in this region. Stretching vibration of the C—C bond in the phenolic group absorption peak occurs between 1500 cm-1 and 1400 cm^{-1} while the peak bands at 1098 cm^{-1} and 995 cm^{-1} are assigned to OH deformation and C—O stretching in the phenolic group, in the fermented beverage (Ayed, Ben Abid, & Hamdi, 2017).

Apparent viscosity is an essential measure that could be utilized to evaluate the quality of dairy products developed using various procedures. The control (unfermented) and fermented samples were tested for rheological behavior, as shown in Fig. S2 (A to C). The apparent viscosity was initially high but gradually decreased as the shear rate increased. The rise in viscosity could be linked to the formation of a gel or network due to physicochemical and biochemical alterations after fermentation of the substrate. On the other hand, LGG fermented products had considerably lowest viscosity compared to LF and co-culture (LF + LGG). The results suggest that LGG culture could not singly ferment the substrate, which is also supported by the TSS, pH, lactic acid, and texture (hardness) data, as previously discussed in the present investigation. In agreement with current investigation results, Ge et al. (2022) reported apparent viscosity in similar ranges for the fermented milk product supplemented with sea buckthorn. Similarly, the effect of pattern on apparent viscosity against shear rate was demonstrated by a millet-based yogurt-like product (Song et al., 2020). Our results are supported by an earlier study, which reported that as the shear rate increased, the apparent viscosity of the skim milk cereal-based substrates (fermented and unfermented) decreased, representing a non-Newtonian fluid behavior. Both samples displayed shear thinning properties, as demonstrated by the fact that the apparent viscosity of both substrates reduced as the shear rate increased at a particular temperature (Ganguly et al., 2022). Similarly, symbiotic lactic beverage (De Castro et al., 2009) and inulin-enriched yogurt (Donkor, Henriksson, Vasiljevic, & Shah, 2007) obtained results aligned with the current findings. As shear time increased, viscosity decreased, demonstrating that the shear dilution condition was correlated with an interaction between apparent viscosity and shear duration. As a result, the FMSMP obtained from LF fermentation was more stable and suited for industrial dairy product manufacturing.

Table S2 shows the color parameters of control and fermented samples. Results showed that the lightness color parameter (L*) value was substantially ($p < 0.05$) increased in all the fermented substrates when compared to the unfermented sample, except the LF (8 h) fermented sample, which had a slightly high but not significant ($p > 0.05$) L* value. The increased L* value might be defined as the oxidation of pigmented components, which results in improved light color of the fermented products. Similar to our findings, Ganguly et al. (2022) reported a considerable rise in the L* value because of skim milk cereal-based composite substrate fermentation compared to the unfermented product. The findings are similar to the outcomes stated by Gong et al. (2020), who found that potato flour lightness intensity gradually rose with fermenting duration. Our findings are corroborated by a recent study by Navyashree, Buvaneswaran, Sunil, Rawson, and Natarajan (2022), who reported L* and b* value color profiles in the white finger millet probiotic beverage that are similar to the range obtained in the current study. In the present study, no significant changes were measured for a* value in fermented samples compared to the control, except in the LGG (8 h) sample. Numerous investigations reported color values for L*, a*, and b* in non-dairy or dairy functional products (Ge et al., 2022; Öztürk et al., 2018; Park et al., 2005; Sharma, Singh, Deshwal, Rao, & Kumar, 2021) that are similar to those obtained in the current study.

4. Conclusion

The present study was conducted to determine the best possible fermentation combination for biofortified HHB-311 PM and skim milk mixtures that yield fermented probiotic products with enhanced

micronutrient bioavailability, protein digestibility, and other techno-functional characteristics, in addition to reduced levels of anti-nutrients. Substrates were fermented with indigenous LF and a known probiotic culture LGG in three selective fermentation combinations. All the singly fermented treatments using LF or LGG resulted in significant ($p < 0.05$) reductions in phytate content as compared to the control (unfermented). Singly fermented products had significantly reduced phytic acid content with concomitant increment in in vitro protein digestibility and bioavailability of micronutrients when compared to the product from co-culture fermentation. A significant negative correlation was observed between phytic acid and in vitro micronutrient bioavailability. The highest tannin reduction was observed in the 24-h fermented product from LF. The results showed improved iron bioavailability for the fermented LGG (24 h) sample. However, at the time of sample fermentation, LGG could not singly produce an organoleptically good sour taste and did not properly ferment the sample even after 24 h. On the other hand, LF 16 h fermentation time showed improved iron (39% increase) and zinc (14% increase) bioavailability and proper fermentation of the sample. The results suggest that the LF 16 h fermentation is an excellent process to make millet-based fermented products as a result of the improved in vitro protein digestibility and micronutrient bioavailability as well as better techno-functional attributes. The study results concluded that fermentation improved the micronutrient bioavailability and protein digestibility by reducing anti-nutritional factors that negatively affect absorption. Overall, we conclude that various low-cost but nutritious millet-based probiotic foods can be produced from fermentation with different microorganisms that were used in this study.

Ethics approval and consent to participate

Not applicable.

Funding

The authors are thankful to the Haryana State Council for Science Innovation and Technology India (Project:HSCSIT/R&D/2021/461) for funding this research work.

CRediT authorship contribution statement

Mrinal Samtiya: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Prarabdh C. Badgujar:** Writing – review & editing, Resources, Conceptualization. **Gauri A. Chandratre:** Writing – review & editing, Resources. **Rotimi E. Aluko:** Writing – review & editing, Data curation. **Ashwani Kumar:** Writing – review & editing, Data curation. **Bharat Bhushan:** Writing – review & editing. **Tejpal Dhewa:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Authors are grateful to the Director, National Institute of Food Technology Entrepreneurship and Management, Kundli (NIFTEM-K), Sonipat and Vice Chancellor, Central University of Haryana, Jant-pali, Mahendergarh for providing necessary support and research facilities. The NIFTEM-K internal manuscript reference number is NIFTEM-P-2024-73.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101483.

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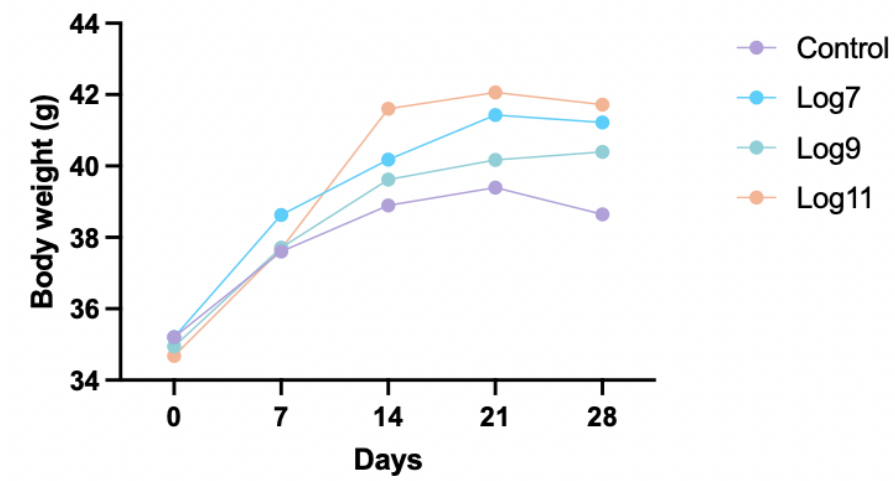

Figure 1. Effect of *L. fermentum* on body weight of mice after 28 days oral exposure

Data is analysed by one-way and two-way ANOVA. * indicate significant difference relative to 0th day at *P<0.05*.

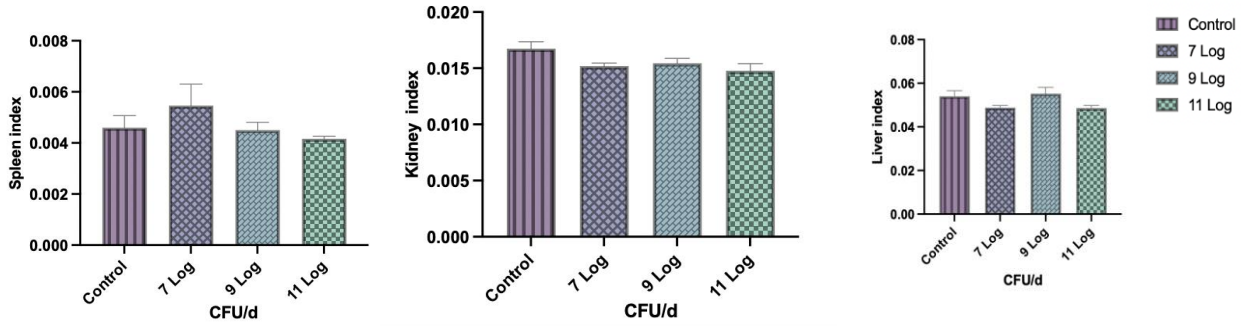

Figure 2. Effect of *L. fermentum* on organ index of mice after 28 days oral exposure

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA.

Table 1: Effect of *L. fermentum* on hematology of mice after 28 days oral exposure

Haematological Parameters	Control	7 Log CFU/d	9 Log CFU/d	11 Log CFU/d
HB (g/dL)	13.97 ± 1.73	14.22 ± 1.07	15.26 ± 0.58	15.46 ± 0.35
RBC (10^3/µL)	9.52 ± 0.66	9.25 ± 0.28	9.75 ± 0.47	9.16 ± 0.11
TLC (10^3/µL)	12.63 ± 2.08	8.81 ± 3.23	9.49 ± 2.03	10.65 ± 3.69
PCV (%)	47.68 ± 1.91	46.86 ± 2.29	49.13 ± 1.93	47.46 ± 2.62
MCV (fl)	50.39 ± 1.25	50.51 ± 1.35	50.12 ± 2.96	51.12 ± 2.52
MCH (pg/mL)	16.58 ± 1.99	15.79 ± 0.52	15.69 ± 1.32	16.89 ± 0.46
MCHC (g/dL)	31 ± 0.99	31.31 ± 1.33	31.29 ± 1.45	32.8 ± 1.55

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. * significant difference relative to control at *P<0.05*

Table 2: Effect of *L. fermentum* on serum biochemical parameters of mice after 28 days oral exposure

Other Biochemical Parameters	Control	7 Log CFU/d	9 Log CFU/d	11 Log CFU/d
Triglycerides	145.7 ± 20.6	118.3 ± 15.3	97.7 ± 13.6	306 ± 26
Cholesterol	99.7 ± 29.3	96.3 ± 9.3	83.3 ± 18.8	102 ± 14
Uric acid	2.81 ± 0.45	2.61 ± 0.304	2.97 ± 0.44	2.09 ± 0.15
Albumin	2 ± 0.62	2.18 ± 0.15	2.21 ± 0.2	2.8 ± 0.67
Protein	155.3 ± 18.8	155.1 ± 7.6	161.2 ± 17.5	146.7 ± 6.9
HDL Cholesterol	5.77 ± 1.4	6.29 ± 0.83	6.42 ± 0.28	6.4 ± 1.12

Values are expressed as mean ± SD (n=3). *Data is analysed by one-way ANOVA. * significant difference relative to control at P<0.05.*

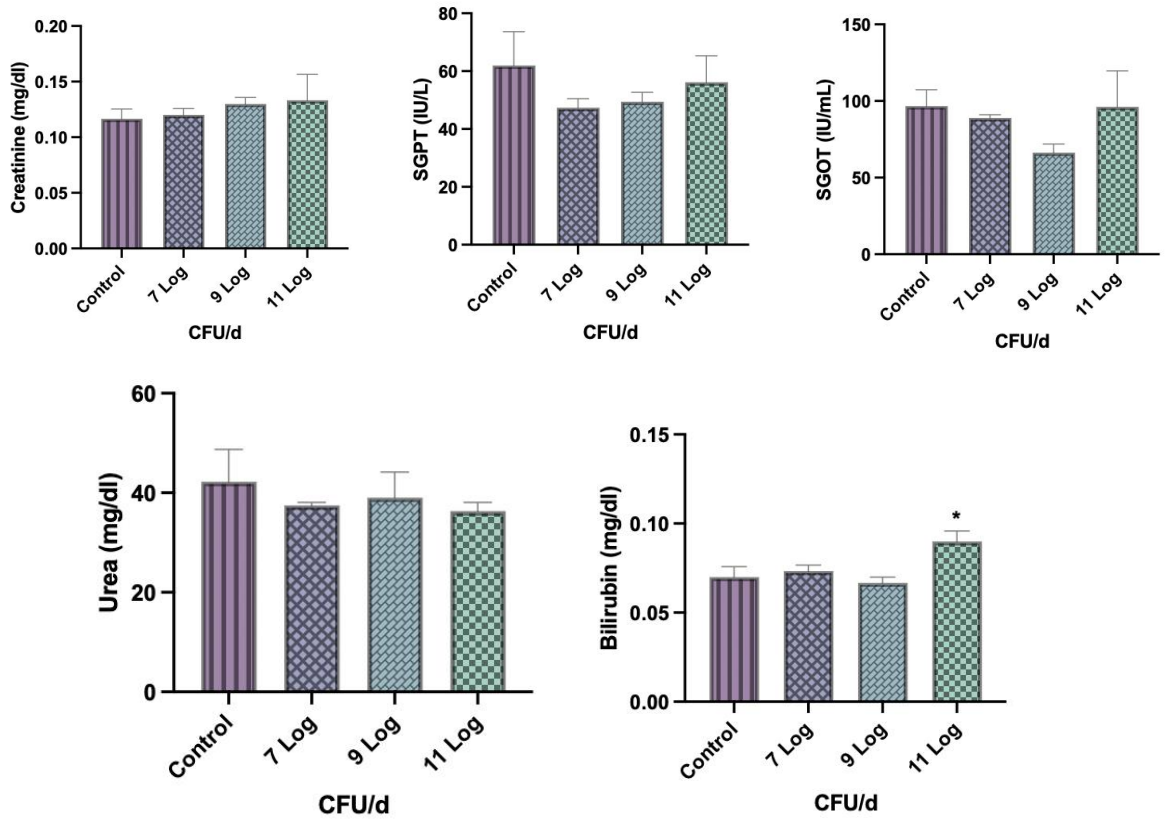

Figure 3. Effect of *L. fermentum* on serum biochemical parameters of mice after 28 days oral exposure

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. * significant difference relative to control at *P<0.05*

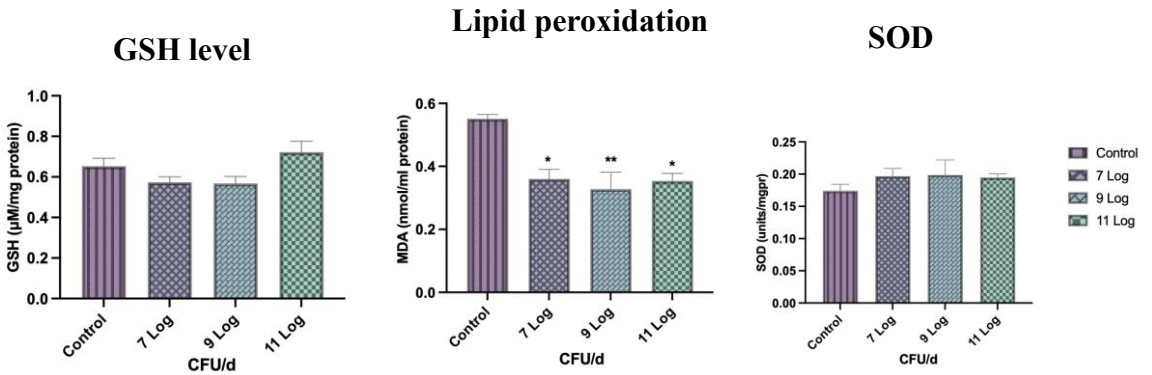

Figure 4. Effect of *L. fermentum* on oxidative stress markers in liver of mice after 28 days oral exposure

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. * and ** indicate significant difference relative to control at *P<0.05 and P<0.01.*

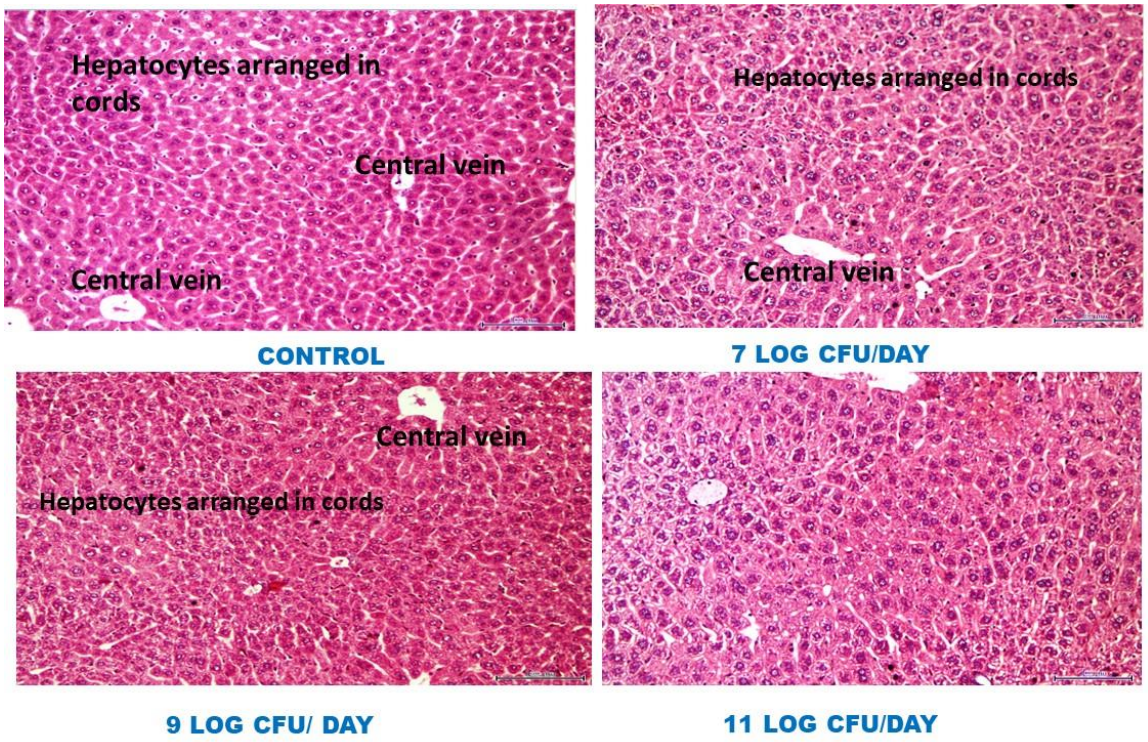

Figure 5. Liver: Normal histological architecture containing central vein and arranged hepatic cords in hepatic parenchyma in **control as well as in mice administered with 7, 9 & 11 log CFU/d lactobacilli e*x 200 H & E stain***

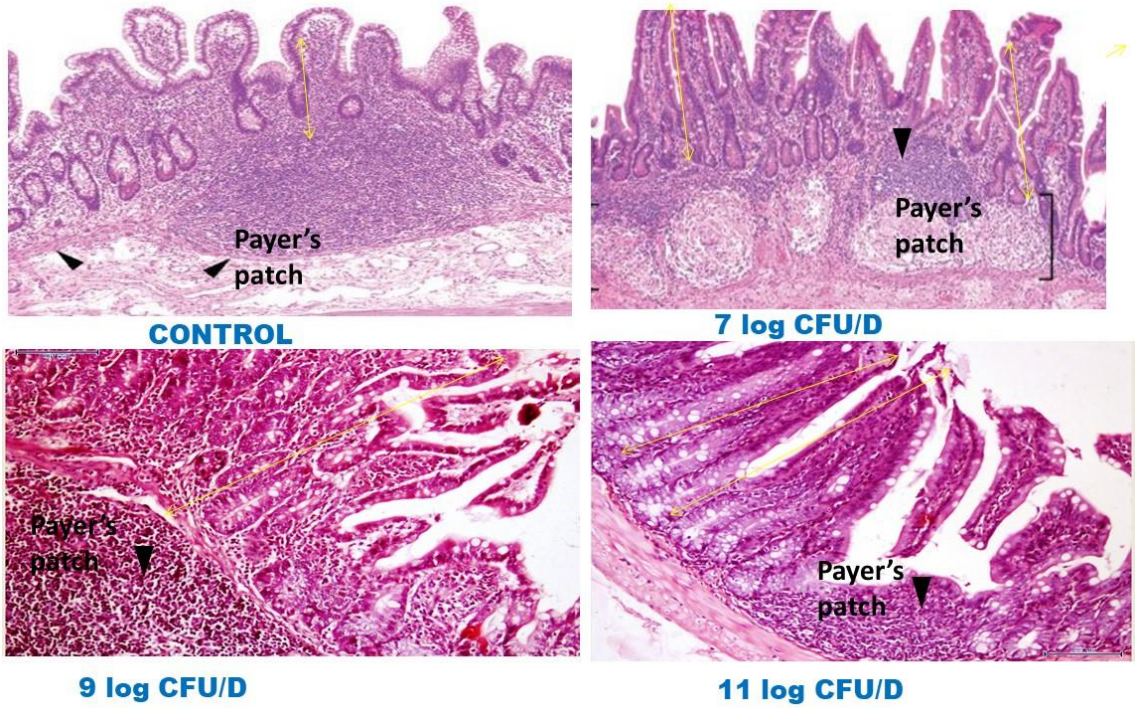

Figure 6. Ileum: Adequate length of villi and enough lymphocytes in Payer's patches of control mice, increase **in length of villi and dense population of lymphocytes in payer's patch** of mice dosed 7, 9, & 11 log CFU/d lactobacilli ***x 200 H & E stain***

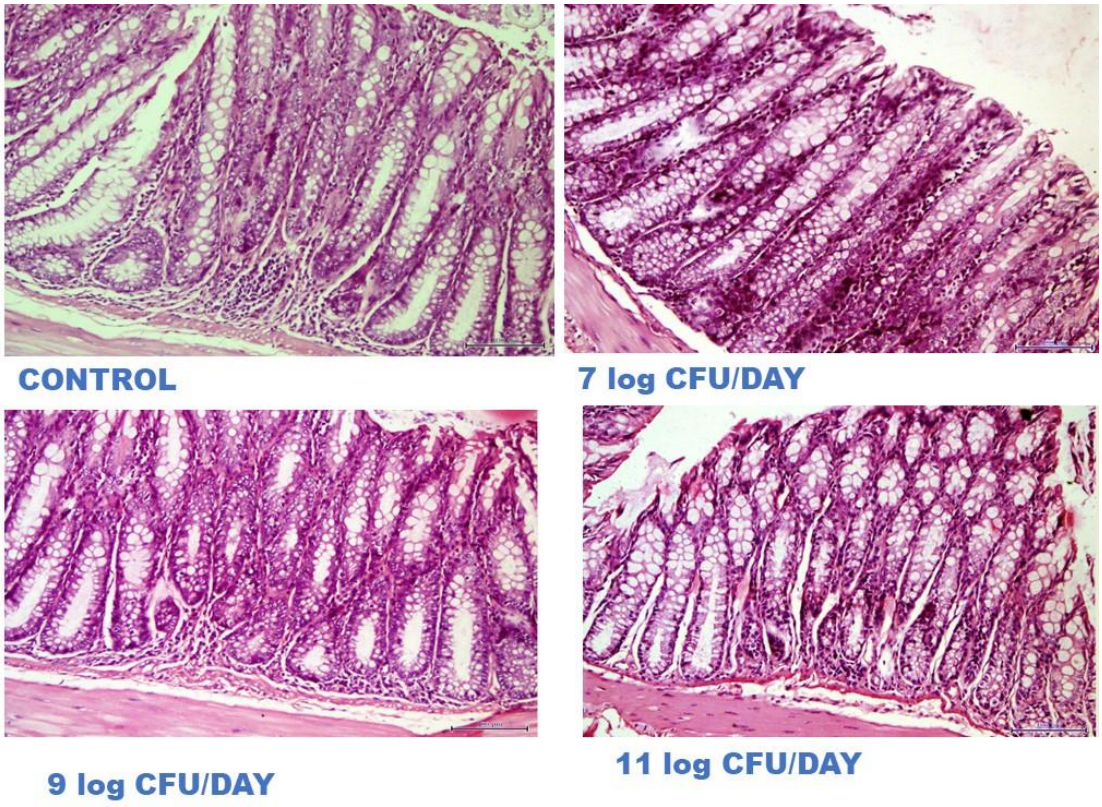

Figure 7 Colon: Normal histological structure of colon **in control and mice administered with 7, 9 & 11 log CFU/d Lactobacilli** *x 200 H & E stain*

Immunomodulation study

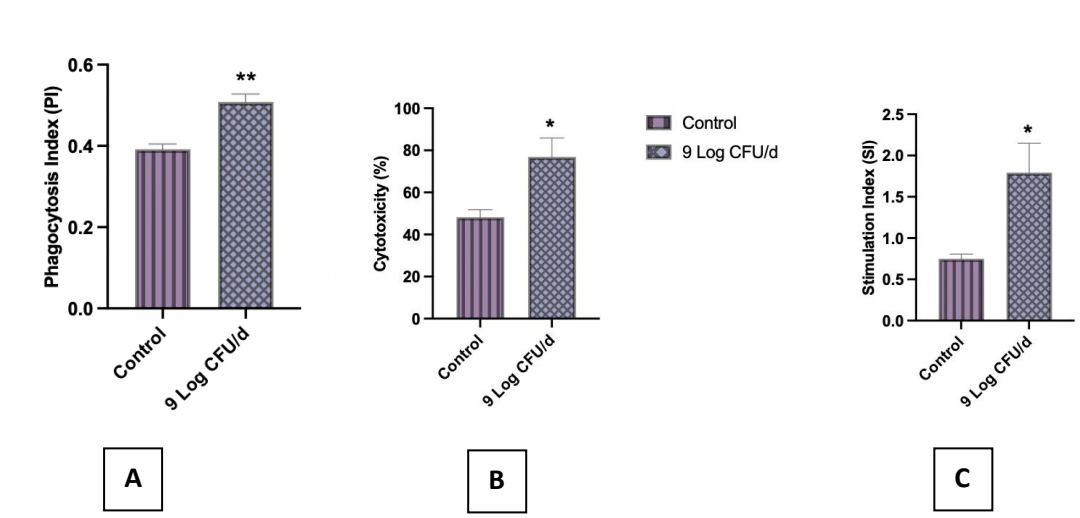

Figure 8. Effect of *L. fermentum* on (A) Macrophage phagocytosis capacity, (B) NK cell activity and (C) Lymphocyte proliferation assay of mice after 28 days oral exposure

Data (n=3/gr) is analysed by unpaired t test. * and ** indicate significant difference relative to control at *P<0.05* and *P<0.01*, respectively.

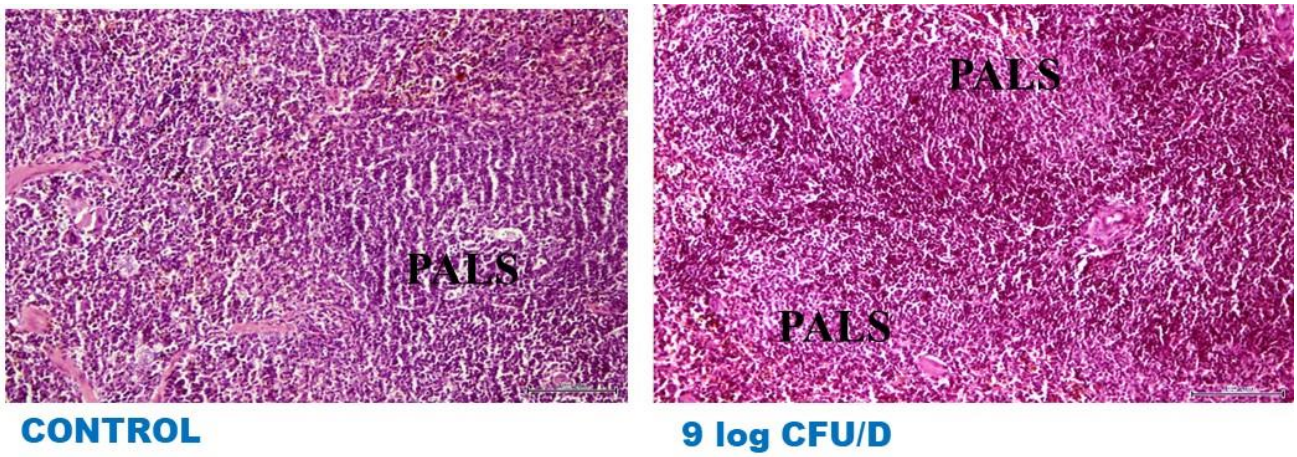

Figure 9. Spleen: adequate population of lymphocytes in periarteriolar lymphoid sheath (PALS) of white pulp in **control group Comparatively dense population of lymphocytes in PALS** of spleen in mice dosed **7, 9, & 11 log CFU/d lactobacilli** indicating improved immune status. x ***200 H & E stain***

Table 1 Effect of Bio-fortified and nonbio-fortified Pearl Millet Based Rabadi on body weight in Dietary Induced Anaemia in Sprague Dawley Rats

Body Weight:

	DAY 1	DAY 35	DAY 66
G1	43.00 ± 1.90[a]	155.05 ± 21.77[a]	191.75 ± 28.46[a]
G2	41.33 ± 4.60[a]	**121.33 ± 6.74[b]**	180.83 ± 12.88[a]
G3	43.83 ± 5.31[a]	125.08 ± 14.97[b]	187.83 ± 26.94[a]
G4	44.17 ± 3.40[a]	115.42 ± 10.58[b]	184.75 ± 9.64[a]

Values are expressed as mean ± SD (n=5). Data is analysed byone-way ANOVA. Different superscript letters (a, b,…) significant difference relative to control at *P<0.05.*

Table 2 Effect of Bio-fortified and nonbio-fortified pearl millet based Raabadi on feed consumption in dietary induced anaemia in Sprague Dawley rats

GROUP	7	14	21	28	35	42	50	56	63	65
G1 **Normal diet**	189.00 ±15.56	205.25 ±7.42	214.25 ±10.25	218.75 ±12.37	217.50 ±20.51	216.25 ± 14.50	222.75 ±22.27	216.25 ±14.50	222.75 ±22.27	235.00 ±2.83
G2 **Iron Deficient Diet**	170.00 ±14.14	175.25 ±13.79	183.25 ±6.01	185.00 ±7.07	186.00 ±4.24	**185.25± 3.18***	**192.75 ± 17.32***	**185.25 ± 3.18***	**192.75 ± 17.32***	**218.25 ± 3.89***
G3 **ND + BFPM Raabadi**	174.00 ±9.90	195.75 ±6.01	199.50 ±1.41	201.25 ±5.30	200.75 ±0.35	198.00 ± 2.83*	218.25 ± 23.69*	198.00 ± 2.83*	218.25 ± 23.69*	234.00 ± 7.07*
G4 **ND+NFPM Raabadi**	161.50 ±28.99	165.50 ±6.36	174.75 ±12.37	182.00 ±14.14	184.00 ±9.19	181.00 ±5.66	194.75 ±8.13	181.00 ±5.66	194.75 ±8.13	207.25 ± 9.55*

Key: N.= Number of animals SD = Standard Deviation; * = Mean value of group significantly different from control group at p<0.05. **Note**: ND + BFPM= Normal Diet+ Bio-fortified pearl millet based fermented Raabadi, ND+NFPM= Normal Diet+ Non- fortified pearl millet based fermented Raabadi.

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. * significant difference relative to control at *P<0.05*

Table 3. A. Effect of feeding low iron containing feed till day 38 and B. effect of feeding bio-fortified and non-bio-fortified Pearl Millet Based Rabadi on haematological parameters in dietary induced anaemia in Sprague Dawley rats at post day 66

A.

DAY 38								
GROUP	**WBC (10^3/L)**	**RBC (10^6/L)**	**HGB (g/dL)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**	**PLT (10^3/L)**
G1 **Normal diet**	18.67 ± 7.30^{a}	7.12 ± 0.43^{a}	**12.78 ± 0.53^{a}**	40.62 ± 2.78^{a}	57.02 ± 1.27^{a}	18.00 ± 0.88^{a}	31.55 ± 1.50^{a}	947 ±81.7^{a}
G2 **Iron Deficient Diet**	17.12 ± 3.62^{a}	6.69 ± 0.59^{a}	**9.62 ± 1.95^{b}**	**32.40 ± 5.64^{b}**	**51.13 ± 5.12^{b}**	15.78 ±2.00^{a}	30.77 ± 1.05^{a}	656.80 ± 780.7^{b}
G3 **ND + BFPM Raabadi**	16.00 ± 9.18^{a}	6.65 ± 0.37^{a}	10.78 ± 0.76^{ab}	35.10 ± 1.57^{ab}	52.9 ± 3.89^{ab}	16.28 ± 1.73^{a}	30.85 ± 1.03^{a}	1029.2 ± 170.5^{a}
G4 **ND+NFPM Raabadi**	15.98 ± 5.76^{a}	6.20 ± 1.07^{a}	10.62 ± 1.51^{b}	32.85 ±5.15^{b}	54.15 ± 2.72^{ab}	17.47 ± 1.20^{a}	32.41 ± 1.10$_{a}$	883.6 ± 134.5^{ab}

B.

DAY 66								
GROUP	**WBC (10^3/L)**	**RBC (10^6/L)**	**HGB (g/dL)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**	**PLT (10^3/L)**
G1 **Normal diet**	13.08 ± 6.30^{a}	7.57 ± 0.44^{a}	12.35 ± 1.43^{b}	43.23 ± 2.83^{a}	57.15 ± 0.90^{a}	16.31 ± 1.64^{a}	28.60 ± 3.11^{b}	876.30 ± 187.3^{a}
G2 **Iron Deficient Diet**	18.12 ± 4.93^{a}	7.47 ± 0.34^{a}	**11.48 ± 0.78^{a}**	41.82 ± 2.11^{a}	55.98 ± 1.07^{a}	16.70 ± 0.56^{a}	29.83 ± 0.81^{ab}	727.00 ± 396^{a}
G3 **ND + BFPM Raabadi**	16.78 ± 7.80^{a}	7.80 ± 0.41^{a}	13.02 ± 1.26^{ab}	43.47 ± 2.13^{a}	56.78 ± 0.38^{a}	16.68 ± 1.46^{a}	29.98 ± 2.57^{ab}	984.7 ± 142.4^{a}
G4 **ND+NFPM Raabadi**	11.57 ± 2.82^{a}	7.98 ± 0.76^{a}	14.57 ± 1.42^{b}	44.93 ± 3.67^{a}	56.417 ± 1.47^{a}	18.43 ± 1.42^{a}	32.63 ± 1.89^{a}	949.7 ± 232.5^{a}

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. Different superscript letters (a, b,…) indicate significant difference relative to control at $P<0.05$.

Table 4. Effect of Biofortified and non-biofortified Pearl Millet Based Raabadi on iron profile and serum biochemicals in dietary induced anaemia in Sprague Dawley rats

	Iron	TIBC	Transferrin	UIBC
G1	307.7 ± 39.8[a]	410.67 ± 9.61[a]	74.88 ± 9.03[a]	131 ± 40.8[a]
G2	**183 ± 21.7[b]**	410.33 ± 14.36[a]	61.84 ± 14.92[a]	202 ± 71[a]
G3	228 ± 13.89[ab]	400 ± 20.7[a]	71.23 ± 5.03[a]	141.3 ± 29.1[a]
G4	269 ± 58.8[ab]	372.7 ± 41.4[a]	71.83 ± 9.95[a]	103.7 ± 29.3[a]

	GPT (IU/L)	GOT (IU/L)	BID (IU/L)	BIT (IU/L)	UREA (mg/dl)	PRO (g/dl)
G1	45.47 ± 2.31[a]	109.43 ± 11.41[a]	0.017 ± 0.012[a]	0.043 ± 0.015[a]	34.27 ± 03.50[b]	6.20 ± 0.28[a]
G2	59.30 ± 28.8[a]	90.00 ± 30.9[a]	0.020 ± 0.010[a]	0.046 ± 0.005[a]	**61.30 ± 11.55[a]**	7.11 ± 0.91[a]
G3	42.90 ± 18.4[a]	107.97 ± 8.68[a]	0.017 ± 0.006[a]	0.043 ± 0.006[a]	33.30 ± 04.61[b]	6.50 ± 0.11[a]
G4	52.03 ± 3.85[a]	113.40 ±1.65[a]	0.013 ± 0.006[a]	0.037 ± 0.006[a]	35.43 ± 07.70[b]	6.38 ± 0.36[a]

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. Different superscript letters (a, b,…) indicate significant difference relative to control at *P<0.05.*

Table 5. Effect of Biofortified and non-biofortified Pearl Millet Based Raabadi on relative organ weight and absolute organ weight in dietary induced anaemia in Sprague Dawley rats

Relative Organ weight				
	BODY	**LIVER**	**SPLEEN**	**KIDNEYS**
G1	186.75 ± 9.03[a]	7.12 ± 0.36[a]	0.56 ± 0.12[b]	1.77 ± 0.19[a]
G2	181.50 ± 4.20[a]	6.30 ± 0.20[a]	**0.84 ± 0.09[a]**	1.66 ± 0.14[a]
G3	174.25 ± 10.21[a]	6.22 ± 0.72[a]	0.75 ± 0.19[ab]	1.80 ± 0.21[a]
G4	184.25 ± 3.69[a]	6.60 ± 0.45[a]	0.62 ± 0.90[ab]	1.56 ± 0.10[a]
Absolute Organ weight				
	LIVER	**SPLEEN**	**KIDNEYS**	
G1	3.31 ± 0.41[a]	0.32 ± 0.06[a]	0.88 ± 0.10[a]	
G2	3.82 ± 4.90[a]	0.48 ± 0.04[a]	1.00 ± 0.05[a]	
G3	4.08 ± 0.61[a]	0.46 ± 0.08[a]	0.98 ± 0.05[a]	
G4	3.94 ± 0.60[a]	0.42 ± 0.14[a]	1.56 ± 0.10[a]	

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. Different superscript letters (a, b,…) indicate significant difference relative to control at *P<0.05.*

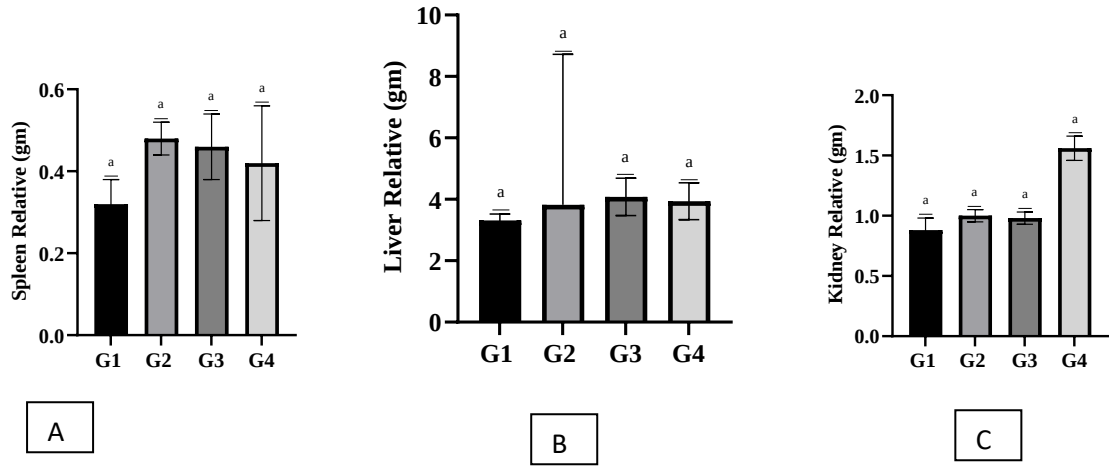

Fig 1 Effect of Biofortified and non-biofortified Pearl Millet Based Raabadi on relative organ weight of spleen (A), Liver (B) and Kidney (C) in dietary induced anaemia in Sprague Dawley rats

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. Different superscript letters (a, b,…) indicate significant difference relative to control at *P<0.05.*

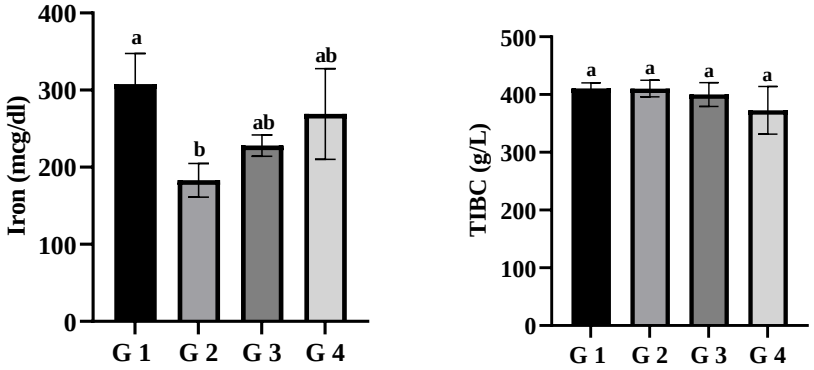

Fig 2 Effect of Biofortified and non-biofortified Pearl Millet Based Raabadi on serum iron and TIBC level in dietary induced anaemia in Sprague Dawley rats

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. Different superscript letters (a, b,…) indicate significant difference relative to control at *P<0.05.*

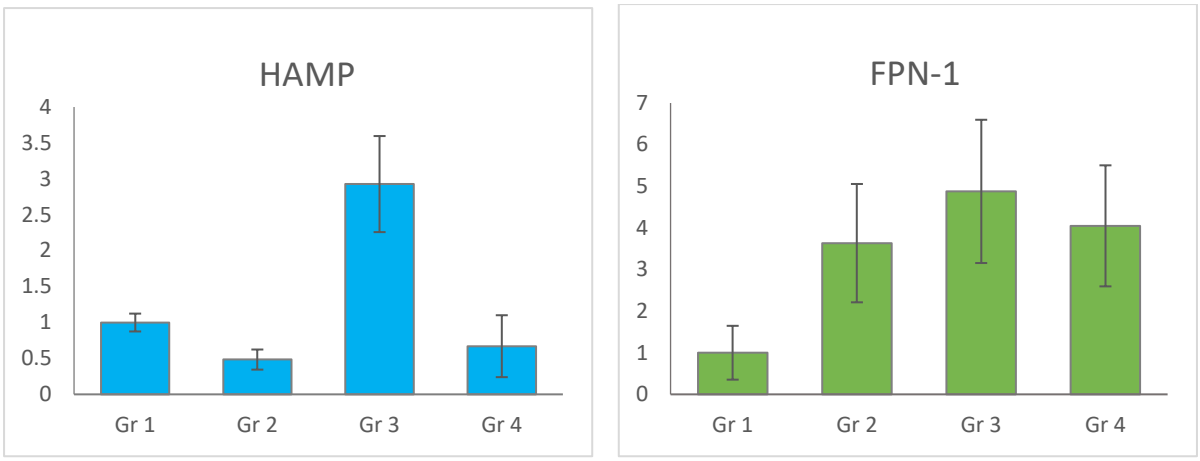

Fig 3. Effect of Biofortified and non-biofortified Pearl Millet Based Raabadi on mRNA levels of ferroportin 1(FPN1) and hepcidin (HAMP) in livers of control (G1) and anaemic (G2) rats, fed biofortified (G3) non-biofortified (G4) pearl millet based Rabadi

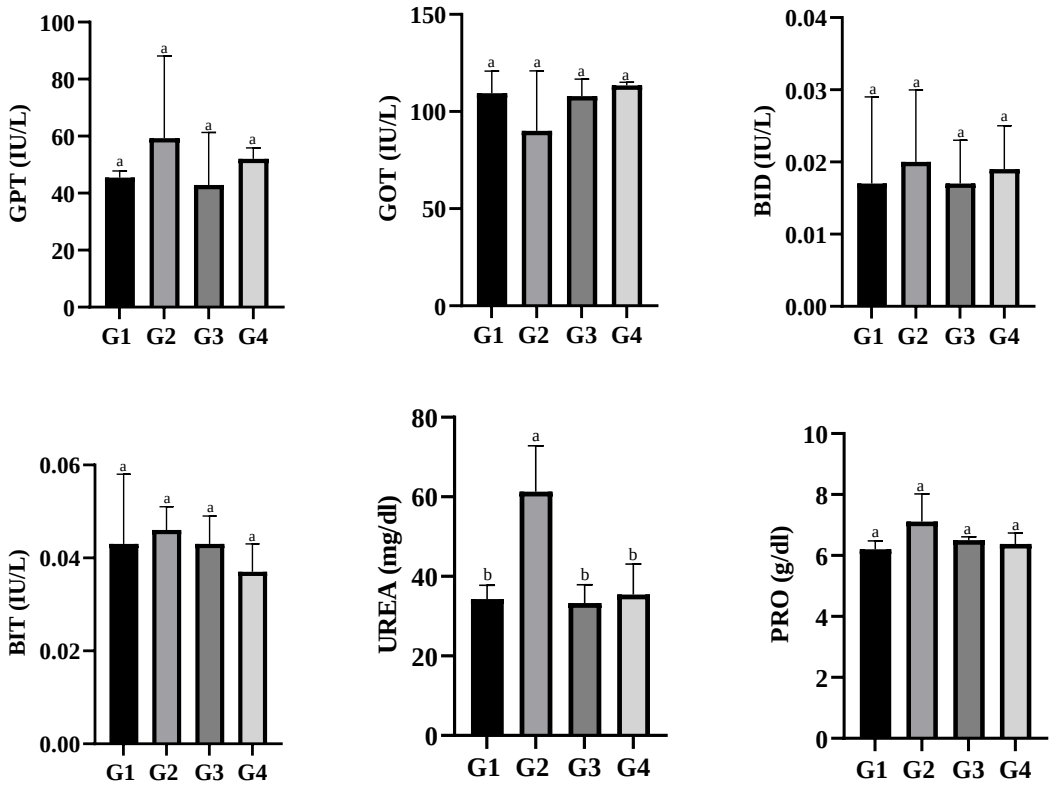

Fig 4. Effect of Biofortified and non-biofortified Pearl Millet Based Raabadi on serum biochemical in dietary induced anaemia in Sprague Dawley rats

Values are expressed as mean ± SD (n=5). Data is analysed by one-way ANOVA. Different superscript letters (a, b,…) indicate significant difference relative to control at *P<0.05.*

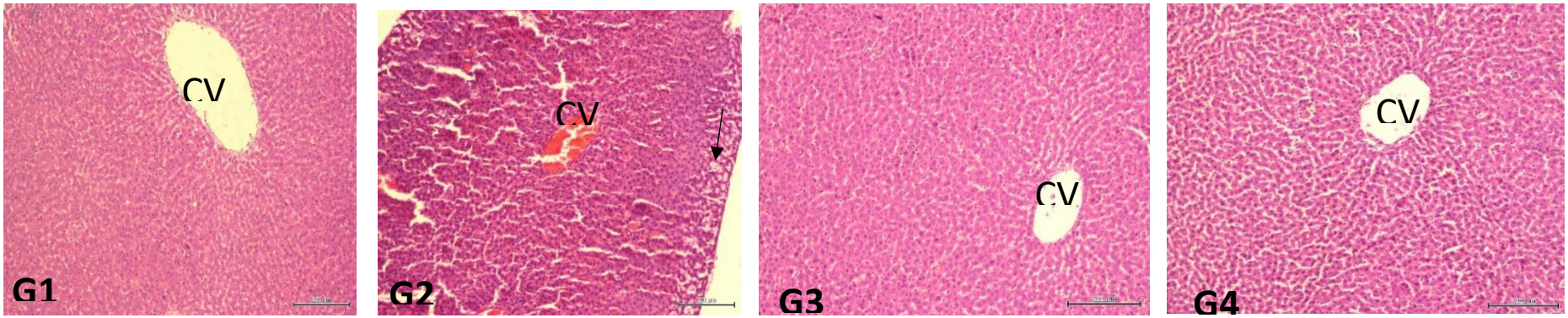

Fig. 1. Representative images of liver sections from rats of (G1) normal control showing normal architecture of liver tissue; (G2) anemic control indicating degenerative changes of hepatocytes, congestion of central vein sinusoidal space. Normal appearance of hepatocytes in (G3) biofortified PM based product treated rats and (G4) focal area of cellular swelling nonbiofortified PM based product treated group (H&E 200 x)

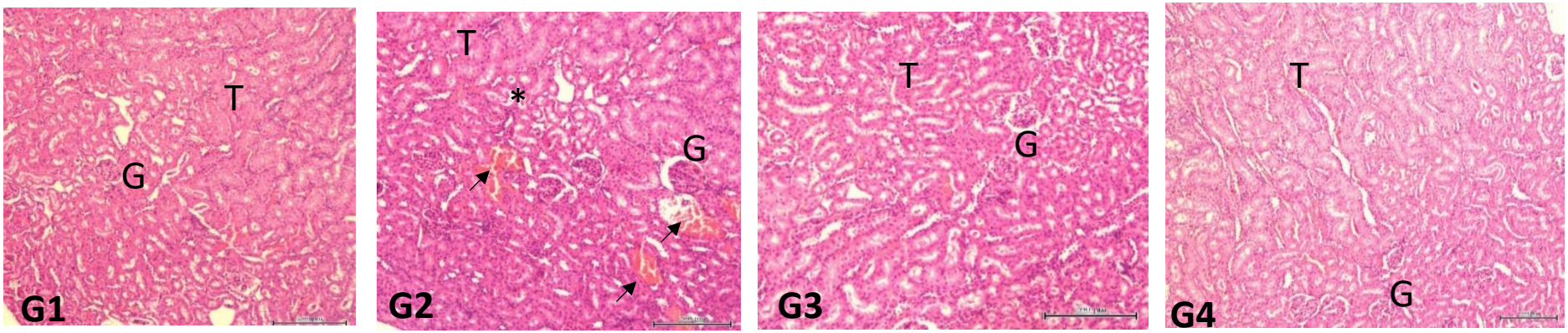

Fig. 2. Representative images of kidney sections from rats of (G1) normal control showing normal architecture of glomeruli (G) and Tubules (T) in kidney tissue; (G2) anemic control showing hemorrhages (arrow) and formation of hyaline cast in tubules (asterisk). Normal appearance of glomeruli and tubules (G3) biofortified PM based product treated and (G4) focal of cellular swelling of tubules in non-biofortified PM based product treated group (H & E 200 x)

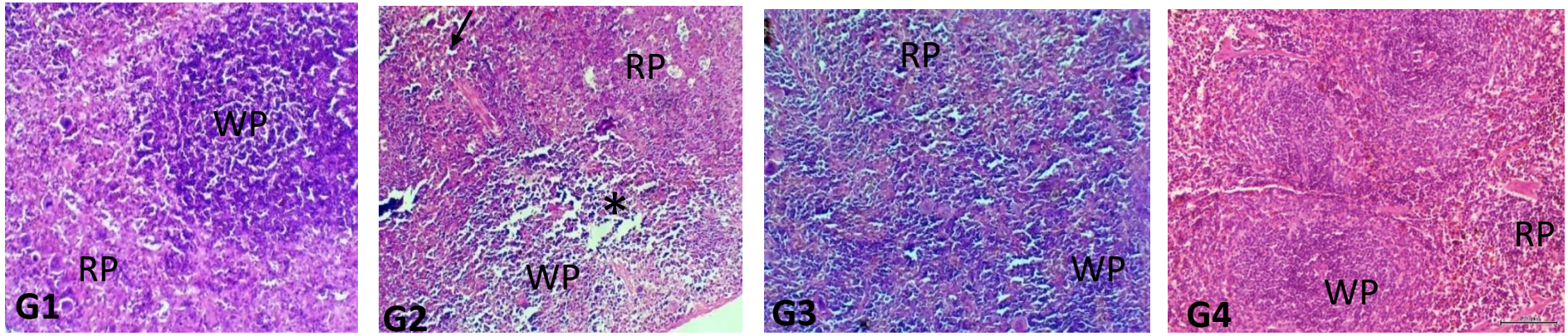

Fig. 3. **Representative images of spleen sections** from rats of (G1) normal control showing normal architecture of spleen tissue white pulp (WP) and red pulp (RP); (G2) anemic control indicating loss of lymphocytes in white pulp and hemorrhages (asterisk) in red pulp, abundant lymphocytes in white pulp and normal appearance of red pulp (G3) biofortified PM based product treated and (G4) lymphoid follicles in white pulp and adequate red pulp in non-biofortified PM based product treated group (H & E 200 x)

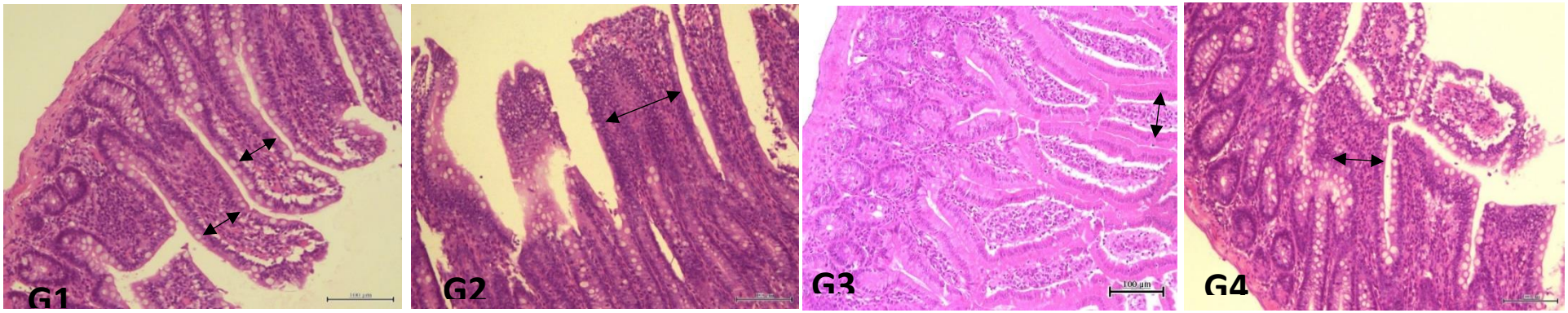

Fig. 4. **Representative images of illeum sections** from rats of (G1) normal control showing normal architecture of villi with adequate length and thickness and mucosal epithelium (double arrow), (G2) anemic control indicating increased thickness of villi and loss of goblet cells , normal appearance of villi (double arrow) and sufficient goblet cells (G3) biofortified PM based product treated and (G4) adequate number of villi (double arrow) and sufficient goblet cells in non-biofortified PM based product treated group (H and E 200 x)

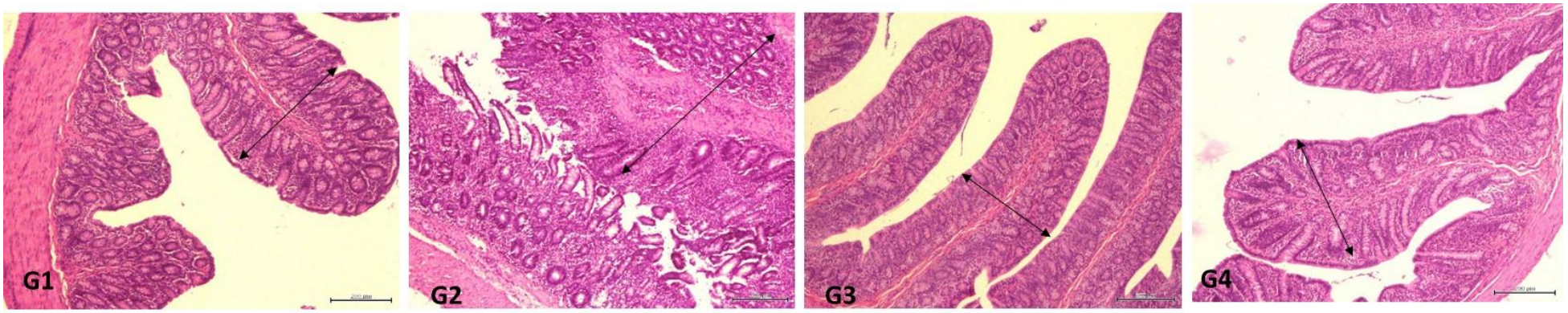

Fig. 5. **Representative images of colon sections** from rats of (G1) normal control showing normal architecture of crypts with adequate length and thickness and mucosal epithelium (double arrow), (G2) anemic control indicating increased thickness of crypts, loss of goblet cells and distorted mucosal epithelium , normal appearance of crypts (double arrow), intact epithelium and sufficient goblet cells (G3) biofortified PM based product treated and (G4) adequate number of crypts (double arrow) and sufficient goblet cells in nonbiofortified PM based product treated group (H and E 200 x).

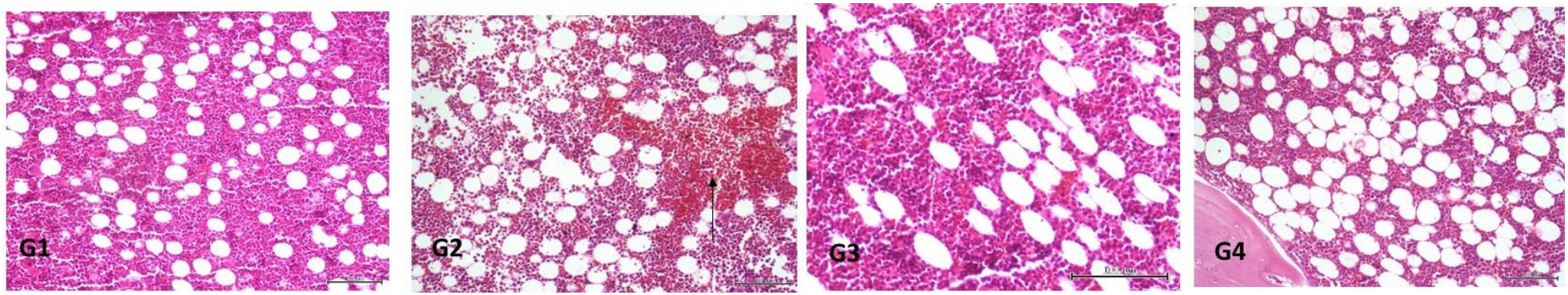

Fig. 6 Representative images of bone marrow from rats of (G1) normal control showing normal cellularity (double arrow), (G2) anemic control indicating severe hemorrhages (arrow) and hypocellularity, adequate cellularity in (G3) biofortified PM based product treated and (G4) sufficient number of cells in nonbiofortified PM based product treated group (H and E 200 x).

RESEARCH & DEVELOPMENT PROJECT

Genome scanning for identification of economically important traits related markers in goat: a step towards improving economy of goat farmers in Haryana

SUBMITTED BY

Dr. Ankit Magotra

To

HARYANA STATE COUNCIL OF SCIENCE, INNOVATION AND TECHNOLOGY, PANCHKULA

HSCSIT Reference No. HSCSIT/R&D/2021/537 Dated: 05.03.2021

Project Duration: 3-years

Final Project Report

Genome scanning for identification of economically important traits related markers in goat: a step towards improving economy of goat farmers in Haryana

Funding agency

HARYANA STATE COUNCIL FOR SCIENCE, INNOVATION & TECHNOLOGY (HSCSIT),
Govt. Of Haryana

Principal Investigator
Dr. Ankit Magotra
Dept. of Animal Genetics & Breeding

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FINAL PROJECT COMPLETION REPORT

1) **Title of the Project**

Genome scanning for identification of economically important traits related markers in goat: a step towards improving economy of goat farmers in Haryana

2) **Date of start of Project**: 05/03/2021

3) **Name of Principal Investigator and address**:

1. Dr. Ankit Magotra, Assistant Professor, Department of Animal Genetics and Breeding, LUVAS, Hisar

4) **Name of Co-Investigators (if any)**

1. Dr. Yogesh C Bangar, Scientist, Department of Animal Genetics and Breeding, LUVAS, Hisar

2. Dr. Sandeep Kumar, Assistant Professor, Department of VGO, LUVAS, Hisar

5) **Place of work:** Hisar, Haryana

6) Objectives:

1. To identify and characterize genomic regions of candidate genes associated with economically important traits in Beetal, Jakhrana, Barbari and Sirohi goats.
2. Genetic and molecular evaluation of identified SNPs and their association with performance traits.
3. Genome wide SNP profiling of selected animals of respective breeds using Caprine SNP chip to evaluate breedwise variation.
4. Molecular tagging and dissemination of superior germplasm at farmer's goat flock.

Detailed Project Report

1.Introduction

This project aimed to investigate the genetic and phenotypic characteristics of indigenous goat breeds in India with a focus on growth, reproduction, and survivability traits. Field-based sampling, phenotypic data collection, and molecular analysis were conducted on selected goat breeds across different regions of Haryana.

2. Sampling and Data Collection

Blood samples were collected from various locations representing both farmers' flocks and institutional farms:

S.N.	Location
1	Gangwa & LUVAS
2	Durjanpur, Bhiwani
3	Adampur, Hisar
4	Basra/Chaudhriwas, Hisar
5	Budain, Mahendergarh
6	Depal (Hansi)
7	Karnal

3. Blood Collection and DNA Isolation

The blood samples were collected from five goat breeds:

Breed	Sample Size
Beetal	136
Jakhrana	50
Barbari	56
Sirohi	52
Sojat	41

DNA was isolated using the Phenol Chloroform Method and Maxwell Promega Blood DNA Isolation Kit with an Automatic DNA Purification System.

4. In-silico and Phenotypic Data Analysis

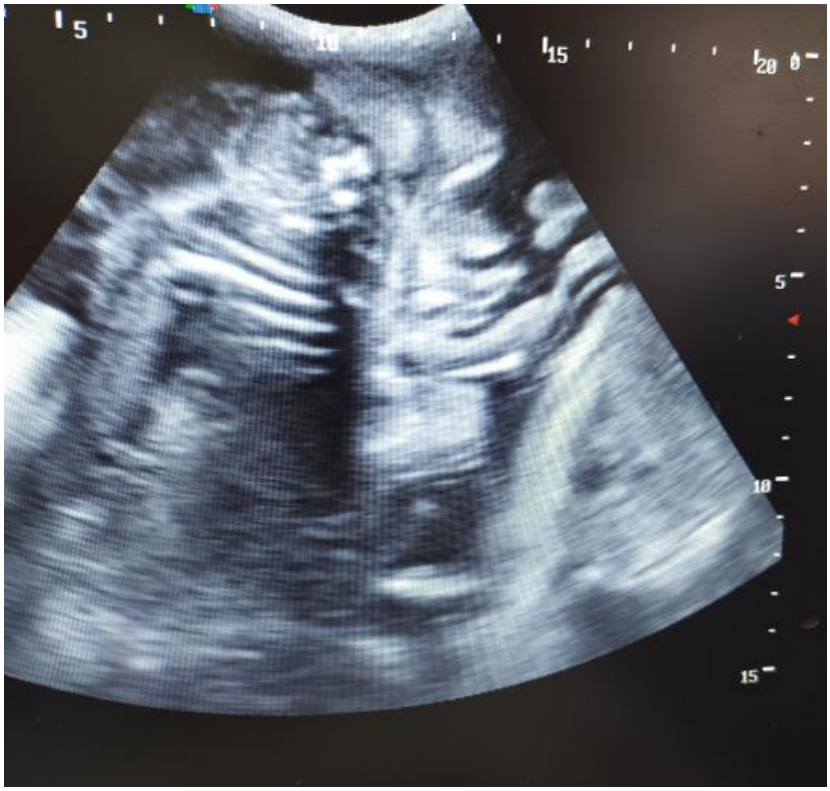
- In-silico analysis was conducted to retrieve candidate SNPs associated with growth, litter size, and disease resistance traits.
- Phenotypic data relevant to these traits were also recorded from the sampled goats for association studies.

5. Ultrasonographic Grouping and Litter Size Recording

Ultrasound-based prenatal litter size estimation was carried out on **university farm goats**. These animals were also used for detailed phenotypic trait measurement.

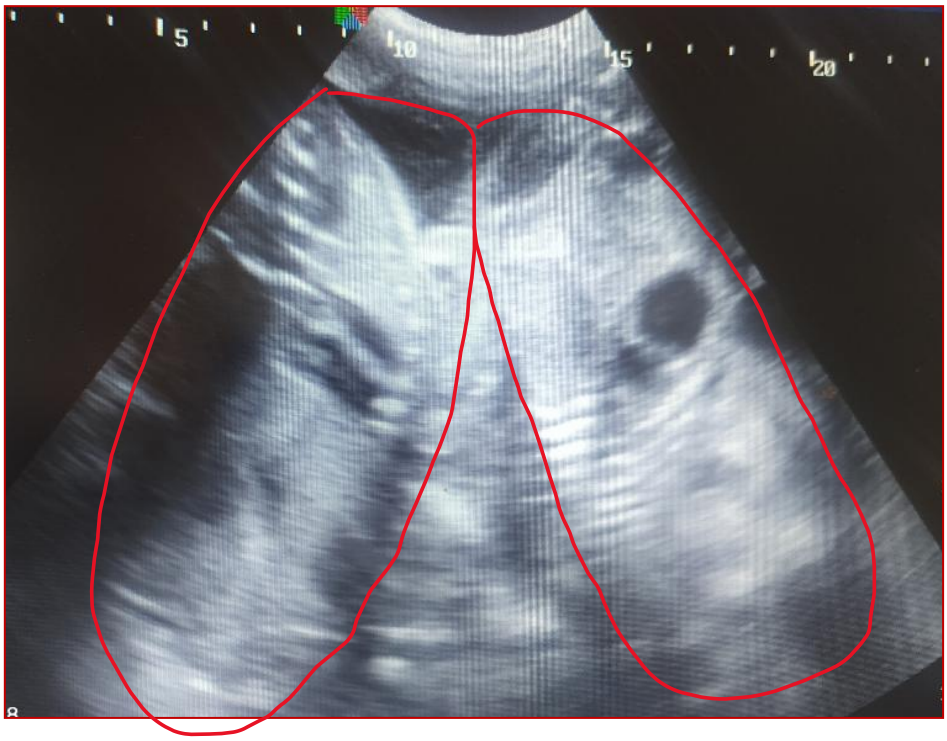
Real-Time Ultrasonographic Imaging in Goat

Ultrasonographic Examination of a Goat



Ultrasonographic imaging of goats used for prenatal litter size prediction

A.



Ultrasound Image Confirming Twin Pregnancy in a Goat

A. Phenotypic data of University farm animals:

1. Beetal Goat (University Farm)

Trait	Mean ± S.E. (kg)	Observations
Birth weight	2.98 ± 0.07	77
3-month weight	10.09 ± 0.35	71
6-month weight	18.91 ± 0.62	39
9-month weight	25.15 ± 0.81	34
1-year weight	28.00 ± 1.38	22
Weight at service	35.24 ± 0.89	51
Weight at kidding	37.96 ± 0.95	51
Kids per kidding	**1.51**	

2. Comparative Performance Over Five Years – Beetal

Year	3-month wt (kg)	6-month wt (kg)	1-year wt (kg)	Kids/kidding
2019–20	11.56	20.28	26.43	1.15
2020–21	10.56	19.38	28.64	1.41
2021–22	11.29	19.63	27.34	1.63
2022–23	10.19	19.80	27.19	1.63
2023–24	10.09	18.91	28.00	1.51

3. Jakhrana Goat

Trait	Mean ± S.E. (kg)	Observations
Birth weight	2.91 ± 0.08	35
3-month weight	10.71 ± 0.55	29
6-month weight	19.22 ± 0.86	23
9-month weight	28.64 ± 1.50	11
1-year weight	34.33 ± 1.97	9
Weight at service	33.20 ± 1.13	25
Weight at kidding	36.48 ± 1.23	25
Kids per kidding	**1.40**	

4. Comparative Performance Over Five Years – Jakhrana

Year	3-month wt (kg)	6-month wt (kg)	1-year wt (kg)	Kids/kidding
2019–20	10.54	19.13	26.60	1.13
2020–21	10.67	19.88	25.17	1.28
2021–22	11.86	20.04	26.35	1.43
2022–23	12.02	23.04	26.37	1.26
2023–24	10.71	19.22	34.33	1.40

5. Survivability of Goat Flocks (All Breeds Combined)

Year	Survivability (%)
2019–20	94.49
2020–21	96.07
2021–22	93.26
2022–23	97.13
2023–24	87.32

B. Phenotypic and Reproductive Performance of Field-Goat Populations

Field-based data collection was conducted to assess growth, reproductive efficiency, and overall health status of goats across multiple farmer-managed flocks and institutional sources. The results are summarized as follows:

1. Growth Performance

Phenotypic observations were recorded for body weights at key developmental stages across breeds. The average body weights (Mean ± S.E.) observed were:

Trait	Mean ± S.E. (kg)
Birth weight	2.76 ± 0.05
3-month weight	11.29 ± 0.31
6-month weight	19.63 ± 0.49
9-month weight	24.58 ± 0.59
1-year weight	27.34 ± 0.74
Weight at service	34.20 ± 0.50
Weight at kidding	37.40 ± 0.64

The data revealed a steady increase in body weight with age, indicating satisfactory growth trends under existing field conditions. Such performance benchmarks are crucial for breed evaluation and management planning.

2. Reproductive Performance (Litter Size)

Breed-wise evaluation of litter size showed significant variation, suggesting genetic influence on reproductive traits. The average litter size (Mean ± S.E.) recorded in different breeds was as follows:

Breed	**Litter Size (Mean ± S.E.)**
Beetal	$1.89^{a} \pm 0.06$
Sirohi	$1.71^{a} \pm 0.09$
Sojat	$1.17^{c} \pm 0.06$
Barbari	$2.86^{b} \pm 0.08$

Note: Superscripts denote statistically significant differences ($p<0.05$).

Barbari goats exhibited the highest prolificacy among the studied breeds, making them an ideal candidate for selection in genetic improvement programs targeting enhanced reproductive output. In contrast, Sojat goats demonstrated significantly lower litter size, which may be influenced by breed-specific genetics or local management practices.

3. Disease Occurrence and Health Constraints

Farmers reported a range of health disorders affecting productivity and survivability. The commonly recorded disease conditions included:

- **Respiratory infections**
- **Diarrhea**
- **Diarrhea with abortion**
- **Abortion (isolated cases)**
- **Multiple co-morbid conditions**: Diarrhea + Abortion + ORF (Contagious Ecthyma) + Bloat + Lameness
- **Mastitis with Prolapse**

C. Evaluation of physiological and morphological parameters for early prediction of prenatal litter size in goats

An experimental study was conducted using two indigenous breeds (22 Beetal and 11 Jakhrana) maintained at a goat breeding farm, at the Department of Animal Genetics and Breeding, LUVAS, Hisar (India). Using ultrasonography, these 33 does were categorized into three categories: (1) does bearing twin fetus (n =12); (2) does bearing a single fetus (n = 12); and (3) non-pregnant does (n=9). In the initial statistical analysis, we did not find any significant ($P > 0.05$) differences between the two breeds for various parameters. Therefore, we pooled these two breeds into a combined category to give a sufficient sample size in each litter size category.

Traits targeted:

In the present study, age at service and weight of service were obtained from the service register maintained at a goat breeding farm. Two physiological parameters, i.e. rectal temperature oF (RT) and respiration rate (RR), and two morphological parameters, i.e. abdominal girth in cm (AG) and udder circumference in cm (UC), were recorded at different times of gestation, i.e. 118, 125, 132, and 140 days.

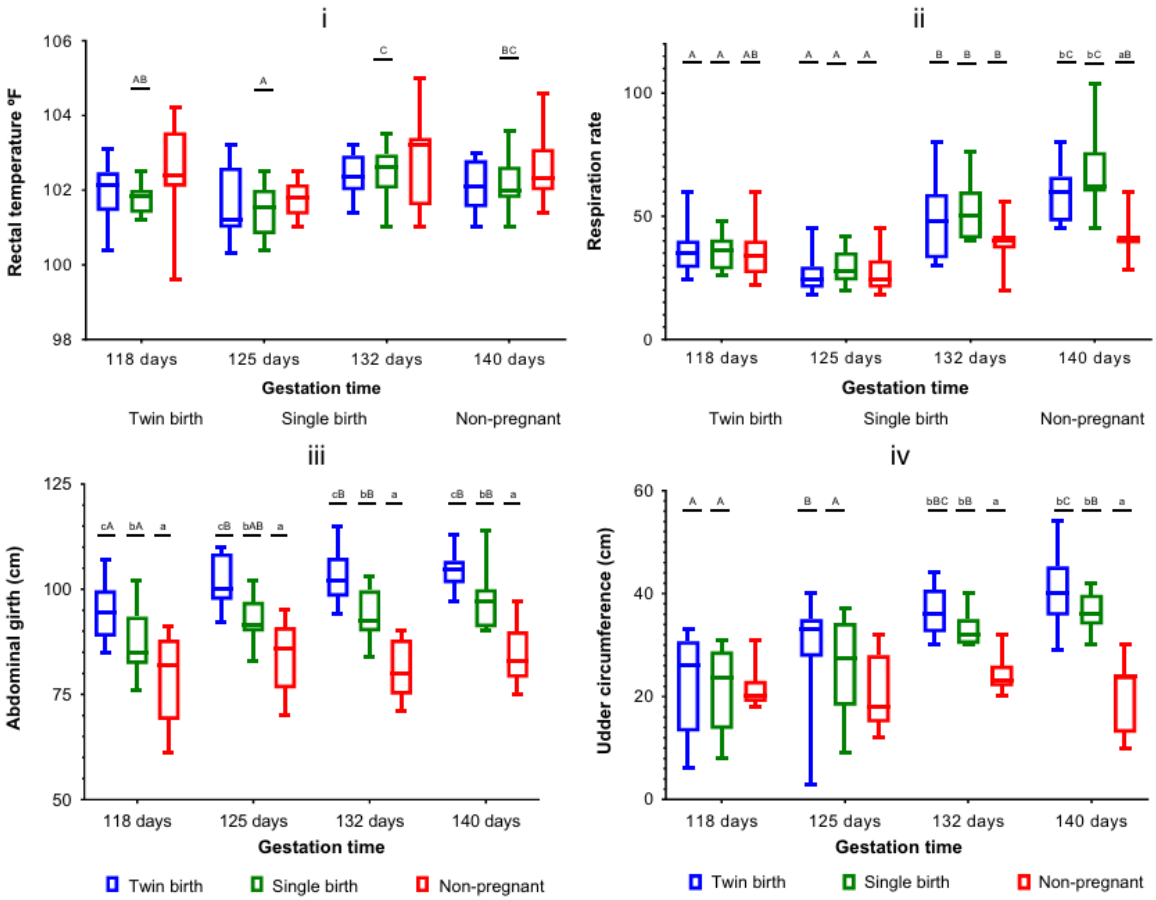
Statistical analysis: All the data from the 33 does were compiled using Microsoft Excel and processed for further statistical analysis. Descriptive statistics and one-way ANOVA were used to determine any significant difference between the three groups of does for age at service (years), weight at service and kidding (kg) and litter weight (kg).

To compare these three groups of does, along with gestation times, a two-way ANOVA with repeated measures was used to obtain a significant difference between the three groups of does and the four gestation time intervals (within groups). Pairwise comparisons were made using Duncan's test between groups and Bonferroni test within groups. To predict in advance whether pregnant does would have single or twin litter size, a linear discriminant analysis (LDA) was used in this study. Considering two categories of does, i.e. single and twin litter size as a dependent variable and six variables, and i.e. age at service, weight at service, RT, RR, AG and UC as predictors, four discriminant functions were developed separately for each gestation time. The strength of the estimated discriminant function was checked using Wilk's lambda, which is the proportion of unexplained variability out of total variability; smaller values indicate the better ability of the function to classify the individuals into the correct

category of litter size. The significance of Wilk'slambda was determined using the chi-squared test. The homogeneity of covariances under each LDA was confirmed using Box's M statistic. Furthermore, a classification table of observed and predicted catego ries of does bearing single and twins was used to estimate the accu racy, sensitivity, and specificity of the estimated discriminant function at each gestation time. For all analyses, the significance was considered for P-value.

Descriptive analysis

A detailed data structure and descriptive statistics for three groups of does that were sampled initially for the current study are given in Table 1. The overall body weight gain in kg was1.50 and 2.50kg for does bearing twin and single kids respectively. The average litter weight for the respective groups was 2.59 and 2.91 kg, respectively. The does with twins were a statistically similar ($P > 0.05$) age at service, but had significantly ($P < 0.05$) different weights at service compared with does with a single fetus. However, non-pregnant does had a significantly ($P < 0.05$) lower age and weight at service than did both pregnant groups, i.e. single and twin fetuses.



Although weight at kidding was higher in does with twins compared with does with a single fetus, the litter weight in both groups was statistically (P > 0.05) on par

Physiological and morphological parameters The association of three groups of does with physiological (RT and RR) and morphological (AG and UC) parameters at different times of gestation, i.e. 118, 125, 132 and 140 days, was obtained and the results are depicted in Figure 1. It was observed that there was no significant (P > 0.05) difference for both physiological parameters between the three groups at different gestation times, except at 140 days of gestation. RR was found to be significantly (P < 0.05) different among the three groups at 140 days and was higher in does bearing a single fetus, followed by twins compared with non-preg nant does. Between different times of gestation, it was observed that RT was significantly (P0.05) show this difference in RT over time. For RR, all three groups had significantly (P < 0.05) differences were observed between the three groups at all gestation times and that a wider AG was found among does bearing twins, followed by a single fetus and non-pregnant does. Similarly, UC (cm) showed a significantly increasing (~40%) trend from 118 to 140 days among the pregnant does and ranged from 22.50 to 40.70 cm in does bearing twins and 21.67–36.15 cm in does bearing a single fetus. Although UC was non-significantly different between the three groups upto 125 days, it was significantly(P<0.05)different among pregnant does compared with non-pregnantdoesat132and140days.However, it was not statistically significant (P>0.05) between does bearing single/twin fetuses at advanced gestation. Furthermore, there was a non significant (P>0.05) difference in gestation time among the non-pregnant does.

Table 1. Data structure and descriptive statistics for different litter size of does

Litter size of does	Sample size	Age at service (years)	Weight at service (kg)	Weight at kidding (kg)	Weight gain (kg)	Litter weight (kg)
Twins[#]	12	4.51 ± 0.48[b]	37.75 ± 1.48[b]	39.25 ± 1.21[b]	1.50	2.59± 0.58 (25)
Single	12	4.20 ± 0.45[b]	32.67 ± 0.87[a]	35.17 ± 1.20[a]	2.50	2.91 ± 0.05 (12)
Non-pregnant	9	2.82 ± 0.41[a]	30.67 ± 1.23[a]	–	–	–

[#]One kidding gave triplets that was included under twin type of litter size.
Different superscripts (a, b) in the same column indicates that values differ significantly ($P < 0.05$).

Discriminant function

A stepwise LDA was performed to check if all predictors contributed to function. It was revealed that all six predictors, i.e. age, weight at service, RR, RT, AG and UC, were important for developing the discriminant function. The estimated unstandardized coefficients under discriminant analysis are presented in Table2. The

associated values of Wilks' lambda that measured the discriminating power of each function for separating animals into two groups were low and ranged from 0.46 to 0.64. As a proportion of the total variance in the discriminant scores was not explained by differences among the groups, smaller values of Wilks' lambda in this study indicated the greater discriminatory power of the estimated functions. The significance of Wilk's lambda was tested using chi-square statistical tests and it was revealed that respective functions had significantly better power for separating the two groups, i.e. single and twin litter sizes at 125 days onwards. Table 3 shows the classification for does bearing single and twin fetuses using estimated discriminant functions based on six predic tors at each gestation time. The accuracy (%) of classifying does into the correct category at days 118, 125, 132, and 140 days was obtained as 75.00, 87.50, 83.30 and 86.40%, respectively. The sensitivity (%) and

Table 2. Discriminant function for prediction of litter size in goats

Gestation time	Eigen values	Wilk's lambda	Discriminant function
118 days	0.56	0.64	−0.13*Age + 0.214*WTS + −0.124*RT + −0.022*RR + 0.055*AG + 0.028*UC + 0.807
125 days	1.17	0.46*	−0.321*Age + 0.076*WTS + 0.464*RT + −0.071*RR + 0.149*AG + 0.024*UC + −61.471
132 days	1.07	0.48*	−0.298*Age + 0.058*WTS + −0.105*RT + −0.01*RR + 0.146*AG + 0.128*UC + −8.285
140 days	1.08	0.48*	−0.08*Age + 0.068*WTS + 0.233*RT + −0.055*RR + 0.077*AG + 0.141*UC + −35.438

Age: age at service (years); AG: Abdominal girth (cm); RR: Respiration rate; RT: rectal temperature °F; UC: Udder circumference (cm); WTS: weight at service (kg).
*Significant chi-squared distribution at the 5% level.

Table 3. Classification of pregnant does into two groups of litter size

		Predicted group membership			
Gestation time	Litter size of dam	Single	Twin	Total	Accuracy (%)
118 days	Single	9 (75.00)	3 (25.00)	12	75.00
	Twin	3 (25.00)	9 (75.00)	12	
125 days	Single	10 (83.30)	2 (16.70)	12	87.50
	Twin	1 (8.30)	11 (91.70)	12	
132 days	Single	10 (83.30)	2 (16.70)	12	83.30
	Twin	2 (16.70)	10 (83.30)	12	
140 days	Single	11 (91.70)	1 (8.30)	12	86.40
	Twin	2 (20.00)	8 (80.00)	10	

specificity (%) for respective gestation times were 75.00 and 75.00%, 83.30 and 91.70%, 83.30 and 83.30% and 91.70 and 80.00%, respectively

D. In-silico SNP Screening

To identify candidate polymorphisms associated with economically important traits such as growth, reproduction, and disease resistance, an in-silico approach was adopted. Publicly available gene sequences were analyzed using bioinformatics tools to detect single nucleotide polymorphisms (SNPs) and to select suitable restriction enzymes (RE) for downstream genotyping assays.

Software and Tools Used

1. **BLAST (Basic Local Alignment Search Tool)**

   URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi

   Utilized to retrieve and align nucleotide sequences of target genes from the NCBI database and identify potential SNP loci within the gene regions.

2. **NEBcutter V2.0**

   URL: http://tools.neb.com/NEBcutter2/

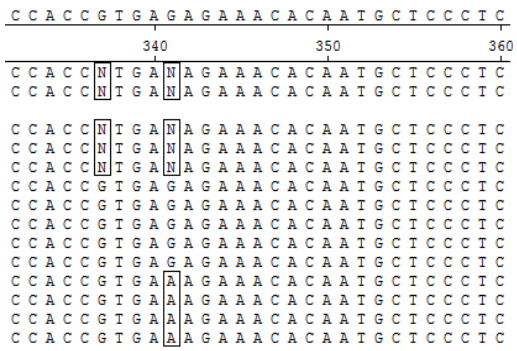
   Used to analyze the candidate gene sequences for the presence of **restriction enzyme recognition sites** and select enzymes capable of differentiating allelic variants (RFLP analysis).

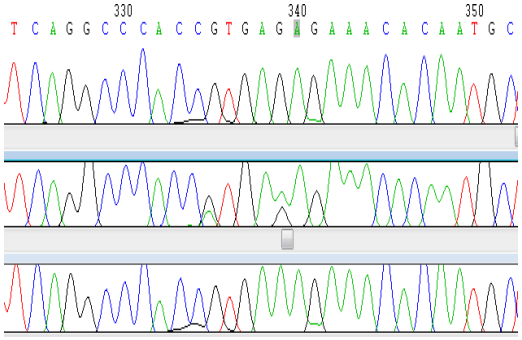
Candidate Genes Identified for SNP Analysis

Based on a literature survey and functional relevance to growth, litter size, and disease resistance, the following genes were shortlisted for SNP screening:

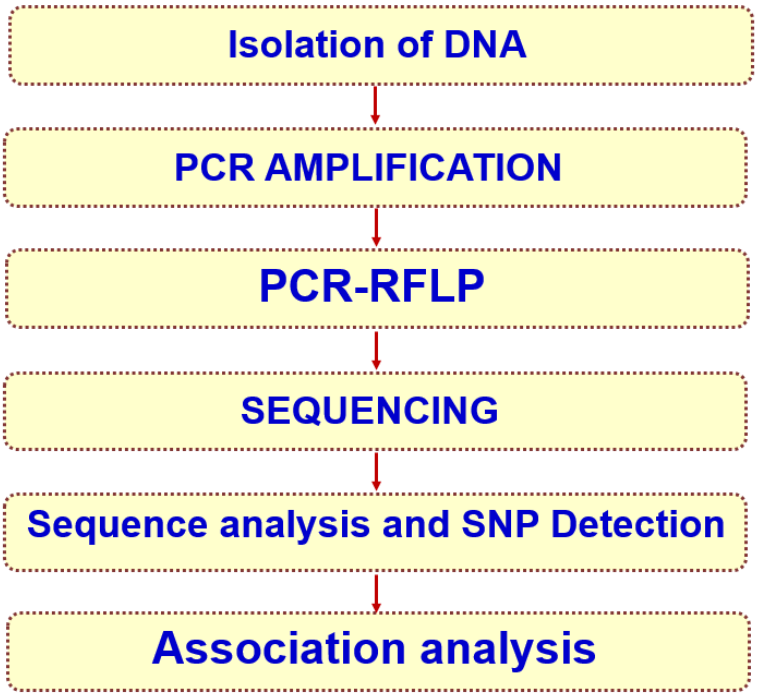
Gene Symbol	Function
Kiss1	Reproductive hormone regulation (puberty onset)
GDF9	Ovarian folliculogenesis and ovulation rate
GH	Growth hormone; impacts body weight and growth traits
GHR	Growth hormone receptor; affects response to GH
IGF	Insulin-like growth factor; regulates body development
LEP	Leptin; involved in energy balance and fertility

POU1F1	Pituitary development and growth regulation
BMP	Bone morphogenetic proteins; reproductive traits
FecB	Fecundity gene associated with prolificacy
FSHβ	Follicle-stimulating hormone beta-subunit; reproductive function
MHC	Major histocompatibility complex; immune response and disease resistance
MNTR	Melatonin receptor gene; reproductive seasonality
DGAT1	Diacylglycerol O-acyltransferase 1; fat metabolism
Beta-LG	Beta-lactoglobulin; milk production traits





Materials and Methods



3. DNA EXTRACTION FROM BLOOD SAMPLES

3.1 Collection and storage of blood

5 ml of blood was aseptically collected from the jugular vein in vacutainer tube containing EDTA (2.7%). The tube was rolled gently in between palms to ensure proper mixing of blood and EDTA. The samples were suitably labelled and were stored at -20°Ctill the isolation of genomic DNA. DNA was isolated from blood using standard phenol chloroform method as described in Sambrook and Russell (2001) with minor modifications.

3.2 DNA Extraction

Phenol-chloroform method, as described by Sambrook and Russel (2001) was used for DNA isolation from experimental animals. The steps involved in DNA isolation were as follows:

3.3 Cell Lysis

1. Total 3 ml Blood was taken in centrifuge tube with two volumes of ice-cold RBC lysis buffer, mixed well by vortexing and the tubes were kept at 4°C for 30 minutes.
2. Centrifuged at 4000 rpm for 4 minutes at 4°C and the supernatant were decanted.
3. WBC pellet was resuspended in one volume of RBC lysis buffer and kept on ice for 10 minutes and centrifuged as given in above step.
4. Step 2 and 3 were repeated for complete lysis of red blood cells until a clear pellet of white blood cells was obtained.
5. WBC pellet was resuspended in DNA extraction buffer (1ml for 3ml blood) by vortexing and incubated at 37°C for 30 min.
6. SDS (10%) was added at 60 µl per 10ml of whole blood. The mixture was gently mixed, by inverting the tubes 2-3 times. Proteinase-K was added at 8µl (20 mg/ml stock solution) per 3 ml of blood and incubated at 56°C overnight.

3.4 Phenol Extraction:

1. Equal volume of Tris (pH 8.0) saturated phenol was added to the above mixture and mixed gently by inversion to form a uniform suspension (10 minutes).
2. The mixture was centrifuged at 4000 rpm for 10 minutes at 20°C.
3. Upper aqueous phase was gently aspirated using a wide bore sterile Pasteur

4. A mixture of saturated phenol, chloroform and isoamyl alcohol in a ratio of 25:24:1 was added in equal volume and mixed gently by inverting the tube until a uniform suspension was formed.
5. The mixture was centrifuged at 4000 rpm for 10 minutes at 20°C and the upper aqueous phase was gently aspirated using a wide bore sterile Pasteur pipette without disturbing the interphase of protein and transferred into a fresh Oakridge tube.
6. A mixture of chloroform and isoamyl alcohol at a ratio of 24:1 was added and mixed properly by inverting the tube. The mixture was centrifuged again at 4000 rpm for 10 minutes and the aqueous phase was aspirated into a fresh sterile glass tube.

3.5 DNA Precipitation:

1. Sodium acetate (3 M, pH 5.2) was added to the aqueous layer and mixed gently, and two volumes of ice-cold absolute ethanol was added and tilted gently for DNA precipitation.
2. The precipitated DNA was spooled out with the help of bent Pasteur pipette and transferred to eppendorf tube with 100µl of 70% ethanol, mixed well and centrifuged at 4000 rpm for 5 minutes at 4°C.
3. The DNA was dried by keeping the eppendorf tubes open in a sterile incubator at 37°C. The DNA was dissolved in 50µl nuclease free water the tubes were kept at 40 °C for 4-5 days and finally stored at -20 °C.

3.6 Quality and Quantity Check of DNA

DNA quality was evaluated on 1% agarose (w/v) suspension in 1 X TBE electrophoresis buffer (Thermo scientific), Ethidium bromide (1%) was added at the rate of 0.5µl/100 ml of agarose gel solution. Further, 3µl DNA mixed with 2µl of 6X gel-loading dye and loaded gradually in separate well. Electrophoresis was carried out at 90V for 30 minutes at room temperature and gel was carefully removed from the chamber and placed on gel documentation system (Bio-Rad). Clear distinct single bands were considered as accurate concentration of the DNA. Quality and quantity of DNA was also assessed by Scandrop Nano-Volume Spectrophotometer (Analytikajena). Optical density (O.D.) was determined at wavelengths 260 nm and 280 nm in UV-VIS spectrophotometer against distilled water as blank sample. Quantity of DNA was calculated using the following formula:

3.7 Quantity of DNA in µg/ml = OD_{260} x 50 x Dilution Factor

The ratio between OD_{260} and OD_{280} was observed for each sample. DNA sample with ratio of 1.8 was considered good and taken for further analysis.

3.8 Primers

The primers were designed from Sigma Aldrich to amplify the target genes using Primer 3 online software. The primer sequence, annealing temperature (A.T.) and product size were given in Table 3.1.

Primer sets designed for amplification of the target region of Kiss1 and GDF9 Genes

Gene Name	Primer Sequence
GH	CTC TGC CTG CCC TGG ACT
GH	GGA GAA GCA GAA GGC AAC C
IGF1	TGAGGGGAGCCAATTACAAAGC
IGF1	CCGGGCATGAAGACACACACAT
GHR	CATTCCCACCACTGCATGAC
GHR	ACAGCAGCTCTGAAGCTATGG
Leptin	TGCAGTCTGTCTCCTCCAAA
Leptin	CGATAATTGGATCACATTTCTG
POU1F1	GTATTGCTG CTAAAGACGCC
POU1F1	GAGGGAAAGA TATAGTGAAAGGG
BMPR1B	CCAGAGGACAATAGCAAAGCAAA
BMPR1B	CAAGATGTTTTCATGCCTCATCAACAGGTC
MHC Class II DRB3	TAT CCC GTC TCT GCA GCA CAT TTC
MHC-DRB1	CCG GAA TTC CCG TCT CTG CAG CAC ATT TCT T
	TTT AAA TTC GCG CTC ACC TCG CCG CT
	AGC TCG AGC GCT GCA CAG TGA AAC TC
DQB1 FWD	CCC CGC AGA GGA TTT CGT G
DQB1 REV	ACC TCG CCG CTG CCA GGT
MHC class II DRB3	TATCCCGTCTCTGCAGCACATTTC
MHC class II DRB3	TCGCCGCTGCACACTGAAACTCTC
DGAT1	GCGGGGGAAGTTGAGCTCGTAG
DGAT1	CCGACTGGCGCCTGCCGCTTGCTCGTAG
Beta-lacto globulin	CGGGAGCCTTGGCCCCTCTG
Beta-lacto globulin	CCTTTGTCGAGTTTGGGTGT
BMP 4	ACGAAGGTCAGTCCCTACC
BMP 4	ACCAAACATTTCCCCAGCGA

PCR amplification

The working solutions (10pmol/µl) of both forward and reverse primers of respective genes were prepared from stock solutions by adding primer and Milli Q water in 1:9 ratios separately for both forward and reverse primers. Each time PCR reaction mixture was prepared using PCR Master Mix (2X) (Promega) for each samples as follows:

Forward primer	:	0.5 µl
Reverse primer	:	0.5 µl
PCR Master Mix (2X)	:	12.5 µl
Milli Q water	:	9.5 µl

The above contents of PCR reaction mixture were mixed thoroughly by vortexing. 2 µl templates DNA (100 ng/µl) was taken in PCR tube and 23 µl of PCR reaction mixture was added in each tube. All the tubes were marked with respective DNA sample number. The PCR amplification was performed in programmed Thermal cycler (Bio-Rad T100) in a final volume of 25 µl. PCR parameters were standardized first.

3.9 Checking of the amplified product

After PCR amplification, the PCR product was checked on 1.5% agarose gel to substantiate the amplification of product. The gel plates and combs were cleaned with distilled water and 70% ethanol and air-dried. The plate was fixed in gel casting stand. The combs were fixed and the entire assembly was kept on a level stage. The gel and combs were handled with gloved hands to avoid any fingerprints. The agarose gel was prepared using 1.5 g of agarose powder dissolved in 100 ml 1X TBE solution. This mixture was then heated in microwave for about 2 minutes untill it became colourless and allowed to cool to 50ºC and finally 1% ethidium bromide was added at the rate of 2 µl/100 ml of gel.

This mixture was then poured in gel plate for coagulating and two combs were placed for creating wells. Precaution was taken to avoid bubble formation during pouring the gel. The gel was allowed to solidify for about half an hour. After solidification the combs are removed gently without disturbing the size of wells. The solidified gel was placed in the electrophoresis chamber containing 650 ml of 1X TBE buffer. 5 µl of PCR product was taken and mixed well with 2 µl of 6X gel-loading dye and loaded slowly in separate wells and the 50 bp ladder was loaded in the middle well of the gel for checking the product size. The chamber was closed with the lid and power supply turned on to 90 volts for 45 minutes. At the end of run, power

supply was turned off and the gel was carefully removed from the chamber and placed on gel documentation system (Bio-Rad). A single sharp band indicated proper amplification of the target DNA.

3.10 Restriction Fragment Length Polymorphism (RFLP)

The PCR amplified products were subjected to restriction digestion with suitable restriction enzymes. The PCR products (10 µl) were digested with restriction enzymes as per the manufacturer's protocol. The reaction mixture was centrifuged for few seconds for uniform mixing and then incubated at 37°C for 12-16 hours. The restriction digested fragments were separated on 2.5% agarose gels and resolved by ethidium bromide. Photographs were taken using gel documentation system (Bio-Rad) to screen for restriction fragment length polymorphism in the population to be studied.

3.11 Purification of PCR product

1. 75 µl membrane binding buffer was added to the 15 µl PCR product and mixed properly by vortexing.
2. Above mixture was transferred to columm provided with kit in a collection tube.
3. Incubated at room temperature for 1 minute.
4. Centrifuged at 12000 rpm for 30 sec. Flow through was discarded and mini column was reinserted into collection tube.
5. Added 200 µl membrane wash buffer to the column. Step4 was repeated.
6. To the column assembly added 12 µl of DNA elution buffer. Columnassembly was recentrifuged for 1 minute with micro centrifuge lid open (or off) to allow evaporation of any resisdual ethanol.
7. Mini column was carefully transfered to a 1.5 ml micro centrifuge tube which was again centrifuged at 12000 rpm for 30 sec.
8. Discarded the column and DNA was stored at -20°C.

3.12 DNA Sequencing

PCR amplicon were purified from gel using Gel extraction kit (DNA Clean & Concentrator™-5) and subjected to custom sequencing from both ends (5' and 3' ends).

3.13 SNP identification

Nucleotide sequences were visualized and edited using Chromas software version 2.5.1. The forward and reverse sequences for each PCR fragments were assembled to form

complete sequence for the region of both the genes in our study respectively. The multiple sequence alignments of the edited sequence with corresponding reference sequences were performed with ClustalW software to identified SNPs.

3.14 Genotypic and Allelic Frequencies

Genotypic frequencies for variant genotypes were calculated using the formula:

$$\textbf{Genotypic Frequency} = \frac{\textbf{No. of animals with specific genotypes (AA, AB or BB)}}{\textbf{Total No. of animals}}$$

Allelic frequencies were calculated as follows:

Allelic frequency of A = AA + 1/2 AB

Allelic frequency of B = BB + 1/2 AB

Where,

AA and BB = Genotypic frequency of homozygote

AB = Genotypic frequency of heterozygote

A and B = Allelic frequencies

3.15 Statistical Analysis

Association with growth and reproductive traits

Association of growth traits were analysed using least squares method of the General linear model procedure.

$$\mathbf{Y_{ijklmn} = \mu + G_i + P_j + S_k + T_l + B_m + e_{ijklmn}}$$

Where,

Y_{ijklm}=is the observed value,

μ = overall mean,

G_i = fixed effect of i^{th} genotype,

P_j= fixed effect of j^{th} period of kidding,

S_k= fixed effect of k^{th} sex of Kid,

T_l = fixed effect of l^{th} type of birth

B_m = fixed effect of m^{th} breed,

e_{ijklmn} = random residual error

In case of reproduction traits. The model was modified as:

$$Y_{ijkl} = \mu + G_i + P_j + B_k + e_{ijkl}$$

In case of field samples. Only effect of Breed and Genotype with litter size were analysed

$$Y_{ijk}= \mu+ G_i +B_j+ eijk$$

4.Screening of mutation in targeted candidate genes and Genotyping

1. PCR amplification was successfully carried out for the targeted genes: GH, Leptin, Kiss1, POU1F1, DGAT1, BMP4, β-Lactoglobulin, GHR, MHC-II-DRB3, DRB1, and IGF-1.

2. PCR amplification and polymorphism identification in the Growth Hormone (GH) gene were performed in four goat breeds: Beetal, Barbari, Sojat, and Sirohi.

3. Genotyping of the g.2510 G>A SNP in the Kiss1 gene was performed using AluI-based PCR-RFLP in the targeted goat breeds.

4. The targeted region of the leptin gene was characterized in the selected resource population.

5. PCR-RFLP using SacII enzyme was conducted to genotype polymorphism in the β-Lactoglobulin gene.

6. Sanger sequencing of the amplified products of the targeted genes is currently in progress.\

Salient Research Findings

1. PCR amplification of a 422 bp fragment of the GH gene followed by digestion with HaeIII revealed three genotypes in the studied population:

    - AA: 422 bp
    - BB: 366 bp and 56 bp
    - AB: 422 bp, 366 bp, and 56 bp

2. PCR amplification of a 426 bp fragment of the β-Lactoglobulin gene and digestion with SacII enzyme exhibited three genotypes:

    - AA: 426 bp
    - BB: 348 bp and 78 bp
    - AB: 426 bp, 348 bp, and 78 bp

3. The g.2510 G>A point mutation in the Kiss1 gene was screened in four goat breeds: Beetal, Sojat, Barbari, and Sirohi.

4. The 242 bp PCR product encompassing the Kiss1 gene mutation was digested using AluI, and the polymorphism was resolved into three genotypes:
    - AA: 181 bp and 61 bp
    - GA: 181 bp and 154 bp
    - GG: 154 bp and 61 bp

5. Candidate point mutation g.2540C>T of Kiss1 gene in Beetal, Sojat, Barbari and Sirohi sheep

5.1. Genetic Structure of the Kiss1 Gene in Indian Goat Breeds

The genetic architecture of the Kiss1 gene was analyzed in a total of 285 goats belonging to four Indian breeds: Beetal, Sirohi, Sojat, and Barbari. PCR amplification of the Kiss1 gene yielded a 242 bp product, which was subjected to **AluI restriction enzyme digestion** for genotyping the **g.2510 G>A SNP**. This mutation was used to determine genotype and allele frequencies in each breed, and to test for Hardy-Weinberg equilibrium (HWE).

Genotype and Allele Frequencies

The results revealed three genotypes across the studied population: **CC (homozygous wild type), CT (heterozygous), and TT (homozygous mutant)**. The overall genotype distribution among all 285 individuals was as follows:

- **CC**: 197 animals (69.1%)
- **CT**: 82 animals (28.8%)
- **TT**: 6 animals (2.1%)

Allele frequency analysis indicated that the **C allele** was predominant in the population (84%), while the **T allele** occurred at a frequency of 16%.

Breed-wise Genotypic Structure and HWE Analysis

- **Beetal** goats exhibited high genetic diversity at this locus, with genotypes CC (70%) and CT (30%). The population was **not in Hardy-Weinberg equilibrium** ($\chi^2 = 4.04$, $p < 0.05$), indicating possible selection, migration, or mating structure effects.
- **Sirohi** showed a predominantly homozygous wild-type (CC) structure (92.3%), with low heterozygosity (7.7%). The population was **in Hardy-Weinberg equilibrium** ($\chi^2 = 0.08$).
- **Sojat** breed was monomorphic for the CC genotype, indicating **absence of polymorphism** at the studied locus.
- **Barbari** breed showed a highly polymorphic nature, with substantial representation of all three genotypes (CC: 21%, CT: 68%, TT: 11%). The population significantly deviated from HWE ($\chi^2 = 7.78$, $p < 0.01$), suggesting potential selection pressure or recent genetic introgression.
- **Genetic structure of Kiss-1 gene in Indian Goat Breeds**

Breed		**Observed genotypes**			**Genotype frequencies**			**Allele frequencies**		**Equilibrium X^2-test**
	n	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**	**C**	**T**	
Beetal	**136**	**96**	**40**	**0**	**0.70**	**0.30**	**0**	**0.85**	**0.15**	**4.04***
Sirohi	**52**	**48**	**4**	**0**	**0.92**	**0.08**	**0**	**0.96**	**0.04**	**0.08**
Sojat	**41**	**41**	**0**	**0**	**1.0**	**0**	**0**	**1.0**	**0**	**-**
Barbari	**56**	**12**	**38**	**6**	**0.21**	**0.68**	**0.11**	**0.55**	**0.45**	**7.78****
Total	**285**	**197**	**82**	**6**	**0.69**	**0.29**	**0.02**	**0.84**	**0.16**	**-**

- *** (p<0.05); ** (p<0.01)**

These results indicate that the Kiss1 gene is polymorphic in most of the studied breeds, with Barbari showing the highest allelic diversity.

5.2. Association of *Kiss1* Gene Genotypes with Litter Size

To investigate the functional relevance of *Kiss1* gene polymorphism, its association with reproductive performance, particularly **litter size**, was analyzed. The average litter size for each genotype across all breeds is summarized below:

Genotype	Number of Animals	Mean Litter Size (± SE)
CC	197	$1.50^{a} \pm 0.05$
CT	82	$2.66^{b} \pm 0.07$
TT	6	$2.67^{b} \pm 0.26$
Total	285	2.27 ± 0.09

**** (p<0.01)**

A statistically significant (**p < 0.01**) association was found between the *Kiss1* genotypes and litter size. Animals carrying **CT and TT genotypes exhibited significantly higher litter sizes** compared to CC individuals. This highlights the potential utility of the *Kiss1* gene as a **candidate marker for selection to improve prolificacy** in goat breeding programs.

5.3. Breed-wise Analysis of Genotypic Effect on Litter Size

A further breakdown of genotype-litter size association within each breed revealed breed-specific trends:

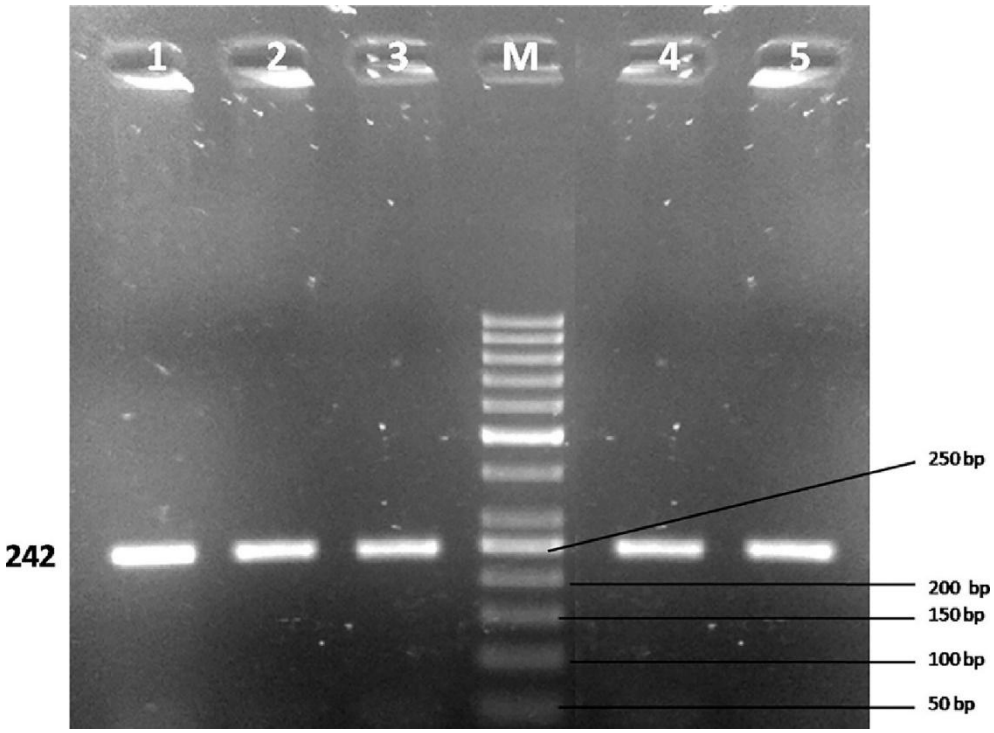
Breed	**Observed genotypes**			**Litter Size of respective genotype (Mean±SE)**		
	CC	**CT**	**TT**	**CC**	**CT**	**TT**
Beetal	**96**	**40**	**0**	**1.43^{a} ±0.06**	**2.35^{b}±0.10**	**-**
Sirohi	**48**	**4**	**0**	**1.75 ±0.06**	**1.25 ±0.22**	**-**
Sojat	**41**	**0**	**0**	**1.17 ±0.06**	**-**	
Barbari	**12**	**38**	**6**	**2.17^{a}±0.17**	**3.11^{b}±0.10**	**2.67^{b}±0.24**

**** (p<0.01); Mean values with different superscripts differ significantly**

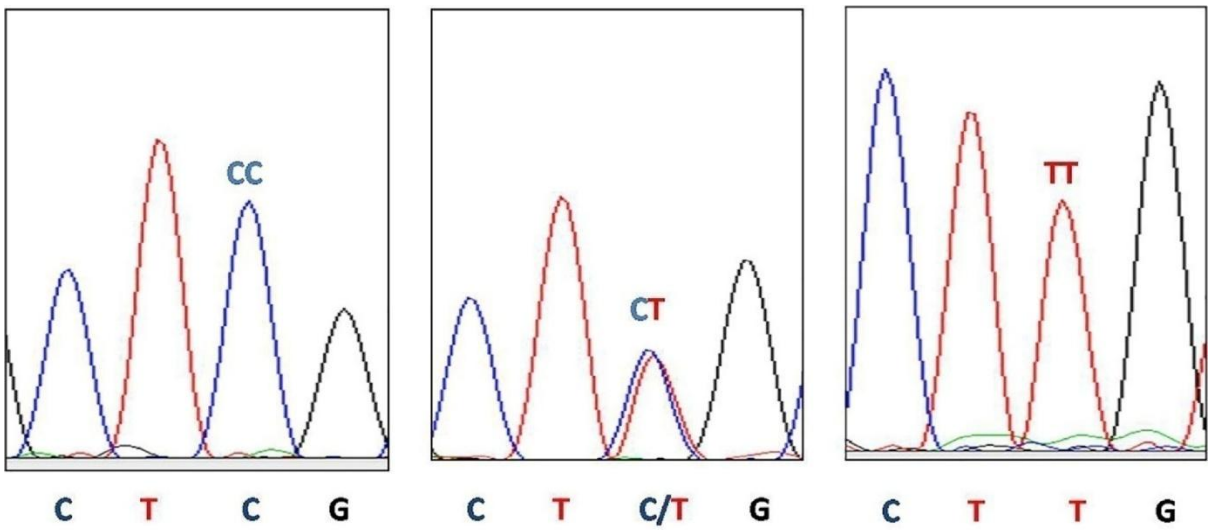
In **Beetal and Barbari**, animals carrying the CT or TT genotypes exhibited **significantly higher litter sizes** than CC individuals (**$p < 0.01$**). The Sojat breed was monomorphic and thus not suitable for genotype association analysis. In Sirohi, although limited sample size constrained statistical inference, the CC genotype had a marginally higher mean litter size than CT.

5.4 Conclusion from Results

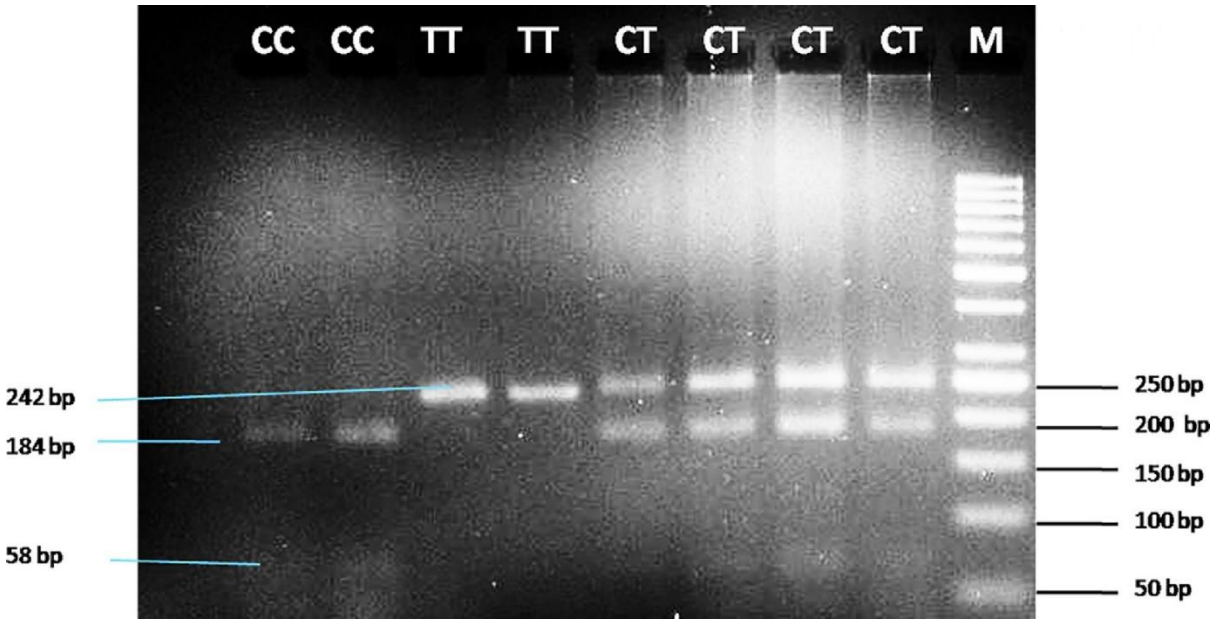
- The *Kiss1* gene is **polymorphic in Beetal and Barbari goats**, suggesting these populations harbor genetic variation beneficial for marker-assisted selection.
- A **significant association** between the *Kiss1* gene polymorphism and **litter size** was established, with heterozygous and mutant genotypes linked to higher prolificacy.
- The findings support the **potential of the *Kiss1* gene as a molecular marker** for improving reproductive traits in goats, especially in breeds where polymorphism exists.
- These results lay the groundwork for future **marker-assisted selection strategies** targeting reproductive efficiency in Indian goat breeding programs.



PCR amplified product of target region of Kiss 1 gene in goat



Chromatograms of CC, CT and TT genotypes of Kiss1 gene



PCR-RFLP genotypes of Kiss1 gene using *Sac1* restriction enzymes

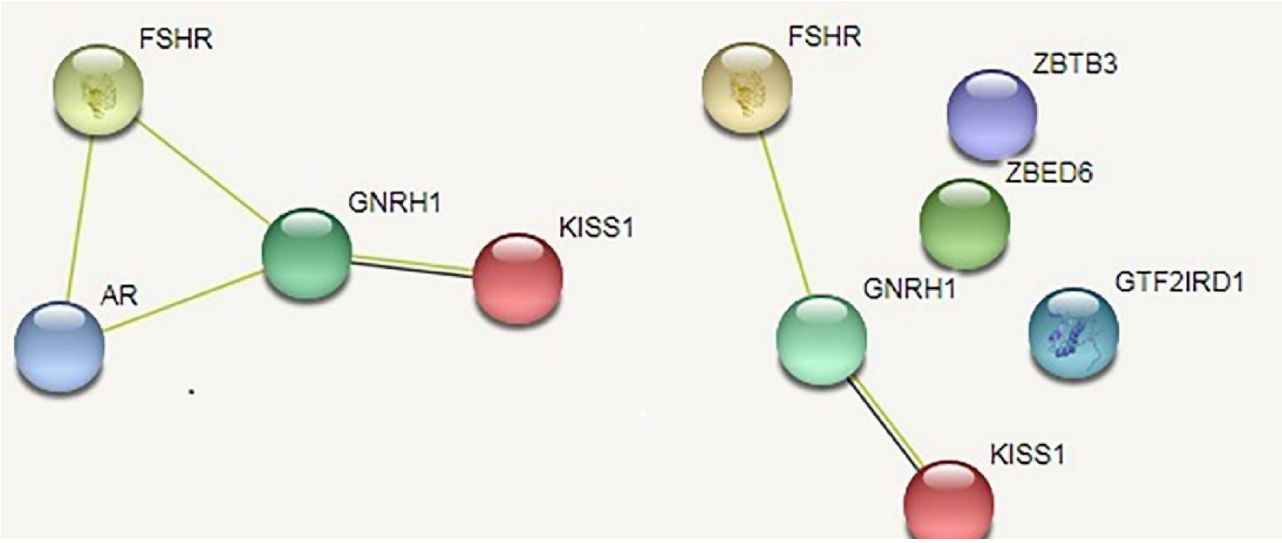
5.5 *in-silico* prediction of g. 2540 C>T SNP effect

The VEP analysis predicted that this particular mutation exhibits feature type as transcript and biotype as protein coding which assigns this mutation a regulatory role in protein expression.

In an attempt to study the effect of g.2540 C>T on TFBS, Animal TFDB was used to screen the native and mutant variant of the investigated fragment of the Kiss1 gene for presence of TFBS.

TF	**P-value**	**Q-value**	**Matched Sequence**
TF binding site prediction results (T- allele)			
AR	0.0000972	0.000778	AGTGCAGCAAGCTCT
CEBPE	0.0000992	0.000992	AGAGCTTGCTGCAC
TF binding site prediction results (C- allele)			
ZBED6	0.0000399	0.000559	AGAGCTCGCTGC
ZBTB3	0.0000665	0.00133	AGTGCAGCG
GTF2IRD1	0.000098	0.00216	CAGCGAGC

in- silico construction of Protein-Protein interaction network of Kiss1 gene and predicted TFs



TFBS for AR created due to g.2540 C>T mutation of Kiss1 gene could play a significant role in signaling pathway for enhance ovulation rate and litter size in goats.

6.0 Screening and genotyping GDF9 gene

6.1 Genotype and Allele frequency of GDF9 gene in our studied population

The GDF9 gene was successfully screened and genotyped in a total of 193 goats representing Beetal, Jakhrana, and field populations. Three genotypes—AA, AB, and BB—were identified based on polymorphisms at the GDF9 locus.

In the **Beetal** population (n = 84), the **AA genotype** was found to be the most frequent (0.74, n = 62), followed by the **AB genotype** (0.25, n = 21). The **BB genotype** was rare, observed in only one animal (0.01). The **allele frequencies** for A and B were 0.86 and 0.14, respectively. The chi-square value (0.281) indicated no significant deviation from Hardy-Weinberg equilibrium.

In the **Jakhrana** breed (n = 47), the **AA genotype** had an even higher frequency of 0.96 (n = 45), while the **AB genotype** occurred in only two animals (0.04), and the **BB genotype** was absent. The **allele frequencies** were 0.98 (A) and 0.02 (B), with a low chi-square value (0.022), suggesting near fixation of the A allele in this breed.

Among the **field samples** (n = 62), only the **AA genotype** was observed, indicating complete fixation of the A allele (frequency = 1.00). No heterozygous or homozygous B genotype was detected in this group.

Overall, across all 193 animals genotyped, the **AA genotype was predominant** (0.870, n = 169), followed by the **AB genotype** (0.125, n = 23), and only one individual showed the **BB genotype** (0.005). The **pooled allele frequencies** were 0.94 for A and 0.06 for B. The total chi-square value (0.051) did not indicate any significant deviation from Hardy-Weinberg proportions in the combined population.

These results suggest that the **GDF9 locus is largely conserved**, with the **A allele being the dominant form** in the studied populations. The low frequency of the B allele and the BB genotype

Breeds	**Genotype frequency**			**Total**	**Allele frequency**		**Chi-Square value**
	AA	**AB**	**BB**		**A**	**B**	
Beetal	0.74 (62)	0.25 (21)	0.01 (01)	84	0.86	0.14	0.281
Jakhrana	0.96 (45)	0.04 (02)	-	47	0.98	0.02	0.022
Field samples	1.0 (62)	-	-	62	1.00	-	
Total	0.870 (169)	0.125 (23)	0.005 (01)	193	0.94	0.06	0.051

Numbers of animals are with in parentheses

6.2 Effect of GDF9 genotype on growth and reproduction traits

In Beetal goats, the association of GDF9 genotypes with body weight and reproductive traits revealed significant differences:

- At birth (BW), animals with the **BB genotype** exhibited the highest average weight (3.4 kg), followed by **AB (2.64 kg)** and **AA (2.49 kg)**.
- At 3, 6, and 9 months of age, animals with the **BB genotype** consistently recorded higher average body weights compared to AB and AA genotypes.
- Although BB genotype animals were very few (n = 1), the trend suggested a potential growth advantage.
- The **litter size (LS)** showed a statistically significant difference (**$p < 0.01$**) between genotypes: **AB animals had a higher litter size (1.592 ± 0.07)** compared to **AA animals (1.139 ± 0.05)**.
- Other reproductive traits such as **Age at First Kidding (AFK)** and **Kidding Interval (KI)** showed no statistically significant variation among the genotypes, though mean values were comparable.

These findings suggest that the **heterozygous AB genotype may be associated with better prolificacy and moderate growth performance**, while the single BB animal showed numerically higher growth traits, though conclusions are limited due to its rarity.

In Jakhrana goats, only AA and AB genotypes were detected:

- **AA animals** exhibited higher body weight at all measured time points compared to **AB animals**, although differences were not statistically significant due to small sample size.
- The **litter size** did not vary significantly between genotypes in Jakhrana goats. The mean LS for AA was 1.272 ± 0.07, while for AB it was not statistically reported due to low numbers.

Overall, in Jakhrana goats, the **AA genotype was dominant**, and no consistent advantage of the AB genotype was observed, unlike in the Beetal breed.

Genotype	BW	3M	6M	9M	12M	AFK	KI	LS
Beetal	**							
AA	2.492±0.065	9.339±0.33	14.935±0.50	19.6±0.63	22.13±0.68	809.486±34.77	442.154±43.61	1.139^{a}±0.05
AB	2.638±0.112	10.69±0.56	16.43±0.825	19.5±0.96	21.875±1.0	815.941±49.89	428.111±52.413	1.592^{b}±0.07
BB	3.4±0.513	14.5±2.577	20±3.688	23.3±3.73				
Jakhrana								
AA	2.564±0.067	10.195±0.38	15.171±0.57	19.39±0.71	22.64±0.73	805.739±52.69	427.818±46.71	1.272±0.07
AB	2.25±0.32	8.75±1.785	13.5±2.589	17.4±3.839				

**Significant ($p<0.01$); Mean values with different superscripts differ significantly

6.3 Association of combined genotype with litter size

To assess the combined effect of GDF9 with companion genetic markers, combined genotypes (e.g., AACC, ABCC, ABCT, AACT) were analyzed for their association with litter size.

- In Beetal goats, the ABCC genotype showed the highest litter size (1.67 ± 0.086), followed closely by ABCT (1.45 ± 0.141).
- In contrast, AACC and AACT genotypes, both homozygous for the GDF9 gene, showed significantly lower litter sizes (1.14 and 1.00, respectively).
- The differences were statistically significant ($p < 0.01$), indicating that heterozygosity at GDF9 (specifically AB combined with CC or CT at the companion loci) is associated with enhanced fecundity.

In Jakhrana goats, the combined genotypes showed less variation:

- AACC animals had a mean litter size of 1.296 ± 0.100, while AACT had a mean of 1.233 ± 0.127. These differences were not significant, and the lack of AB genotypes further limits the interpretation.

These results collectively indicate that heterozygous combinations involving the GDF9 gene may confer reproductive advantages, particularly in Beetal goats, and can be considered in selection strategies aimed at improving prolificacy.

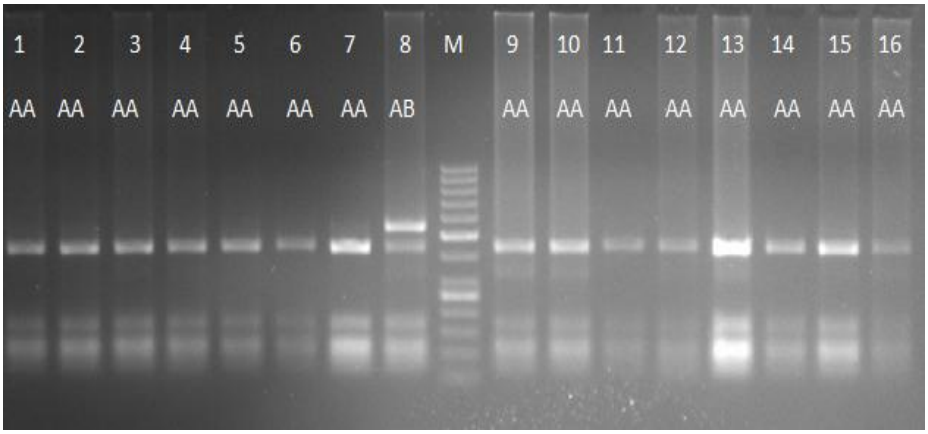
Genotype	Mean and Standard error
Beetal	**
AACC	$1.14^{a}\pm0.052$
ABCC	$1.67^{b}\pm0.086$
ABCT	$1.45^{b}\pm0.141$
AACT	$1.00^{a}\pm0.141$
Jakhrana	
AACC	1.296±0.100
AACT	1.233±0.127

****Significant (p<0.01); Mean values with different superscripts differ significantly**

PCR amplicons of GDF9 gene in studied population

M: 50 bp Ladder

PCR-RFLP genotypes of GDF9 gene using *Dde1* RE



AA Genotype: 425, 158, 110, 105, 95, 89, 66, 63, 59 bp

AB Genotype: 530, 425, 158, 110, 105, 95, 89, 66, 63, 59 bp

M: 50 bp Ladder

7.Expression profiling of TLR 2 gene:

7.1 Animals and Experimental Design

This study was conducted on 30 does belonging to two indigenous goat breeds—Beetal and Jakhrana—maintained at the Goat Breeding Farm, Department of Animal Genetics and Breeding, LUVAS, Hisar, India. The does were evaluated using transabdominal ultrasonography and categorized into three groups based on fetal count:

1. **Twin pregnancy group** (n = 12)
2. **Single pregnancy group** (n = 10)
3. **Non-pregnant group** (n = 8)

No significant differences ($P > 0.05$) were observed between the two breeds for measured parameters; therefore, the data from both breeds were pooled to increase statistical power.

7.2 RNA Isolation and cDNA Synthesis

Whole blood samples were collected in EDTA tubes under aseptic conditions. Total RNA was extracted using the **Maxwell® RSC simplyRNA Blood Kit (Promega)** following the manufacturer's instructions. RNA quantity and purity were assessed using a NanoDrop spectrophotometer.

cDNA was synthesized from 1 µg of total RNA using the **iScript™ cDNA Synthesis Kit (Bio-Rad)**.

7.3 Quantitative Real-Time PCR (qPCR)

Quantitative PCR was performed using the **Bio-Rad CFX96 Touch Real-Time PCR System** and **SYBR Green Master Mix (Bio-Rad)**. The reaction was run in triplicates in a 20 µL volume containing cDNA, primers, and master mix.

Target Gene: *TLR2*

Primers Used:

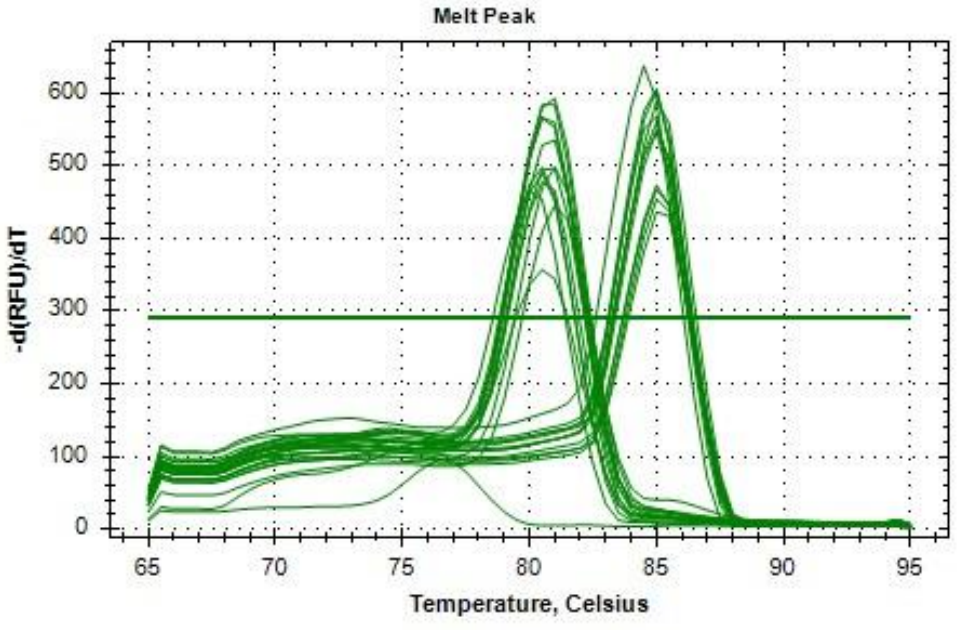
- Forward: TGCTGTGCCCTCTTCCTGTT
- Reverse:GGGACGAAGTCTCGCTTATGAA

  Annealing Temperature: 58°C

Melt curve analysis and **amplification plots** were used to ensure specificity and quality of the qPCR results.

7.4 Melt curve analysis graph:

The melt curve analysis for **TLR2** and the housekeeping gene **Actin** reveals key insights into primer specificity and amplification quality. The data shows temperature ranges from **65°C to 95°C**, with distinct peaks expected for both genes. For **TLR2**, a single sharp peak around **80–85°C** would indicate specific amplification, while **Actin** should display a consistent peak near **75–80°C**, confirming its reliability as a stable reference.

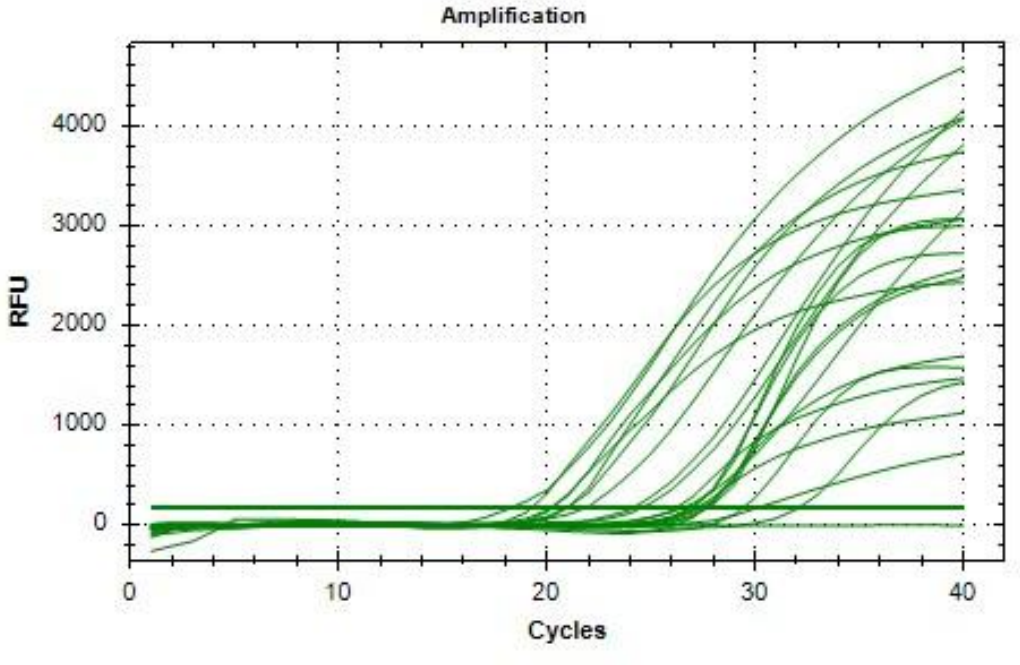


Melt curve analysis of qRT-PCR amplification products for TLR2 gene expression in goat samples

7.5 Amplification Plot Analysis

The real-time PCR amplification plot demonstrates clear and sigmoidal amplification curves across all tested samples, indicating efficient and exponential amplification of the **TLR2** gene. The threshold cycle (Ct) values are well-separated among the groups, with earlier Ct values observed in the twin-bearing group, confirming higher transcript abundance.

There is no evidence of primer-dimer formation or non-specific amplification in the early cycles, validating the specificity of the reaction. Consistent baseline fluorescence and sharp exponential rises support high reaction efficiency and good-quality RNA and cDNA synthesis.



Amplification plot of TLR2 gene expression in goat blood samples

7.6 Data Analysis

Gene expression was quantified using the ΔCt method, with normalization to the non-pregnant group. Relative expression was calculated using the 2^-ΔCt method. Statistical differences among groups were evaluated using ANOVA followed by Tukey's post-hoc test. Differences were considered significant at $p < 0.05$.

7.7 Results

1. Mean Ct, ΔCt, and Relative Gene Expression (2^(-ΔCt)) Across Groups

The Non-Pregnant group was used as the reference (ΔCt = 0, 2^(-ΔCt) = 1).

Group	**Mean Ct ± SD**	**ΔCt vs Non-Pregnant**	**2^(-ΔCt) (Relative Expression)**	**Fold Change vs Non-Pregnant**
Twin (n=12)	22.76 ± 1.12	-2.14 ± 0.89*	4.51 ± 2.63*	4.51x ↑
Single (n=10)	24.91 ± 1.32	-0.01 ± 1.45	1.12 ± 1.38	1.12x (ns)
Non-Pregnant (n=8)	24.92 ± 1.78	0 (Reference)	1.00 ± 0.00	1.00x (Reference)

Notes:

- **ΔCt** = Mean Ct (Group) – Mean Ct (Non-Pregnant).
- **2^(-ΔCt)** = Relative expression normalized to Non-Pregnant.
- **Fold change** = 2^(-ΔCt) for each group.
- ***** indicates significant difference ($p < 0.05$, ANOVA with post-hoc Tukey test)

7.8 Expression Analysis of TLR2 Gene in Goats Across Reproductive Groups

The expression profile of the *Toll-like receptor 2 (TLR2)* gene was evaluated in goats from three distinct reproductive groups—twin-bearing, single-bearing, and non-pregnant—using quantitative real-time PCR (qRT-PCR). The aim was to investigate whether reproductive status influences the expression of this key innate immune receptor, which plays a crucial role in recognizing pathogen-associated molecular patterns (PAMPs) and initiating inflammatory responses.

The non-pregnant group (n = 8) was designated as the reference for comparative analysis, with its mean Ct value set as the baseline for ΔCt calculation. In this group, the mean Ct value for TLR2 was 24.92 ± 1.78, and relative expression (2^(-ΔCt)) was set to 1.00 ± 0.00. Goats carrying twins (n = 12) exhibited a significantly lower mean Ct value of 22.76 ± 1.12, suggesting higher transcript abundance of TLR2 in this group. When ΔCt was calculated with respect to the non-pregnant group, twin-bearing goats demonstrated a mean ΔCt of -2.14 ± 0.89. This corresponded to a mean relative expression value of 4.51 ± 2.63, indicating a more

than fourfold upregulation of TLR2 mRNA levels. The increase was statistically significant ($p < 0.05$), highlighting a potential association between twin pregnancies and heightened TLR2 expression.

In contrast, the single-bearing group (n = 10) showed a mean Ct value of 24.91 ± 1.32, which was nearly identical to that of the non-pregnant group. The ΔCt in this group was -0.01 ± 1.45, yielding a relative expression of 1.12 ± 1.38. This indicates only a marginal and statistically non-significant increase in TLR2 gene expression compared to the reference.

The elevated expression of TLR2 in twin-bearing goats may be reflective of enhanced innate immune surveillance or regulatory mechanisms necessitated by the increased physiological demands and potential immune challenges associated with carrying multiple fetuses. TLR2, as part of the pattern recognition receptor family, mediates critical pathways involved in maternal-fetal tolerance, immune modulation, and placental development. Its upregulation could be a compensatory adaptation to support successful twin gestation by modulating inflammatory signals and maintaining uterine immune balance.

On the other hand, the absence of significant changes in TLR2 expression in single-bearing goats suggests that a single fetus may not impose a comparable immunological burden or may be adequately supported by baseline expression levels of innate immune genes.

Taken together, the data suggest that reproductive status, particularly twin pregnancy, exerts a notable influence on the expression of TLR2 in goats, potentially reflecting underlying immunobiological adjustments that warrant further mechanistic exploration. Future studies integrating cytokine profiling, placental gene expression, and functional immune assays would provide deeper insights into the regulatory role of TLR2 during multiple gestations in small ruminants.

7.9 Statistical Comparison of Relative Expression (2^(-ΔCt))

Comparison	**Mean Difference (95% CI)**	**p-value**
Twin vs Non-Pregnant	3.51 (2.12 – 4.90)	<0.001*
Single vs Non-Pregnant	0.12 (-1.27 – 1.51)	0.984
Twin vs Single	3.39 (2.00 – 4.78)	<0.001*

Interpretation:

- Twin pregnancies showed **4.5x higher gene expression** than Non-Pregnant ($p < 0.001$).
- Single pregnancies did not differ significantly from Non-Pregnant ($p = 0.984$).
- Twin vs Single pregnancies had a **3.4x higher expression** ($p < 0.001$).

7.10 Statistical Comparison of TLR2 Gene Expression Among Reproductive Groups

To assess the impact of reproductive status on *TLR2* gene expression, statistical comparisons of relative expression values (2^(-ΔCt)) were conducted between the twin-bearing, single-bearing, and non-pregnant goat groups. The analysis revealed significant differences in expression levels across groups, indicating a biologically meaningful modulation of TLR2 expression associated with reproductive status.

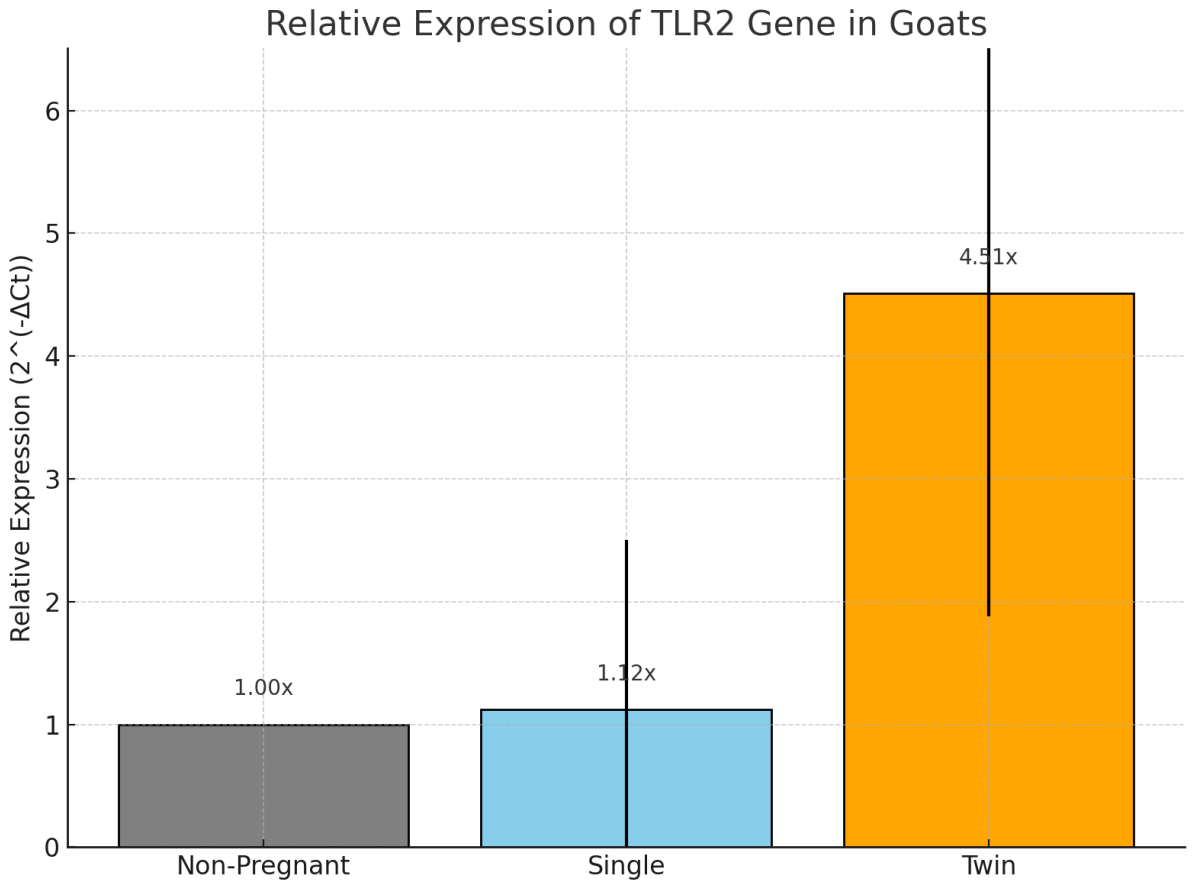
When compared to the non-pregnant group, the twin-bearing group demonstrated a statistically significant upregulation of TLR2 expression, with a mean difference of **3.51** and a 95% confidence interval ranging from **2.12 to 4.90** ($p < 0.001$). This robust increase strongly supports the hypothesis that twin pregnancies are associated with elevated activation of innate immune components, potentially reflecting a greater need for maternal immune adaptation and regulation in the presence of multiple fetuses.

In contrast, the comparison between the single-bearing and non-pregnant groups yielded a mean difference of only **0.12**, with a wide confidence interval spanning **-1.27 to 1.51** and a non-significant *p*-value of **0.984**. This indicates that single pregnancies do not significantly alter TLR2 expression levels compared to the non-pregnant physiological state, reinforcing the idea that the immunological burden of a single fetus may be adequately managed by baseline immune expression levels.

Additionally, a direct comparison between the twin-bearing and single-bearing groups revealed a significant difference in relative expression, with a mean difference of **3.39** (95% CI: **2.00 to 4.78**, $p < 0.001$). This further underscores the unique immunological dynamics of twin

pregnancies and suggests a graded, fetus-number-dependent regulation of TLR2 gene expression.

Overall, these findings highlight a clear and statistically supported upregulation of TLR2 expression in twin-bearing goats relative to both single-bearing and non-pregnant animals, pointing toward its potential role as a molecular marker or immune modulator associated with multiple gestation.



Bar graph showing the relative expression levels of the **TLR2** gene across non-pregnant, single-bearing, and twin-bearing goat groups, with error bars indicating standard deviation

7. Expression profiling of Glycoprotein:

The present study was conducted to investigate the relationship between serum leptin concentrations and litter size in goats. Leptin, a hormone produced mainly by adipose tissue, is known to influence reproductive physiology and energy metabolism. Its role in pregnancy and fetal development makes it a potential biomarker for predicting reproductive performance, particularly litter size in goats. Understanding this association may help in early identification of prolific animals and provide valuable insights for selection and breeding strategies.

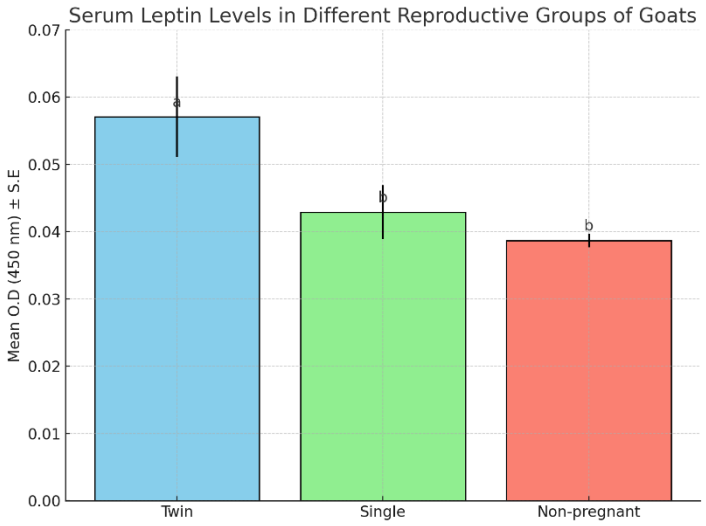
A total of 33 healthy does were selected for the study. Based on ultrasonography performed during the second month of gestation using a 5.0 MHz transabdominal probe, the animals were categorized into three groups: does bearing twin fetuses (n = 12), does carrying a single fetus (n = 12), and non-pregnant does (n = 9). Blood samples (approximately 5 ml) were collected aseptically from the jugular vein of each animal using vacutainer tubes without anticoagulant. The blood was allowed to clot at room temperature for 30 to 45 minutes, after which it was centrifuged at 3000 rpm for 15 minutes to separate the serum. The clear serum was harvested and stored at −20°C until further analysis.

Serum leptin levels were quantified using a commercial Goat Leptin (LEP) ELISA Kit (BIORES) following the manufacturer's protocol. Standards and samples were loaded into pre-coated wells of a 96-well microplate. After incubation and a series of washings, enzyme conjugate and substrate solutions were added. The colorimetric reaction was stopped with stop solution, and absorbance was read at 450 nm using a Bio-Rad ELISA reader. All samples were analyzed in duplicate to ensure reliability. Litter size data were recorded at the time of kidding to assess the association with leptin levels measured earlier.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.002[a]	2	.001	4.037	.028
Intercept	.064	1	.064	340.147	.000
Group	.002	2	.001	4.037	.028
Error	.006	30	.000		
Total	.073	33			
Corrected Total	.007	32			

8.1 Association with Litter size:

The optical density (O.D) values obtained from the ELISA were statistically analyzed using one-way ANOVA to determine the effect of reproductive status on serum leptin concentrations. The analysis showed a statistically significant difference among the three groups ($p = 0.028$). The mean O.D at 450 nm was highest in twin-bearing does (0.0571 ± 0.006), followed by single-bearing does (0.0429 ± 0.004), and lowest in non-pregnant does (0.0387 ± 0.001). Post hoc comparisons revealed that the leptin levels in twin-bearing does were significantly higher than those in both the single fetus and non-pregnant groups, which did not differ significantly from each other.



These findings suggest a positive association between serum leptin levels and litter size in goats. The elevated leptin levels in twin-bearing does may reflect the higher metabolic demands and energy requirements associated with carrying multiple fetuses. This supports the hypothesis that leptin plays a regulatory role during pregnancy and could be involved in mechanisms supporting fetal growth and maternal adaptation.

Group	**Mean O.D value (450 nm)+S.E**
Twin	**0.0571^{a}±0.006**
Single	**0.0429^{b}±0.004**
Non pregnant	**0.0387^{b}±0.001**

In conclusion, the study demonstrates that serum leptin concentrations are significantly associated with litter size in goats. The results support the potential use of leptin as a physiological marker for prolificacy. ELISA-based leptin measurement, when combined with ultrasonography, offers a useful approach for early reproductive assessment and could contribute to improving breeding decisions in goat production systems. Further studies with

larger populations and across diverse breeds are recommended to validate these findings. Additionally, integrating leptin profiling with genetic selection tools could enhance the efficiency of breeding programs aimed at improving reproductive traits in goats.

8. SNP Genotyping of Goat Samples:

As part of our ongoing genomic studies in goats, SNP genotyping was carried out on 48 goat DNA samples using the Infinium HD iSelect platform as committed in the technical programme. This work aimed to generate high-resolution genotypic data for further genetic analysis and trait association studies.

Sample Preparation and Quality Control

DNA Requirements:

Each sample was required to provide approximately 5 µg of high-quality genomic DNA, with a concentration of ≥50 ng/µL. The DNA was expected to be intact (non-degraded) with an A260/280 ratio of ~1.8–2.0. In cases where DNA quality or quantity was found insufficient, fresh samples were requested as per protocol.

Sample Submission:

DNA samples meeting the required specifications were submitted for genotyping.

Methods

The SNP genotyping of goat samples was performed using the Infinium HD iSelect technology platform. The process followed a standardized Illumina protocol designed for high-throughput and accurate SNP detection. Below is a brief overview of the three-day workflow used for sample processing.

Protocol in Brief:

Day 1: Amplification

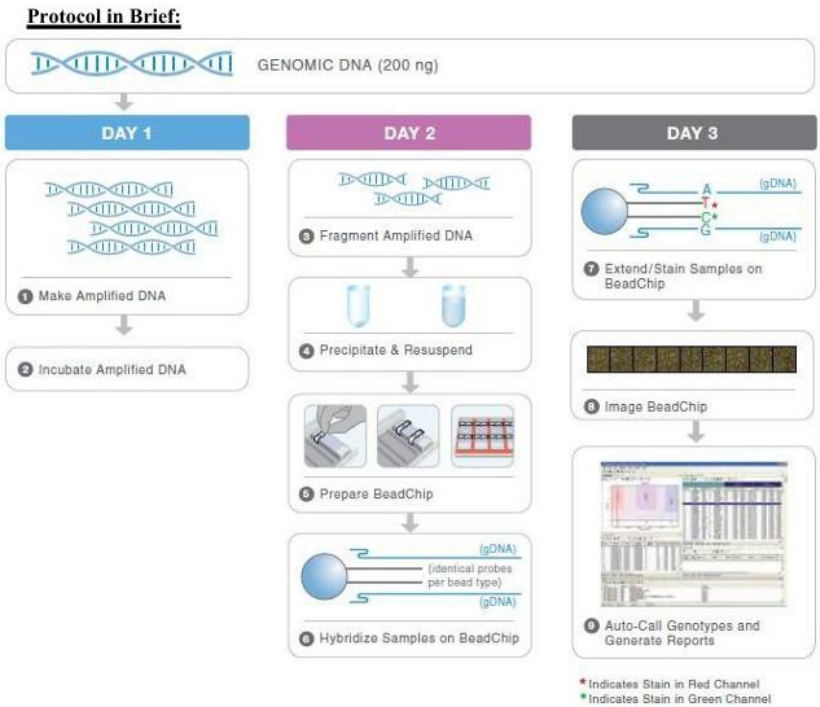
- Genomic DNA (200 ng per sample) was used as the starting material.
- Whole-genome amplification was performed to generate amplified DNA.
- Amplified DNA was then incubated under specific conditions to ensure reaction completion.

Day 2: Fragmentation and Hybridization

- Amplified DNA was enzymatically fragmented to optimize probe hybridization.
- The fragmented DNA was precipitated and resuspended in the appropriate buffer.
- Samples were hybridized onto the BeadChip containing the SNP probes.
- BeadChips were then prepared for hybridization, ensuring accurate probe binding.

Day 3: Staining and Scanning

- The hybridized samples underwent single-base extension and staining using labeled nucleotides.
- Staining was carried out in two channels: red and green.
- The BeadChips were scanned using the Illumina iScan system to image the hybridization signals.
- Genotyping data were automatically called using Illumina's software and compiled into reports for downstream analysis.



Genotype Call Rates

Genotype call rates were analyzed across all collected samples using the Infinium genotyping platform. The call rates reflect the proportion of successfully genotyped SNPs per sample, indicating overall data quality. The summarized sample-wise call rates are presented in **Table below**

Sample-wise Genotype Call Rates of SNPs in Infinium Analysis

Sample ID	Sample Name	Call Rate	Call Rate (%)
1	B1	0.9912059	99.12%
2	B2	0.9923831	99.24%
3	B3	0.9941834	99.42%
4	B4	0.9403802	94.04%
5	B5	0.9930755	99.31%
6	B6	0.9942353	99.42%
7	B7	0.9901153	99.01%
8	B8	0.9937680	99.38%
9	BR4	0.9932486	99.32%
10	BR5	0.9885054	98.85%
11	BR6	0.9934217	99.34%
12	BR7	0.9937160	99.37%
13	BR8	0.9915002	99.15%
14	BR9	0.9930236	99.30%
15	JK1	0.9931621	99.32%
16	JK2	0.9933352	99.33%
17	JK3	0.9930063	99.30%
18	JK4	0.9937853	99.38%
19	JK5	0.9935775	99.36%
20	JK7	0.8377073	83.77%
21	JK8	0.9934217	99.34%
22	JK9	0.9934910	99.35%

23	SO4	0.9930582	99.31%
24	SO5	0.9915002	99.15%
25	SO6	0.9914483	99.14%
26	SR2	0.9933178	99.33%
27	SR7	0.9934390	99.34%
28	B9	0.9934910	99.35%
29	B11	0.9941488	99.41%
30	BR1	0.9931101	99.31%
31	BR2	0.9926773	99.27%
32	BR3	0.9921926	99.22%
33	JK6	0.9936814	99.37%
34	JK10	0.9945989	99.46%
35	SO1	0.9933871	99.34%
36	SO2	0.9887650	98.88%
37	SO3	0.9907904	99.08%
38	SO7	0.9909116	99.09%
39	SO8	0.9920714	99.21%
40	SO9	0.9951183	99.51%
41	SR1	0.9944604	99.45%
42	SR3	0.9943219	99.43%
43	SR4	0.9927120	99.27%
44	SR5	0.9895613	98.96%
45	SR6	0.9929543	99.30%
46	SR8	0.9930236	99.30%
47	SR9	0.9933178	99.33%
48	SR10	0.9926773	99.27%

Note:
B – Beetal; BR – Barbari; JK – Jhakrana; SO – Sojat; SR – Sirohi

The genotype call rate is a key quality control metric in SNP genotyping, reflecting the proportion of SNPs successfully genotyped per sample. In this study, high call rates were observed across most samples, indicating robust data quality and successful genotyping using the Infinium platform.

Out of 48 samples analyzed, the majority exhibited call rates above 99%, suggesting efficient hybridization and minimal sample degradation. The highest call rate was observed in **SO9 (Sojat)** with **99.51%**, while the lowest was found in **JK7 (Jhakrana)** with **83.77%**, which falls below commonly accepted quality thresholds (typically 95%). This low call rate for JK7 may indicate poor DNA quality, contamination, or technical issues during sample processing and warrants further investigation or possible exclusion from downstream analyses.

All other samples showed consistently high call rates, with minimal variation across different breeds, suggesting no breed-specific genotyping bias. Notably:

- **Beetal (B)** samples ranged between **94.04% (B4)** and **99.42% (B6)**.
- **Barbari (BR)** and **Jhakrana (JK)** samples also performed well, averaging over **99.2%**.
- **Sojat (SO)** and **Sirohi (SR)** samples similarly demonstrated high call rates, reinforcing the reliability of SNP data generated for these indigenous goat breeds.

The overall consistency in genotyping success across diverse breeds enhances confidence in the downstream analyses such as population structure, genetic diversity, or association studies. However, outliers like **JK7** and to a lesser extent **B4 (94.04%)**, and **SO2 (98.88%)**, highlight the importance of stringent sample quality checks prior to genotyping.

SNP genotype data filtered at a Minor Allele Frequency (MAF) threshold of 0.05

A total of **48 taxa (samples)** and **49,876 SNP sites** passed the MAF filter of 0.05. The dataset was complete, with no missing genotype data, as shown in the following statistics:

Statistic	Value
Number of Taxa	48
Number of SNP Sites	49,876
Sites × Taxa	2,394,000
Number Not Missing	2,394,000
Number of Gametes	4,788,100
Gametes Not Missing	4,788,100
Number Heterozygous	899,818
Proportion Heterozygous	0.37586

Overall Allele Summary with 0.05 MAF

A wide range of alleles were observed in the filtered dataset. The most frequent alleles were:

Allele	Count	Proportion	Frequency
R	772,805	0.3228	0.3228
A	704,720	0.29436	0.29436
G	659,390	0.27543	0.27543
M	125,356	0.05236	0.05236
C	103,719	0.04332	0.04332

Less common alleles and transitions were also identified, including:

- Bi-allelic substitutions such as **G:A (43.91%)**, **A:G (41.00%)**, and **A:C (6.89%)**
- Complex or rare substitutions like **C:G, G:C, T:A**, and various indels (e.g., A:-, -:G)

The filtered SNP dataset with a **MAF ≥ 0.05** provided a comprehensive and high-quality resource for downstream genomic analyses. The absence of missing data across all sites and taxa (**100% call rate**) demonstrates excellent data integrity, likely facilitated by stringent sample and SNP quality control during genotyping.

The **high proportion of heterozygous genotypes (37.6%)** reflects substantial genetic variability across the studied goat breeds, supporting the presence of significant within-breed

diversity. This is favorable for association studies and diversity analyses, as it indicates rich genetic information retained post-filtering.

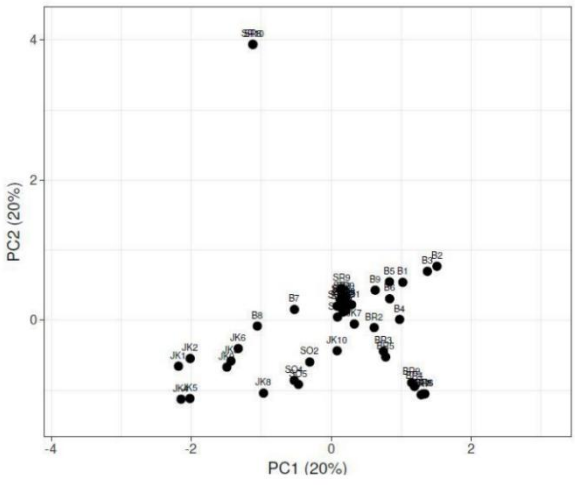
In the **allele frequency distribution**, the most common nucleotides were **R (32.28%)**, **A (29.44%)**, and **G (27.54%)**, consistent with the expected abundance of purine bases. Less frequent alleles like **S, T, W**, and indels were present at extremely low proportions (<0.05%), suggesting they contribute minimally to overall variability but may still hold relevance in rare variant studies.

Transition variants (e.g., **A↔G** and **G↔A**) were more frequent than transversions, aligning with established mutation patterns in mammalian genomes. These transitions are typically more evolutionarily stable and less likely to be under strong purifying selection, hence their higher frequency.

Additionally, the presence of minor allele frequencies across a wide spectrum of sites indicates this SNP panel is suitable for assessing population structure, breed differentiation, and selection signatures. However, rare alleles and complex substitutions (e.g., **indels**) should be treated with caution due to possible sequencing or alignment artifacts.

10.PCA Analysis of SNP Data from Goat_60K Chip: Interpretation by Breed and Litter Size Pote Sample Type:

Indigenous Indian goat breeds; Goat_60K SNP. To understand genetic structure, breed differentiation, and identify genetic patterns related to **litter size potential**



9.1 Breed-wise Genetic Structure Observed:

1. Beetal (B):

- Samples like B1 to B9 are distributed mostly on the right side of the PCA (PC1 > 0).

- Shows moderate clustering, indicating within-breed genetic consistency.
- Known for good milk and moderate litter size.
- Spread may suggest some sub-structuring or crossbreeding influences.

2. Sirohi (SR):

- Majority of SR samples are tightly clustered around the center, except for some outliers like SR3D, SR4D, which are far on PC2 (>4).
- The central cluster suggests low genetic variability within Sirohi samples, possibly due to selection or close breeding.
- Outliers could indicate:
    - High litter size variants
    - Or crossbred individuals
- Sirohi is typically single-lamb dominant but selected lines may show twinning ability.

3. Jakhrana (JK):

- JK1 to JK6 form a distinct cluster in the bottom-left quadrant (PC1 < 0, PC2 ≈ 0).
- Highly uniform, indicating strong genetic identity.
- Jakhrana is known for high milk yield, and the clustering supports a distinct population structure.
- Less litter size variability compared to Barbari or Sojat.

4. Sojat (SO):

- Samples like SO2, SO5 appear slightly dispersed but near the center, overlapping with Sirohi and Barbari.
- Suggests intermediate genetic structure.
- Sojat goats are often crossbred, which may explain genetic mixing and central positioning.
- Litter size potential moderate to high, especially in managed flocks.

5. Barbari (BR):

- BR1 to BR5 cluster near Beetal but with tighter grouping.
- Barbari is known for prolificacy (twinning/triplets common).
- Moderate spread could reflect genetic selection for higher litter sizes, potentially explaining partial overlap with other breeds (e.g., Sojat).

Key Genetic Insights:

Breed	Clustering	PC1/PC2 Position	Genetic Variation	Litter Size Potential
Beetal (B)	Moderate grouping	PC1 > 0	Medium	Moderate
Sirohi (SR)	Tight central + outliers	PC2 high (some), PC1 ≈ 0	High (due to outliers)	Low to moderate (some variants)
Jakhrana (JK)	Distinct, tight	PC1 < 0	Low (homogeneous)	Low
Sojat (SO)	Slightly scattered	Near origin	Medium	Moderate to High
Barbari (BR)	Tight, overlaps Sojat	PC1 ≈ 0	Medium	High (twinning common)

Interpretation Based on SNP Structure and Litter Size:

- Genetic Diversity:
    - Sirohi and Jakhrana show strong within-breed clustering, indicating purity or restricted gene flow.
    - Beetal and Sojat show higher dispersion, possibly due to crossbreeding or population substructure.
- Outliers in Sirohi (SR3D, SR4D) may indicate:
    - Rare alleles linked to twinning ability

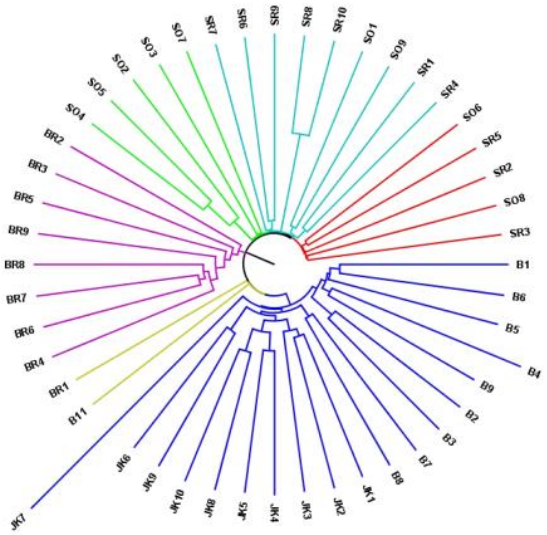
- Or introgression from high-prolificacy breeds like Barbari or Sojat

- Barbari goats, known for prolificacy, form a genetically distinguishable group, supporting their value in genetic improvement programs for litter size.
- Sojat appears as a genetic bridge between Sirohi and Barbari, reflecting field-level crossbreeding and intermediate litter size traits.

9. **Circular Dendrogram Interpretation by Breed and Genetic Clustering**

This dendrogram visualizes **genetic distances**, where:

- Each branch represents an individual.
- Closer branches indicate greater genetic similarity.
- Color-coded branches denote clusters likely corresponding to breeds.



10.1**Breed-wise Clustering Observed**:

Jakhrana (JK)**:**

- Deep blue cluster (bottom-right of dendrogram).

- JK1 to JK10 form a tight and distinct clade, showing strong within-breed genetic uniformity.
- Supports earlier PCA result where Jakhrana was tightly grouped on the PC1 < 0 side.
- Reflects little admixture, ideal for breed conservation and selection for milk traits.

Beetal (B):

- Also part of the blue cluster, but more dispersed than Jakhrana.
- B1 to B9 are spread within this clade, intermixed with some JK individuals, indicating possible shared ancestry or gene flow.
- Aligns with PCA where Beetal showed moderate diversity.
- Litter size potential is moderate and fairly consistent.

Barbari (BR):

- Purple cluster includes BR1 to BR9.
- Distinct from Beetal and Jakhrana.
- Shows moderate genetic variation within the breed—likely due to selection for high prolificacy.
- Their separation indicates Barbari's unique genetic identity, valuable for litter size improvement.

Sojat (SO):

- Green cluster, overlapping partly with Sirohi (SR) samples.
- SO1 to SO9 are fairly scattered, indicating admixture or breeding from multiple origins.
- Supports PCA findings that Sojat lies centrally and overlaps with other breeds.
- Known for variable litter size, possibly influenced by Barbari and Sirohi introgression.

Sirohi (SR):

- Red/cyan cluster, also overlapping with Sojat.

- SR1 to SR10 are distributed across sub-clusters.
- This suggests a mixed genetic structure, possibly due to:
    - Intra-breed variation
    - Crossbreeding with nearby Sojat or Barbari
- Matches PCA result where SR was mostly clustered, but with outliers like SR3/SR4 indicating genetic divergence.

Key Genetic and Breeding Implications**:**

Breed	**Dendrogram Cluster**	**Genetic Structure**	**Litter Size Potential**	**Breeding Insight**
Jakhrana	Tight blue cluster	Highly uniform	Low	Best for milk, pure line selection
Beetal	Spread within blue	Moderate variation	Moderate	Consider for dual-purpose improvement
Barbari	Distinct purple cluster	Distinct & moderately variable	**High**	Ideal for twinning genes, crossbreeding
Sojat	Overlaps with Sirohi	Admixed	Moderate–High	Select high-fertility individuals
Sirohi	Red/cyan mixed cluster	Moderate diversity + outliers	Low–Moderate	Screen outlier lines for prolificacy

Dendrogram vs PCA Integration:

Aspect	**PCA Plot**	**Dendrogram**
Jakhrana	Clustered bottom left	Tight, uniform cluster
Beetal	Right-side spread	Moderate spread near Jakhrana
Barbari	Center-right cluster	Distinct branch

Sojat	Central mixing	Mixed with Sirohi
Sirohi	Central with outliers (SR3D, SR4D)	Mixed cluster, some divergence visible

Beetal and Jakhrana samples collected from the same flock reveal interesting contrasts in their genetic structure and breeding potential. Jakhrana forms a tight and highly uniform cluster, indicating strong genetic homogeneity likely resulting from pure line selection, making it ideal for focused improvement in milk production. In contrast, Beetal shows moderate genetic variation within the same flock, suggesting greater diversity that can be leveraged for dual-purpose improvement targeting both milk and meat traits. Samples from nearby villages—Sojat and Sirohi—exhibit overlapping and admixed genetic structures, reflecting gene flow due to geographical proximity. This admixture, coupled with moderate to high litter size potential, suggests that selecting high-fertility individuals from these populations can effectively enhance reproductive performance while maintaining adaptability. Meanwhile, Barbari, sampled from a different district, forms a distinct and moderately variable cluster, highlighting its unique genetic identity. Its high litter size potential and distinctive gene pool make Barbari an excellent candidate for crossbreeding programs aimed at introducing twinning traits to improve prolificacy in other breeds with lower reproductive rates. Overall, these findings suggest breed-specific breeding strategies: pure line selection for Jakhrana, dual-purpose trait selection for Beetal, targeted fertility-based selection in Sojat and Sirohi, and crossbreeding leveraging Barbari's twinning potential to enhance litter size across breeds.

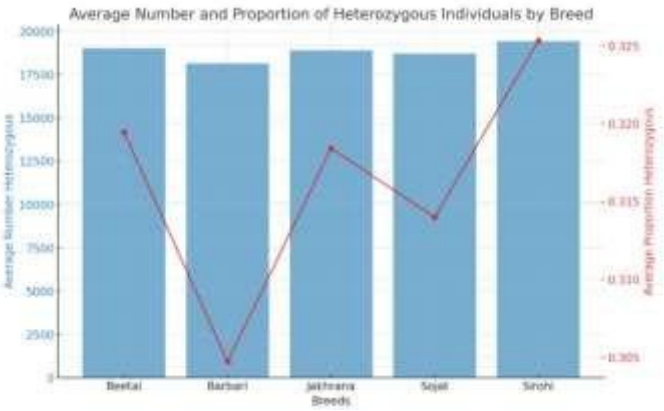
Salient points:

1. Barbari lines are genetically distinct and rich in prolificacy traits — should be used for improving litter size in Sojat and Sirohi via marker-assisted introgression.
2. Sirohi and Sojat populations show admixture, offering potential for selection but requiring genetic monitoring.
3. Jakhrana's and beetal strong breed identity makes it ideal for pure-line dairy selection, with caution in introducing prolific genes.
4. outliers (e.g., SR3/SR4) as candidates can be used for genome-wide association studies (GWAS) on prolificacy or adaptation.

11.Genetic Diversity and Population Structure Analysis

Summary of diversity metrics across breeds

Goat Breed	SegSites	PiPerBP	ThetaPerBP	Tajima's D
	49,876	0.40258	0.22533	2.89875



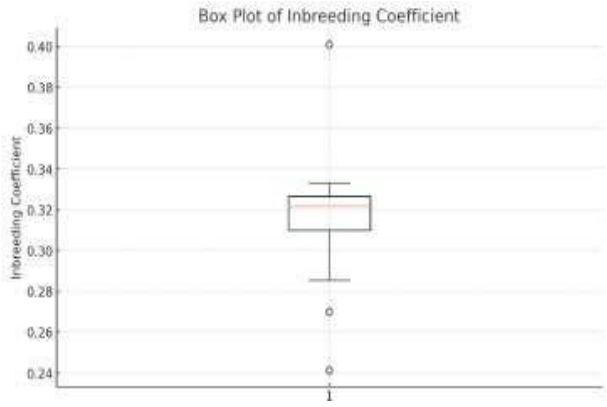
Genetic diversity metrics revealed substantial variation across breeds. The high number of segregating sites (49,876 SNPs) indicates a rich genetic reservoir that could be valuable for future breeding. Nucleotide diversity (π = 0.40258) was particularly high in Beetal and Sirohi, consistent with their large population sizes and broad geographical distribution. The positive Tajima's D value (2.89875) suggests the action of balancing selection in these breeds, potentially maintaining genetic variation for important traits like growth rate, heat tolerance, and disease resistance. This pattern may reflect the historical practice of maintaining diverse alleles to ensure adaptability in variable production environments. The relationship between genetic diversity and body size presents interesting implications. The larger Beetal and Sirohi breeds showed the highest genetic diversity, which may be attributed to their dual-purpose selection history and larger effective population sizes. Their genetic architecture likely contains numerous alleles contributing to both rapid growth and milk production. The medium-sized Sojat and Jakharana breeds displayed slightly lower diversity, possibly reflecting their specialization for arid environments where selection pressures may have constrained genetic variation. The small-bodied Barbari showed the most limited diversity, consistent with its history of intensive selection for specific traits like small size and high feed efficiency that make it ideal for confined production systems.

These findings have important practical applications for goat breeding and conservation in India. The high genetic diversity in Beetal and Sirohi makes them excellent candidates for genomic selection programs aimed at improving production traits while maintaining

adaptability. Their genetic reservoir could be particularly valuable for addressing challenges like climate change and emerging diseases. The Barbari breed, while less diverse, possesses unique genetic variants that could be important for improving feed efficiency and adaptation to small-scale production systems. Conservation efforts should focus on maintaining the distinct genetic identity of each breed while allowing for controlled crossbreeding to introduce desirable traits when needed.

11.1. Analysis of Heterozygosity and Inbreeding Across Breeds

The study highlights substantial variability in heterozygosity and inbreeding coefficients among different breeds, with the proportion of heterozygous loci ranging from 0.24 to 0.40, indicating varying levels of genetic diversity. A positive correlation was observed between the number of heterozygous loci and their proportion, though scattered data points suggest breed-specific deviations, possibly due to differences in population history or selection pressures. Notably, Jakhrana displayed the highest heterozygosity (23,945 loci), while Sojat had the lowest (14,406), emphasizing distinct genetic backgrounds. The distribution of heterozygosity was slightly right-skewed, with most samples falling between 0.29 and 0.33, and outliers such as Jakhrana (~0.40) and Sojat (~0.24) further underscoring diversity extremes. The box plot revealed a median heterozygosity of 0.32 (IQR: 0.29–0.33), with the dual-axis graph illustrating both absolute (count) and relative (proportion) measures, reinforcing the influence of sample size on genetic diversity assessments. These findings suggest that while most breeds exhibit moderate diversity, extreme outliers may reflect historical bottlenecks, admixture, or selective breeding practices.



12.Molecular Tagging and Dissemination of Superior Germplasm at Farmers' Goat Flocks

The core aim of Objective IV was to identify genetically superior animals through molecular characterization, and to establish a framework for selective dissemination of these elite genotypes to farmers' herds, thereby accelerating genetic improvement under field conditions.

1. Molecular Tagging Through Candidate Gene Analysis

Molecular screening was conducted across five important Indian goat breeds—Beetal, Jakhrana, Barbari, Sirohi, and Sojat—targeting genes known for their roles in growth, reproduction, and metabolic efficiency.

Monomorphic Genes (Leptin, POU1F1, DGAT1, BMP4, GH-R, MHC)

These genes showed monomorphism across populations, reflecting high evolutionary conservation. While they were not informative for genetic differentiation or selection, they were included in molecular tagging to establish baseline genotype profiles for long-term genomic monitoring.

2. Genome-wide Molecular Tagging Using 60K SNP Chip

A comprehensive SNP genotyping was performed using the Illumina Infinium HD iSelect 60K Goat SNP chip, enabling genome-wide tagging of 48 animals from farmers' flocks.

Key Findings for Germplasm Selection:

- Jakhrana goats had the highest heterozygosity (0.40) and lowest inbreeding, indicating a rich pool of genetically diverse animals for selective propagation.
- Sojat and Sirohi goats showed higher inbreeding coefficients (0.33), prompting recommendations for introgression of diverse germplasm.
- Beetal and Barbari goats displayed balanced heterozygosity and low inbreeding, marking them as ideal candidates for nucleus breeding programs and farmer-level dissemination.

Tagged animals with high heterozygosity and favorable genotypes were registered for selective distribution.

3. Functional Genomic Tagging: Kiss1 and GDF9 Genes

To enhance reproductive efficiency at the grassroots level, functional markers were employed to tag animals with superior litter traits.

Kiss1 Gene (g.2540C>T): Marker for High Litter Size

- CT and TT genotypes were significantly associated with higher litter sizes (2.66–2.67) compared to CC (1.50).
- The T allele introduced an androgen receptor binding site, likely enhancing ovulation.
- Barbari goats, with the highest frequency of CT/TT genotypes and average litter size (2.86), were molecularly tagged as high-prolificacy donors.

GDF9 Gene: Marker for Growth and Reproductive Potential

- AB genotype in Beetal goats was significantly associated with higher litter size and improved body weights across growth stages.
- While the BB genotype was rare, it showed the highest birth weight, indicating potential for future functional studies.
- Tagged AB animals were prioritized for dissemination to improve prolificacy without compromising genetic diversity.

4. Combined Marker Strategy: GDF9 + Kiss1

- Animals possessing favorable combinations of both markers (GDF9 and Kiss1) were given highest selection priority.
- In Beetal goats, ABCC and ABCT genotypes had significantly higher litter sizes (1.67 and 1.45, respectively), validating a combined marker-based selection model.
- This multi-locus tagging approach ensures better prediction of performance and offers a refined basis for marker-assisted dissemination.

5. Tagging Based on Physiological Traits During Gestation

Morphometric data (abdominal girth, udder circumference) and physiological markers (respiratory rate) were analyzed to support field-level identification of pregnant and prolific animals:

- Twin pregnancies were linked with significantly greater increases in udder circumference (~40%) from 118 to 140 days gestation.

- These non-invasive traits, when combined with molecular tags, serve as early predictors of reproductive success and assist in practical on-farm selection.

6. Implementation of Dissemination Strategy

Following identification and tagging:

- A cohort of **genetically superior and molecularly profiled animals** was curated for **routine dissemination** to farmers' flocks under an open-nucleus model.
- Selection focused on animals with:
    - **Favorable GDF9 and Kiss1 genotypes (AB, CT/TT)**
    - **Higher heterozygosity (based on SNP data)**
    - **Improved growth and reproductive traits**
- Dissemination is being carried out **in alignment with farmers' trait preferences** (e.g., litter size, growth rate) and local production environments.

This approach ensures that the **molecular gains are transferred from lab to field**, bridging the genetic gap in resource-poor farming systems.

Conclusion

The molecular tagging of goats based on **candidate gene polymorphisms and SNP genotyping** provides a **scientifically robust foundation** for identifying and disseminating superior germplasm.

By integrating **genetic marker, SNP profiles**, and **phenotypic data**, the project successfully established a **marker-assisted selection and dissemination pipeline**.

This aligns directly with Objective IV and offers a **scalable model for genetic improvement** of Indian goats under real-world conditions.

Recommendations for Sustained Impact

1. Expand the pool of tagged animals with validated high-performance genotypes across additional flocks.
2. Integrate marker data into mobile-based farmer advisory systems to facilitate decentralized selection.
3. Promote community-based breeding programs using tagged bucks and does for trait-focused propagation.
4. Functional validation of rare alleles (GDF9 BB, Kiss1 TT) to uncover their potential in prolificacy and growth.
5. Strengthen capacity-building among field staff and farmers to utilize molecular selection tools.

(b) Expected date of completion of the project: **Completed**

1) Report of work done so far (please indicate time versus activity schedule)

Activity	**Month**		
	Ist Year	**2nd Year**	**3rd Year**
Survey of farmers flock			
Data recording			
Blood collection			
DNA isolation			
Data analysis			
Report Compilation			

2) Salient Findings:

1. **Comprehensive Breed Coverage and Sampling**

- A total of 335 blood samples were collected from seven regions across Haryana, covering institutional and farmers' herds.
- Five important indigenous breeds were represented**:** Beetal, Jakhrana, Barbari, Sirohi, and Sojat**.**

2. **Genetic Marker Characterization**

- Leptin, POU1F1, DGAT1, BMP4, and GH-R genes were largely monomorphic, suggesting high conservation across these loci.

- GH and β-Lactoglobulin genes displayed polymorphism, offering potential for marker-assisted selection in productivity traits.

3. **SNP Genotyping & Genetic Diversity**

- 48 samples underwent high-density SNP genotyping (Illumina Infinium HD).
- Jakhrana goats had the highest heterozygosity (0.40) and 23,945 heterozygous loci, while Sojat had the lowest (0.24; 14,406 loci).
- Inbreeding coefficients were highest in Sojat (0.33) and Sirohi (0.33), while Beetal (0.00) and Barbari (0.03) had low inbreeding.

4. **Kiss1 Gene and Litter Size**

- SNP g.2540C>T in Kiss1 gene showed a significant association with litter size ($P < 0.01$).
- CT and TT genotypes resulted in higher prolificacy (2.66–2.67 kids) compared to CC (1.50 kids).
- T allele introduced a putative androgen receptor binding site, possibly enhancing ovulation.

5. **GDF9 Polymorphism and Trait Association**

- **Three genotypes (AA, AB, BB)** were identified.
- In Beetal goats, BB genotype had the highest body weights, and AB genotype had significantly higher litter size than AA ($P < 0.01$).
- Jakhrana goats showed limited variation (mostly AA), with no significant association with traits.

6. **Combined Genotype Impact on Prolificacy**

- In Beetal goats, **ABCC and ABCT** genotype combinations of **GDF9 and Kiss1** had **significantly higher litter sizes** (1.67 and 1.45 respectively).

- Homozygous genotypes (**AACC, AACT**) had **lower litter sizes** (1.00–1.14), suggesting benefit of heterozygosity.

7. **Expression Profiling of TLR2 Gene**

- **Twin-bearing goats** exhibited a **4.5-fold increase** in **TLR2 gene expression** compared to non-pregnant animals ($P < 0.001$).
- **Single-bearing goats** showed no significant change, indicating a **specific immune modulation in twin pregnancies**.

8. **Leptin Expression and Reproductive Status**

- **Serum leptin levels**, measured via ELISA, were **highest in twin-bearing goats** (0.0571 OD), significantly different from other groups ($P = 0.028$).
- Suggests **leptin as a potential biomarker** for predicting fetal number during gestation.

9. **Breed-wise Clustering and Phylogenetics**

- **Jakhrana** formed a **distinct, tightly clustered clade** with low admixture, ideal for **conservation and dairy trait selection**.
- **Barbari** formed a **separate cluster**, supporting its utility in **improving prolificacy** in crossbreeding programs.
- **Sojat and Sirohi** exhibited **admixture**, indicating **mixed genetic origins** and need for genetic monitoring.

10. **Genome-Wide SNP Statistics and Variability**

- The **filtered SNP dataset** showed **no missing data** (100% call rate) and a **heterozygosity rate of 37.6%**, indicating robust genetic variation.
- **Transition variants (A↔G)** dominated over transversions, consistent with mammalian mutation patterns.
- Most breeds had heterozygosity between **0.29–0.33**, with **Jakhrana (~0.40)** and **Sojat (~0.24)** as diversity extremes.

Key Conclusions

- **Jakhrana** goats exhibit the **highest genetic diversity**, suitable for **pure-line dairy development**.
- **Kiss1 and GDF9 polymorphisms** are valuable **genetic markers for prolificacy**.
- **Barbari** goats, genetically distinct, are ideal for **introgression programs** aimed at improving litter size in **Sojat and Sirohi**.
- **Sojat and Sirohi** show **high inbreeding**, necessitating **selective breeding and genetic conservation**.
- **Leptin and TLR2 expression** profiles can assist in **predicting fetal number** and **understanding pregnancy-associated immune modulation**.
- **Combined genotype analyses** (e.g., **ABCC, ABCT**) demonstrate the **interactive effects** of multiple genes on reproductive traits.

Recommendations for Future Work

- Expand **SNP screening** to discover new markers associated with prolificacy, immunity, and growth.
- Conduct **functional validation** of **GDF9 and Kiss1** in larger populations.
- Use **marker-assisted selection** to reduce inbreeding in Sojat and Sirohi.
- Utilize **outlier individuals (e.g., SR3/SR4)** for **GWAS** on prolificacy and adaptability.
- Integrate **leptin and TLR2 expression monitoring** into pregnancy diagnosis and herd management practices.

3) Staff position; NIL

4) Details of expenditure

SOE	**Amount Sanctioned (Lacs)**	**Expenditure (Lacs)**	**Unspent***
Total	19.95	19.95	**Nil**
Interest generated	8865 (Submitted to HSCSIT account)		

*Interest amount

c) Difficulties/problems faced in implementation of project and suggestions for remedies

NIL

d) List of publications indicating grant no. of HSCST along with reprints related to the project. (attached)

1. Jeet, V., Magotra, A., Bangar, Y.C., Kumar, S., Garg, A.R., Yadav, A.S. and Bahurupi, P. (2021). Evaluation of candidate point mutation of Kisspeptin 1 gene associated with litter size in Indian Goat breeds and its effect on transcription factor binding sites. Domest. Anim. Endocrinol.78:106676. (Impact factor: 2.1)
2. Magotra, A., Bangar, Y., Kumar, S., & Yadav, A. (2023). Evaluation of physiological and morphological parameters for early prediction of prenatal litter size in goats. Zygote, 1-6. doi:10.1017/S0967199423000035. (Impact factor: 1.89)

Any other relevant information- **NIL**

Signature of PI
(Dr. Ankit Magotra)

Signature of Co- PI
(Dr. Y.C. Bangar)

Signature of Co-PI
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Domestic Animal Endocrinology 78 (2022) 106676

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.journals.elsevier.com/domestic-animal-endocrinology

Evaluation of candidate point mutation of Kisspeptin 1 gene associated with litter size in Indian Goat breeds and its effect on transcription factor binding sites

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ARTICLE INFO

Article history:
Received 23 May 2021
Received in revised form 25 August 2021
Accepted 25 August 2021

Keywords:
Litter size
Kiss1
Genotype
Beetal
Barbari

ABSTRACT

Kisspeptin gene (Kiss1) has a significant role in reproductive processes in mammals. However, only little information is available about the association of Kiss1 gene with litter size in Indian goat breeds. Thus, blood samples from 285 randomly selected animals were collected for DNA isolation and SNP profiling. The PCR product of 242 bp size harboring g.2540C>T mutation of Kiss1 gene was digested with the restriction enzyme *Sac*1. Least squares analysis revealed that Barbari goats showed significantly higher average litter size (2.86±0.08) compared to Beetal, Sirohi and Sojat breeds ($P < 0.01$). SNP locus g.2540C>T of Kiss1 gene also showed significant effect on litter size ($P < 0.01$). Goats with Genotype CT (2.66 ± 0.07) and TT (2.67 ± 0.26) had significantly higher ($P < 0.01$) litter size than CC (1.50 ± 0.05). From the transcription factor binding site analysis, it was predicted that due to g.2540C>T SNP, both native and mutant variant forms coded for putative binding sites for different transcription Factor. Allele T had putative binding sites for the androgen receptor which plays a significant role in the signaling pathway involved in increase in ovulation rate; which consequently can have a tremendous effect on average litter size.

1. Introduction

Goats are an important source of milk and meat. This necessitates setting-up of genetic improvement programs for this species. Over the past few decades, studies in goat genomics were directed towards identifying sequence variants in candidate genes from individual animals, and then exploiting this information about genomic mutations to select for routinely measured traits with considerable effects on the agricultural economics. Several studies have been published reporting a wide spectrum of candidate genes that influence growth, reproduction, milk, wool and disease resistance related traits in goats [1,2]. Reproduction traits possess significant economic importance; litter size and ovulation rate are few such reproduction traits which have been predicted to be controlled by candidate genes [3,4]. However, identifying candidate genes is a cumbersome task both for fitness traits such as litter size and for continuous traits like growth [5]. Thus, there has been little research directed in this direction specifically in goats and understanding of goat genetics has been rather incomplete. As it is established that increase in litter size with adequate birth weight would raise farmer profit substantially, there has been a growing interest in the genetic improvement of performance traits in goats [6].

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0739-7240/$ – see front matter © 2021 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.domaniend.2021.106676

Kiss1 gene in goats has been mapped to chromosome 16. Kiss1 spans around 2.62 kilo bases (kb) and contains single intronic region with two coding exons (transcript length 408bp) and encodes 135 amino acid residues (ENSCHIT00000037363.1). Encoded by Kiss1 gene, Kisspeptins are a family of structurally related peptides which serve as essential regulators of gonadotropin- releasing hormone (GnRH) [7,8] and potent elicitors of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in different mammalian species [5,9,10]. Because of the significance of the Kiss1 as a controller of puberty onset, it is plausible that the polymorphisms in this gene have some association with reproductive traits such as high prolificacy/litter size, sexual precocity and year-round estrus phenotypes of goats [11–13]. Identification of candidate markers can bring a valuable improvement for polygenic traits like litter size [5,14] and it has been previously reported that g.2540 C>T SNP of Kiss1 gene is associated with higher probability for multiple births [5,11,15] but so far, no research has been carried out to check the genetic effect of Kiss1 gene with litter size in Indian goat breeds.

Thus, the present study was carried out to analyze the genomic sequence of Kiss1 gene and perform association analysis of identified SNP with litter size in Beetal, Barbari, Sirohi and Sojat goats.

2. Materials and methods

2.1. Ethical approval

The experimental plan of study was duly approved by the Institutional Animal Ethics Committee (VCC/IAEC/265-93) of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India.

2.2. Resource population

With an objective to find the association of Kiss1 genotypes with litter size, the present study was conducted on four indigenous goat breeds pertaining to first parity to screen for genomic region with variations. Blood samples from 285 animals representing indigenous goat breeds viz., Beetal, Barbari, Sirohi and Sojat were collected from different goat farms of Haryana, India.

2.3. DNA extraction from blood samples

5 ml blood was aseptically collected from the jugular vein in vacutainer tube containing EDTA (2.7%). The samples were suitably labelled and transported to the Animal Genomics laboratory (Department of Animal Genetics and Breeding, LUVAS, Hisar) in an icebox and were stored at -20°C till the isolation of genomic DNA. Phenol-chloroform method, as described by Sambrook and Russel was used for DNA isolation from experimental animals [16]. DNA quality was evaluated on 1% agarose (w/v) gel in 1 X TBE electrophoresis buffer (Thermo Scientific). Quality and quantity of extracted DNA was also assessed by Scandrop Nano-Volume Spectrophotometer (Analytika Jena).

2.4. Primer design and PCR amplification

The goat Kiss1 gene sequence was retrieved from Ensemble Genome Brower. The resulting sequence was assembled in DNA Star to screen for candidate SNPs. Based on reference sequence (GenBank accession no. GU142847) of the goat Kiss1 gene, reported set of PCR primers [5] were used to amplify targeted region encompassing intron 1 of Kiss1 gene in our resource population.PCR reaction mixture was prepared using PCR Master Mix (2X) (Promega) for each sample as follows: (10 pmol/ul) Forward primer 0.5 µl, (10 pmol/ul) Reverse primer 0.5 µl, PCR Master Mix (2X) 12.5 µl and Milli Q water 9.5 µl. PCR was performed in thermal cycler (T-100 Bio Radd) in following stages - initial denaturation at 92°C for 5 min., followed by 34 cycles of 94°C for 30 s, annealing at 51°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gel including 0.5 µg/ml of ethidium bromide, and photographed under gel documentation system (Bio-Rad).

2.5. SNP genotyping

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) was constructed to genotype g.2540C>T mutation in all animals under study following manufacturer's procedures. About 10 µl PCR product was mixed with 2 µl 10X L buffer, 1.0 µl of *Sac* I restriction enzyme (Takara Bio Inc.), and 7 µl sterile ddH_2O, and then incubated at 37°C overnight. The digested DNA fragments were separated through electrophoresis using a 2.5% agarose gel containing ethidium bromide in 1X TAE buffer and visualized under gel documentation system (Bio radd). For estimation of the sizes of digested DNA fragments, we used a 50 bp plus DNA ladder (Fermentas International, Inc.) and the genotypes were recorded. To validate the change in nucleotide sequence, the PCR amplicons corresponding to all three genotypes (five samples per genotype) were purified by DNA Clean and Concentrator kit (ZYMO RESEARCH) and then subjected to Cycle sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Afterwards, excess buffers and dNTPs were removed from the sequence PCR products using the BigDye XTerminator Purification Kit (Applied Biosystems). The final purified products were subjected to capillary electrophoresis on the SeqStudio Genetic Analyzer (Applied Biosystems) from both ends (5' and 3' ends).

2.6. Statistical analysis

To calculate allelic and genotypic frequencies and test the probability of the population of each breed at respective farms to be in Hardy-Weinberg equilibrium GENEPOP Version 1.2 software [17] was used. The results of Chi-square analysis can be used as an indicator of population equilibrium known as Hardy - Weinberg Equilibrium. The general linear model was employed to find association of genotypes with litter size adjusted to effects of breed and farm using SAS software (Statistical Analysis System 9.3, SAS Institute Inc.).

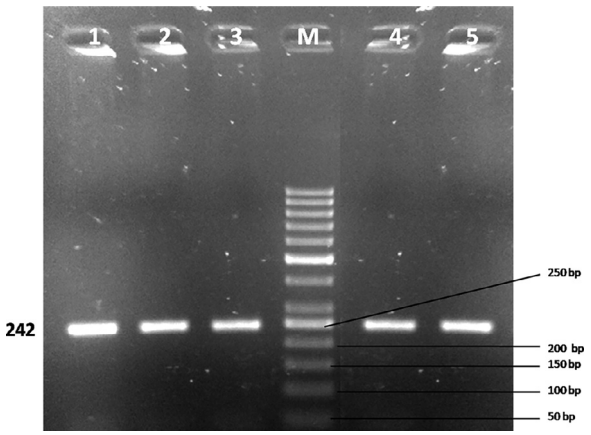

Fig. 1. PCR amplified product of 242 bp; M represents 50bp Ladder.

Following model was used:

$$Y_{ijkl} = \mu + G_i + B_j + F_k + e_{ijkl}$$

Where,

Y_{ijkl} = is the observed value, μ = overall mean, G_i = fixed effect of i^{th} genotype (i=1,2,3), B_j= fixed effect of j^{th} breed (j = 1-4), F_k = fixed effect of k^{th} farm (k =1–4), e_{ijkl} = random residual error. The data were classified according to breed (Beetal, Barbari, Sirohi and Sojat goats).

2.7. Transcription Factor Binding Sites (TFBSs) Prediction

Variant effect predictor (VEP) tool (https://asia.ensembl.org/Bos_taurus/Tools/VEP) was used to predict the nature of targeted SNP. The Animal TFDB (version v3.0) (http://bioinfo.life.hust.edu.cn/AnimalTFDB/) [18] was used to predict changes if any in the TFBSs caused due to the identified SNPs in the intronic region of the Kiss1 gene. To study the impact of predicted Transcription Factors (TFs), we also studied their interaction with Kiss1 gene using STRING version 11.0. STRING is a database of known and predicted protein-protein interactions (PPI) which include physical and functional associations compiled using computational predictions, knowledge transfer between organisms, and aggregations from other databases [19].

3. Results

3.1. SNPs identification and genotypes

As shown in Figure 1, the PCR product of 242 bp size harboring g.2540C>T mutation in intron 1 of Kiss1 gene was successfully amplified. The amplified PCR product was digested with the *Sac*1 enzyme and it resolved into CC, CT and TT genotype in Barbari goats, whereas in Beetal and Sirohi goats CC and CT genotypes were observed. Sojat goats were monomorphic showing only CC genotype. The genotype TT revealed only one band of 242 bp, genotype CT showed three bands of 242 bp, 184 bp, and 58 bp and the genotype CC represented two bands of 184 bp and 58 bp (Fig. 2). Chromatograph representing respective genotypes is shown in Figure 3 which illustrates the sequencing trace of the two homozygotes carrying the allele CTCG and CTTG (underlined bases indicated a polymorphic site) and of the heterozygote with the allele CTC/TG of the Kiss1 gene. Breed wise distribution of genotypic and allelic frequencies of identified SNP are given in Table 1.

3.2. Association of breed and Kiss1 genotype with litter size

The association between different genotypes of g.2540 C>T SNP of Kiss1 gene with litter size was evaluated in our resource population. The different genotypes showed significant association ($P < 0.01$) with litter size (Table 2). The CT (2.66 ± 0.07) and TT (2.67 ± 0.26) genotypes had significantly higher litter size as compared to CC genotype (1.50 ± 0.05) indicating that targeted loci g. 2540 C>T was associated with litter size (Table 2). Within breed association analysis in Beetal and Barbari goats revealed significant association of genotypes on litter size (Table 3). It is apparent from Table 3 that litter size was significantly affected by breed. The average first parity litter size was highest in Barbari (2.86 ± 0.08) compared to Beetal (1.89a ± 0.06), Sirohi (1.71 ± 0.09) and Sojat (1.17^c ± 0.06) goat breeds. Farm did not reveal any significant effect on litter-size in our studied population.

3.3. in silico *prediction of g. 2540 C>T SNP effect*

The VEP analysis predicted that this particular mutation exhibits feature type as transcript and biotype as protein coding which assigns this mutation a regulatory role in protein expression. In an attempt to study the effect of g.2540 C>T on TFBS, Animal TFDB was used to screen the native and mutant variant of the investigated fragment of the Kiss1 gene for presence of TFBS. As shown in Table 4, the fragment with T allele had putative binding sites for the following TFs - Androgen receptor (AR) and Enhancer-binding protein epsilon. Whereas, the fragment with C allele harbored putative binding sites for transcription factor Zinc finger BED domain-containing protein 6 (ZBED6), Zinc finger and BTB domain containing 3 (ZBTB3), General transcription factor II-I repeat domain-containing protein 1 (GTF2IRD1). From PPI analysis, it was revealed that only AR (T variant) had an interaction with Kiss1 and associated genes.

4. Discussion

With respect to g.2540C>T mutation in intron 1 of Kiss1 gene, the pooled frequency of C (0.84) allele was predominant in all the breeds under study. The highest frequency of C allele was observed in Sojat followed by Sirohi, Beetal and Barbari goat (Table 1). The frequency of T allele was highest in Barbari goats (0.45), followed by Beetal goats (0.15). The frequency of CT and TT genotype was also highest in Barbari goats as compared to other three breeds. The variation observed in allelic and genotypic frequencies among different breeds can be explained by interbreed differences and acting selection pressure.

Chi-squared tests showed that g.2540 C>T SNP did not meet with the Hardy–Weinberg equilibrium in Beetal ($P < 0.05$) and Barbari goats ($P < 0.01$). Chi-square value of the targeted locus in Sojat goats could not be estimated

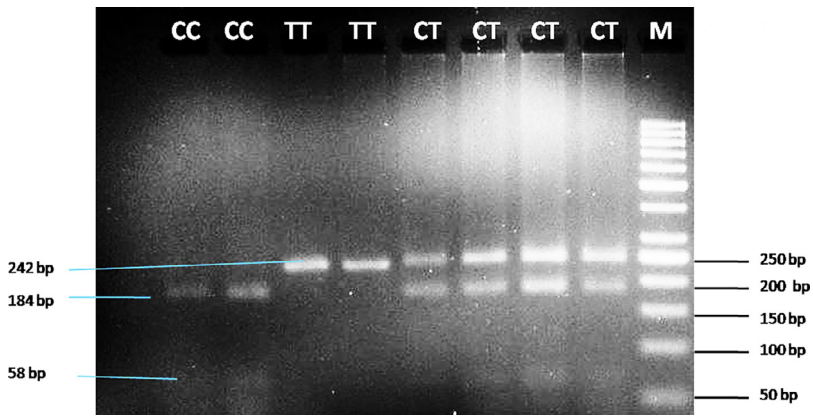

Fig. 2. PCR-RFLP genotypes of Kiss1 gene using Sac1 restriction enzymes; CC genotype = 184, 58bp; CT genotype 242, 184, 58 bp; TT = 242 bp; M = 50 bp ladder.

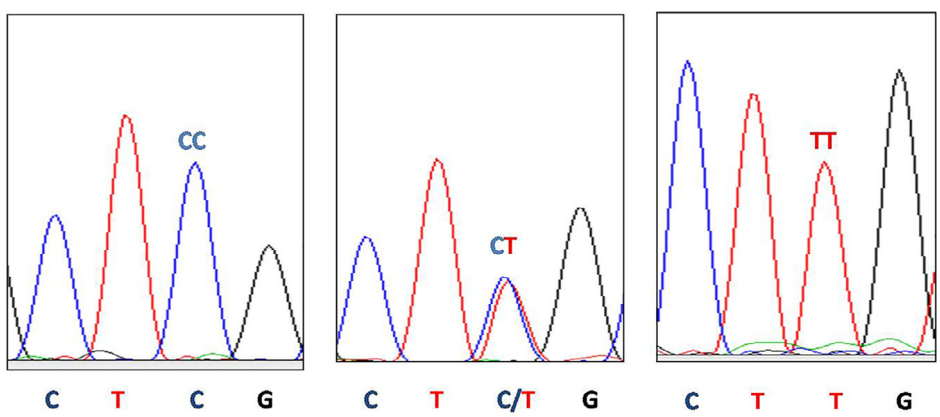

Fig. 3. Chromatograms of CC, CT and TT genotypes of Kiss1 gene; Presence of two peaks at SNP site indicates heterozygosity of the sample.

Table 1
Genetic structure of KiSS-1 gene in Indian goat breeds.

Breed		Observed genotypes			Genotype frequencies			Allele frequencies		Equilibrium X^2-test
	n	CC	CT	TT	CC	CT	TT	C	T	
Beetal	136	96	40	0	0.70	0.30	0	0.85	0.15	4.04[a]
Sirohi	52	48	4	0	0.92	0.08	0	0.96	0.04	0.08
Sojat	41	41	0	0	1.0	0	0	1.0	0	-
Barbari	56	12	38	6	0.21	0.68	0.11	0.55	0.45	7.78[b]
Total	285	197	82	6	0.69	0.29	0.02	0.84	0.16	-

[a] ($P < 0.05$);
[b] ($P < 0.01$)

Table 2
Effect of Kiss 1 genotype on litter size.

Genotype	Number	Litter size[c](Mean ± SE)
CC	197	1.50[a] ± 0.05
CT	82	2.66[b] ± 0.07
TT	06	2.67[b] ± 0.26
Total	285	2.27 ± 0.09

[a] ($P < 0.01$), Mean values with different superscripts differ significantly.

as it was monomorphic. Genotype frequencies in the Sojat goat, therefore, did not meet the assumptions for Hardy-Weinberg equilibrium analysis. Furthermore, Kiss1 polymorphism in Sirohi goats had lower Chi-Square values than table value, so it can be stated that Sirohi goat population under study were in Hardy-Weinberg equilibrium. In contrast to our findings, An *et al* reported high frequencies of T allele at g.2540C>T loci, ranging from 0.56 to 0.60 in their resource population that is Xinong Saanen goats (0.60), Guanzhong goats (0.56) and Boer goats (0.56) [5]. Maitra *et al* also explored targeted locus (g.2540C>T) in indigenous goat. They reported highest frequency of TT

Table 3
Breed-wise association of Kiss 1 genotype (within breed) on litter size.

Breed	Observed genotypes			Litter Size of respective genotype (Mean ± SE)		
	CC	CT	TT	CC	CT	TT
Beetal [c]	96	40	0	1.43[a] ± 0.06	2.35[b]± 0.10	-
Sirohi	48	4	0	1.75 ± 0.06	1.25 ± 0.22	-
Sojat	41	0	0	1.17 ± 0.06	-	
Barbari [c]	12	38	6	2.17[a] ± 0.17	3.11[b] ± 0.10	2.67[b] ± 0.24

($P < 0.01$); Mean values with different superscripts differ significantly.

Table 4
Transcription factor (TF) binding site prediction.

TF	*P*-value	Q-value	Matched Sequence
TF binding site prediction results (T- allele)			
AR	0.0000972	0.000778	AGTGCAGCAAGCTCT
CEBPE	0.0000992	0.000992	AGAGCTTGCTGCAC
TF binding site prediction results (C- allele)			
ZBED6	0.0000399	0.000559	AGAGCTCGCTGC
ZBTB3	0.0000665	0.00133	AGTGCAGCG
GTF2IRD1	0.000098	0.00216	CAGCGAGC

genotype (0.58) and T allele (0.68) in their resource population [11].

The heterozygous signal at the polymorphic site (Fig. 3) showed the decrease in peak height of approximately 50 percent of the C peak (blue line) with the concomitant appearance of the similar peak height of the T peak (red line) underneath when compared to its homozygous counterpart that may be due to simultaneously amplified DNA fragments from duplicated regions of the genome [20]. Furthermore, two alleles exerting different influences on the height of the G nucleotide immediately 3′ to the polymorphic site helped to recognize heterozygotes with confidence. When the sequencing traces of the two allelic homozygotes were compared to that of the heterozygote, a small G peak of sequence homozygous for C allele (larger than the allelic C peak) following the C in the CTCG homozygote, a large G peak of sequence homozygous for T allele (larger than the allelic T peak) following the T in the CTTC homozygote and an intermediate size G peak following the composite C/T peaks in the heterozygote were observed. Based on these criterions, g.2540C>T SNP representing CC, TT and C/T genotypes were validated in the Kiss1 gene. Ukoskit also reported similar criteria for validating SNP genotypes from chromatograph obtained from custom sequencing [20].

Different genotypes of g.2540C>T SNP of Kiss1 gene showed significant association ($P < 0.01$) with litter size. Within breed association analysis in Beetal and Barbari goats revealed significant association of genotypes on litter size (Table 3). In both Beetal and Barbari goats, CT genotype as compared to other genotypes had more significant association with litter size. In Sirohi breed, no significant association with litter size was observed which was expected as the CT genotype was observed in four animals only as opposed to CC genotype in forty eight animals. Association analysis in Sojat breed was not viable as it revealed only one genotype.

From the breed wise distribution pattern of genotypes, it was observed that the frequency of CT and TT genotypes was comparatively higher in Barbari goats (Table 1) and also frequency of T allele was highest in Barbari goats as compared to other breeds, which points towards significant association of T allele with litter size. Similar results were also reported by An *et al* and Maitra *et al* [11] which showed significant association of T allele at g.2450 C>T loci with higher litter size [5,11].

The targeted SNP is located in intron 1 and is of protein biotype of Kiss1 gene. Intronal SNPs have both genetic and epigenetic effects on gene expression [21]. Introns are important in alternative splicing and genome imprinting, as well as tissue-specific gene expression, mRNA transcription, and translation. Transcription factors, enhancers, silencers, and insulators are common intron components that positively influence gene expression. [21,22]. Phenotypic deviation between breed/species is driven by point mutations at transcription factor binding sites that leads to changes in transcriptional regulation [23–32]. Such mutations can increase or decrease the affinity of a transcription factor protein to its binding sites, which in turn modifies the expression of regulated genes. Due to presence of a plethora of individual TFs across the genome, additional proteins recruited by these TFs interact combinatorally to form complex gene regulatory networks; which in turn enables them to influence gene transcription long after the transcription factor itself leaves the region [33].The effect of g.2540 C>T on TFBS predicted that the fragment with T allele had putative binding sites for the Androgen receptor (AR) TF and its interaction with Kiss 1 gene through associated genes from *in-silico* PPI analysis.Kiss1 known to bind G protein-coupled receptor 54 (GPR54) has been implicated to play a pivotal role in reproduction since there have been studies in mice and humans which observed that a lack of functional GPR54 result in failure to undergo puberty in such individuals [3,34–36]. Kiss1 as an essential regulator of GnRH is involved in the stimulation of the production of both LH and FSH. A complex synergistic interaction of FSH and LH governs the process of optimal follicular recruitment, growth, selection and dominance subsequently followed by ovulation. Being recognized as a critical regulator of puberty onset emphasizes the idea that polymorphic nature of Kiss1 gene could have serious repercussions on reproductive system by affecting traits such as high prolificacy/litter size, sexual precocity and yearround estrus phenotypes [11–13]. Additionally, mutations in FSH, LH and GnRH genes have also been reported to be associated with litter size in several species [37–39]. Despite the limited studies in goats, association of Kiss1

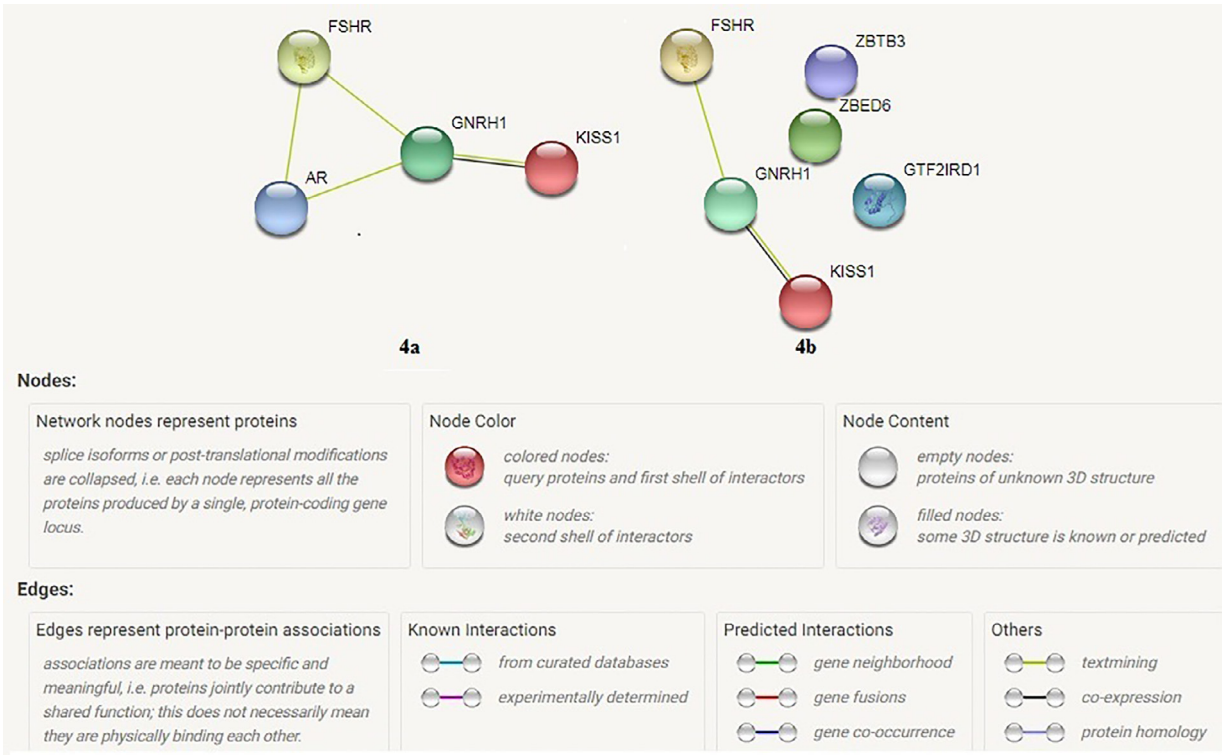

Fig. 4. in- silico construction of Protein-Protein interaction network of Kiss1 gene and predicted TFs; (a) In the TT genotype among the predicted TFs, AR was solely predicted to be a primary interactor of Kiss1, also showing interactions with other critical proteins of the ovulation process. The interaction data was obtained from the STRING database; (b) In the CC genotype, none of the predicted TFs had any interaction with Kiss1.

gene with multiple births has been reported by several other researchers [5,15]. From the TFBS analysis, it was observed that due to a single mutation at g. 2540 C>T, both native and mutant variants harbored putative binding sites for different TFs. The TFs with putative binding sited in C variant had no direct/indirect interaction with Kiss1 gene (Fig. 4). However, AR with a putative binding site in T variant was interacting with Kiss1 gene through other proteins such as GNRH1, Kiss1R and Leptin protein which are involved in sexual differentiation and maturation, spermatogenesis, and gonadotropin regulation (Fig. 4). AR, a steroid hormone receptor, is a member of the nuclear receptor superfamily closely related to progesterone receptor, and glucocorticoid receptor are expressed in various ovarian cell types [40–43]. Androgen is produced and released from cholesterol during folliculogenesis followed by stimulation of the theca cells by LH and FSH.AR signaling is required for the maintenance of follicle development, luteinization, ovulation, and growth of myometrium and endometrium [44,45]. Dysregulation of androgen/AR signaling has been reported to perturb normal reproductive development with prolonged estrous cycle, decreased litter numbers and sizes, and shortened reproductive life [46]. Such changes at or in vicinity of TFs thus have the potential to modify the expression of regulated genes and consequently act as a major driving force for the phenotypic divergence observed between species. Thus, the predicted TFBS for AR created due to g.2540 C>T mutation of Kiss1 gene could play a significant role in signaling pathway for enhance ovulation rate and litter size in goats.

5. Conclusion

Several researchers have undertaken studies to explore the candidate SNPs associated with reproduction traits in goats for their implementation in breeding programs. To this end, we performed SNP profiling of Kiss1 gene in indigenous goat breeds to study the association of mutations in Kiss1 gene with litter size. Significant association of T allele of targeted SNP g.2540C>T of Kiss1 gene was observed with litter size in our resource population ($P < 0.01$) using least square model. Through computational studies, it was predicted that sequence with T allele of targeted SNP g.2540 C>T harbor a putative binding site for AR which is known to alter the gene expression networks involving Kiss1 gene. These predictions provide a basis for further study of differential gene expression and confirmation of change in TFBS by chromatin immunoprecipitation assay in future. Current study provides valuable genotypic data for targeted goat population which can be used for molecular tagging to find applications in marker-assisted selection (MAS) after validation on large number of samples.

Author contributions

Vikram Jeet and Ankit Magotra: lead author as well as implementation of core idea of research work, performed collection of phenotypic data, DNA isolation, PCR standardization, amplification and RFLP. Yogesh Bangar involved in statistical data analysis, Sunil Kumar, Pooja Bahurupi and Asha Rani Garg involved in manuscript writing and helped in wet lab work. A.S.Yadav involved in final revision of the manuscript.

Data availability

Data available on request due to privacy/ethical restrictions.

Acknowledgment

We duly acknowledge the facilities provided by Director of Research, LUVAS, Hisar, India to conduct this research. We also acknowledge funding obtained from HSCSIT, Science and Technology Department (HSCSIT/R&D/2021/538), Haryana, India. Authors declare that there are no conflicts of interest associated with this publication.

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Zygote

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Evaluation of physiological and morphological parameters for early prediction of prenatal litter size in goats

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Research Article

Cite this article: Magotra A *et al*. (2023). Evaluation of physiological and morphological parameters for early prediction of prenatal litter size in goats. *Zygote*. page 1 of 6. doi: 10.1017/S0967199423000035

Received: 27 June 2022
Revised: 30 December 2022
Accepted: 24 January 2023

Keywords
Abdominal girth; Discriminant analysis; Goat; Litter size

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Summary

The aim of the present study was to evaluate the physiological and morphological parameters of pregnant does for early prediction of prenatal litter size. In total, 33 does were screened using ultrasonography and further categorized into three groups based on does bearing twins ($n = 12$), a single fetus ($n = 12$), or non-pregnant does ($n = 9$). The rectal temperature °F (RT) and respiration rate (RR) as physiological parameters, while abdominal girth in cm (AG) and udder circumference in cm (UC) as morphological parameters were recorded at different gestation times, i.e. 118, 125, 132 and 140 days. In addition to this, age (years) and weight at service (kg) were also used. The statistical analyses included analysis of variance (ANOVA) and linear discriminant analysis (LDA). The results indicated that groups had significant ($P < 0.05$) differences among morphological parameters at each gestation time, with higher AG and UC in does bearing twins followed by a single fetus and non-pregnant does. However, both physiological parameters were non-significantly ($P > 0.05$) associated with litter size groups. It was also revealed that the studied parameters showed increasing trends over gestation time in single and twin fetus categories, but they were on par among non-pregnant does. The results of the LDA revealed that estimated function based on age, weight at service, RR, RT, AG and UC had greater (ranging from 75.00 to 91.70%) accuracy, sensitivity and specificity at different gestation times. It was concluded that using an estimated function, future pregnant does may be identified in advance for single or twin litter size, with greater accuracy.

Introduction

India is a vast reserve of 37 indigenous goat breeds with a total population of 148.88 million goats (Livestock Census, 2019; NBAGR, 2022). Goat farming is one of the major enterprises, especially for small and marginal households in India. An increasing demand for goat meat has triggered the interest in farmers to start goat farming and has produced more kids in recent years. Furthermore, the growth and survival of kids have been the most important aspects for goat farmers as they are directly associated with farm income (Kumar *et al.*, 2013; Magotra *et al.*, 2022). In addition to this, an early growth rate is more cost effective for profitable goat farming (Magotra *et al.*, 2021a). It has been reported previously that genetic and environmental factors affect initial growth performance up to slaughter age, and ultimately influence farmer income (Boujenane and Hazzab, 2008; Zhang *et al.*, 2009; Gowane *et al.*, 2011; Lalit *et al.*, 2016; Bangar *et al.*, 2020; Dige *et al.*, 2021; Magotra *et al.*, 2021b; Bangar *et al.*, 2022b).

In addition to growth performance, litter size in goats is also an important parameter in goat production systems that directly contributes to higher economic returns for goat farmers (Song *et al.*, 2006; Bangar *et al.*, 2022a). Genetics, along with breeding and feeding practices, may contribute to larger litter sizes in goats and, furthermore, selection based on molecular markers and stringent breeding plan could achieve the desired genetic progress in flocks (Rashidi *et al.*, 2011; Kebede *et al.*, 2012; Mohammadi *et al.*, 2012; Selvan *et al.*, 2016; Mokhtari *et al.*, 2019; Jeet *et al.*, 2022). However, published literature suggests that litter size in goats is a low-heritable trait and, therefore, it is less favourable for selection for flock improvement (Mellado *et al.*, 2005; Bangar *et al.*, 2022a). This indicated the importance of management practices for rearing by goat farmers and for improving litter size, and also simultaneously litter weight and survival (Song *et al.*, 2006; Lopez-Sebastián *et al.*, 2014). The identification of litter size among pregnant does in advance may give an advantage to goat farmers in optimizing farm practices to increase farm profits (Mellado *et al.*, 2005). The required attention through nutritional and health aspects to pregnant does with twins or more litter size could provide better returns to farmers through higher birth weights, and better survival of kids as well as dams (Pan *et al.*, 2015).

The detection of litter size using ultrasonography is the safest and most reliable technique; however the cost of instrumentation and availability at the farmer's door is not suitable for goat

farmers in developing countries such as India. Other techniques, such as abdominal palpation, are less reliable and have a high risk of damaging or aborting the fetus. The use of linear body measurements could be an alternative method for detecting litter size in goats (Mellado *et al.* 2004). However, there is scarce literature available on the use of physiological and morphological parameters for the prediction of litter size in goats.

Therefore, the aims of the present study were to determine the important physiological and morphological parameters for pregnant does bearing single fetuses for twins at advanced gestation, along with non-pregnant does (as a control) and to develop discriminant functions based on these parameters for classifying the pregnant does into different litter sizes in advance. The results of the present study would indicate the possibility of implementing strategies for improving the growth and survival of kids in farm flocks.

Materials and methods

Animal resource

An experimental study was conducted using two indigenous breeds (22 Beetal and 11 Jakhrana) maintained at a goat breeding farm, at the Department of Animal Genetics and Breeding, LUVAS, Hisar (India). Using ultrasonography, these 33 does were categorized into three categories: (1) does bearing twin fetus ($n = 12$); (2) does bearing a single fetus ($n = 12$); and (3) non-pregnant does ($n = 9$). In the initial statistical analysis, we did not find any significant ($P > 0.05$) differences between the two breeds for various parameters. Therefore, we pooled these two breeds into a combined category to give a sufficient sample size in each litter size category.

Traits targeted

In the present study, age at service and weight of service were obtained from the service register maintained at a goat breeding farm. Two physiological parameters, i.e. rectal temperature °F (RT) and respiration rate (RR), and two morphological parameters, i.e. abdominal girth in cm (AG) and udder circumference in cm (UC), were recorded at different times of gestation, i.e. 118, 125, 132, and 140 days.

Statistical analysis

All the data from the 33 does were compiled using Microsoft Excel and processed for further statistical analysis. Descriptive statistics and one-way ANOVA were used to determine any significant difference between the three groups of does for age at service (years), weight at service and kidding (kg) and litter weight (kg). To compare these three groups of does, along with gestation times, a two-way ANOVA with repeated measures was used to obtain a significant difference between the three groups of does and the four gestation time intervals (within groups). Pairwise comparisons were made using Duncan's test between groups and Bonferroni test within groups.

To predict in advance whether pregnant does would have single or twin litter size, a linear discriminant analysis (LDA) was used in this study. Considering two categories of does, i.e. single and twin litter size as a dependent variable and six variables, and i.e. age at service, weight at service, RT, RR, AG and UC as predictors, four discriminant functions were developed separately for each gestation time. The strength of the estimated discriminant function was checked using Wilk's lambda, which is the proportion of unexplained variability out of total variability; smaller values indicate the better ability of the function to classify the individuals into the correct category of litter size. The significance of Wilk's lambda was determined using the chi-squared test. The homogeneity of covariances under each LDA was confirmed using Box's M statistic. Furthermore, a classification table of observed and predicted categories of does bearing single and twins was used to estimate the accuracy, sensitivity, and specificity of the estimated discriminant function at each gestation time. For all analyses, the significance was considered for a P-value < 0.05. All statistical analyses were performed using SPSS 20.0 version software; graphical representations were produced using GraphPad Prism 9.0 version software.

Results

Descriptive analysis

A detailed data structure and descriptive statistics for three groups of does that were sampled initially for the current study are given in Table 1. The overall body weight gain in kg was 1.50 and 2.50 kg for does bearing twin and single kids respectively. The average litter weight for the respective groups was 2.59 and 2.91 kg, respectively. The does with twins were a statistically similar ($P > 0.05$) age at service, but had significantly ($P < 0.05$) different weights at service compared with does with a single fetus. However, non-pregnant does had a significantly ($P < 0.05$) lower age and weight at service than did both pregnant groups, i.e. single and twin fetuses. Although weight at kidding was higher in does with twins compared with does with a single fetus, the litter weight in both groups was statistically ($P > 0.05$) on par.

Physiological and morphological parameters

The association of three groups of does with physiological (RT and RR) and morphological (AG and UC) parameters at different times of gestation, i.e. 118, 125, 132 and 140 days, was obtained and the results are depicted in Figure 1. It was observed that there was no significant ($P > 0.05$) difference for both physiological parameters between the three groups at different gestation times, except at 140 days of gestation. RR was found to be significantly ($P < 0.05$) different among the three groups at 140 days and was higher in does bearing a single fetus, followed by twins compared with non-pregnant does. Between different times of gestation, it was observed that RT was significantly ($P < 0.05$) increased, especially after 125 days of gestation in does bearing a single fetus. However, does bearing twins and non-pregnant does did not ($P > 0.05$) show this difference in RT over time. For RR, all three groups had significantly ($P < 0.05$) higher values for advanced gestation times, i.e. 132 and 140 days compared with 118 and 125 days.

For morphological parameters, AG (cm) was significantly ($P < 0.05$) increased at advanced gestation time (from 118 to 140 days) and ranged from 94.92 to 104.50 cm for does bearing twins, 87.54–97.58 cm for does bearing a single fetus, and 78.44–84.43 cm for non-pregnant does. It was also revealed that significant ($P < 0.05$) differences were observed between the three groups at all gestation times and that a wider AG was found among does bearing twins, followed by a single fetus and non-pregnant does.

Similarly, UC (cm) showed a significantly increasing (~40%) trend from 118 to 140 days among the pregnant does and ranged from 22.50 to 40.70 cm in does bearing twins and 21.67–36.15 cm in does bearing a single fetus. Although UC was non-significantly

https://doi.org/10.1017/S0967199423000035 Published online by Cambridge University Press

Table 1. Data structure and descriptive statistics for different litter size of does

Litter size of does	Sample size	Age at service (years)	Weight at service (kg)	Weight at kidding (kg)	Weight gain (kg)	Litter weight (kg)
Twins[#]	12	4.51 ± 0.48[b]	37.75 ± 1.48[b]	39.25 ± 1.21[b]	1.50	2.59± 0.58 (25)
Single	12	4.20 ± 0.45[b]	32.67 ± 0.87[a]	35.17 ± 1.20[a]	2.50	2.91 ± 0.05 (12)
Non-pregnant	9	2.82 ± 0.41[a]	30.67 ± 1.23[a]	–	–	–

[#]One kidding gave triplets that was included under twin type of litter size.
Different superscripts (a, b) in the same column indicates that values differ significantly ($P < 0.05$).

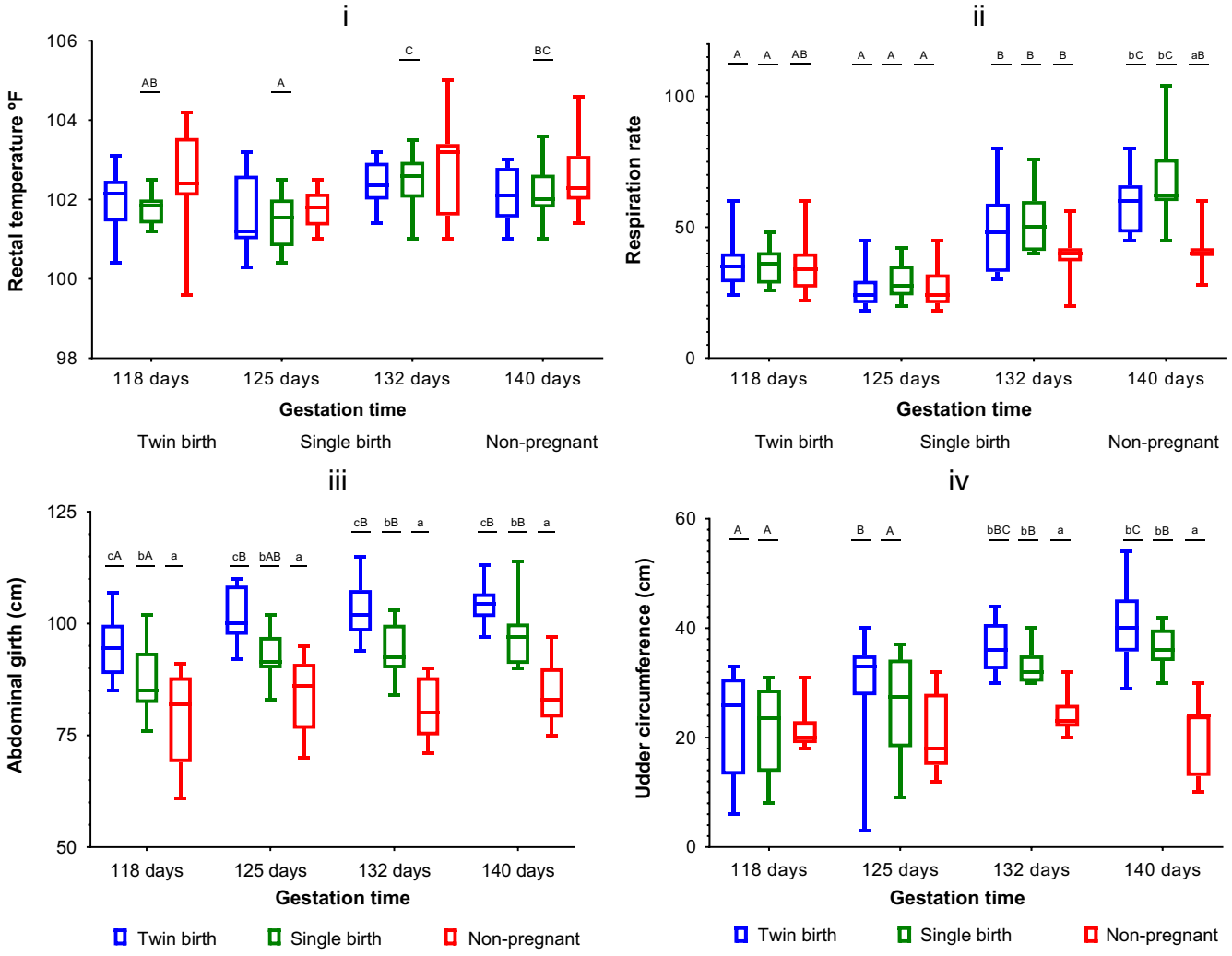

Figure 1. Physiological and morphological parameters with respect to litter size categories in goats. Different superscripts (a–c) differ significantly ($P < 0.05$) between groups among time. Different superscripts (A, B) differ significantly ($P < 0.05$) between times among groups. (i) Rectal temperature °F, (ii) respiration rate, (iii) abdominal girth (cm), and (iv) udder circumference (cm).

different between the three groups up to 125 days, it was significantly ($P < 0.05$) different among pregnant does compared with non-pregnant does at 132 and 140 days. However, it was not statistically significant ($P > 0.05$) between does bearing single/twin fetuses at advanced gestation. Furthermore, there was a non-significant ($P > 0.05$) difference in gestation time among the non-pregnant does.

Discriminant function

A stepwise LDA was performed to check if all predictors contributed to function. It was revealed that all six predictors, i.e. age, weight at service, RR, RT, AG and UC, were important for developing the discriminant function. The estimated unstandardized coefficients under discriminant analysis are presented in Table 2. The associated values of Wilks' lambda that measured the discriminating power of each function for separating animals into two groups were low and ranged from 0.46 to 0.64. As a proportion of the total variance in the discriminant scores was not explained by differences among the groups, smaller values of Wilks' lambda in this study indicated the greater discriminatory power of the estimated functions. The significance of Wilk's lambda was tested using chi-square statistical tests and it was

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Table 2. Discriminant function for prediction of litter size in goats

Gestation time	Eigen values	Wilk's lambda	Discriminant function
118 days	0.56	0.64	−0.13*Age + 0.214*WTS + −0.124*RT + −0.022*RR + 0.055*AG + 0.028*UC + 0.807
125 days	1.17	0.46*	−0.321*Age + 0.076*WTS + 0.464*RT + −0.071*RR + 0.149*AG + 0.024*UC + −61.471
132 days	1.07	0.48*	−0.298*Age + 0.058*WTS + −0.105*RT + −0.01*RR + 0.146*AG + 0.128*UC + −8.285
140 days	1.08	0.48*	−0.08*Age + 0.068*WTS + 0.233*RT + −0.055*RR + 0.077*AG + 0.141*UC + −35.438

Age: age at service (years); AG: Abdominal girth (cm); RR: Respiration rate; RT: rectal temperature °F; UC: Udder circumference (cm); WTS: weight at service (kg).
*Significant chi-squared distribution at the 5% level.

Table 3. Classification of pregnant does into two groups of litter size

		Predicted group membership			
Gestation time	Litter size of dam	Single	Twin	Total	Accuracy (%)
118 days	Single	9 (75.00)	3 (25.00)	12	75.00
	Twin	3 (25.00)	9 (75.00)	12	
125 days	Single	10 (83.30)	2 (16.70)	12	87.50
	Twin	1 (8.30)	11 (91.70)	12	
132 days	Single	10 (83.30)	2 (16.70)	12	83.30
	Twin	2 (16.70)	10 (83.30)	12	
140 days	Single	11 (91.70)	1 (8.30)	12	86.40
	Twin	2 (20.00)	8 (80.00)	10	

revealed that respective functions had significantly better power for separating the two groups, i.e. single and twin litter sizes at 125 days onwards.

Table 3 shows the classification for does bearing single and twin fetuses using estimated discriminant functions based on six predictors at each gestation time. The accuracy (%) of classifying does into the correct category at days 118, 125, 132, and 140 days was obtained as 75.00, 87.50, 83.30 and 86.40%, respectively. The sensitivity (%) and specificity (%) for respective gestation times were 75.00 and 75.00%, 83.30 and 91.70%, 83.30 and 83.30% and 91.70 and 80.00%, respectively.

Discussion

The present study focused on studying various morphological and physiological parameters with the possibility for early prediction of prenatal litter size in goats. The results suggested that physiological parameters such as RT and RR were statistically similar among pregnant and non-pregnant does up to 132 days of gestation. However, there was a significant difference between does bearing single and twin litter size for RR at 140 days of gestation. Also, RR was found to be increasing in all three groups of does as gestation advanced. Contrary to the present findings, Atmoko *et al.* (2020) reported that RR and RT remained non-significant between the early and late pregnancies of Etawah Grade does. From the findings of the present study, it was concluded that those physiological parameters increased in advanced pregnancy might be due to physiological and gestation stress, and might not be useful for differentiating between single or twin litter size among pregnant goats.

Conversely, morphological parameters showed significant changes over gestation duration, as well as between does bearing different litter sizes. Similar to the current findings, Mellado *et al.* (2004) reported 9.3 and 10.6 cm increases in abdominal circumference for single and twin-bearing does, respectively. It is also worth mentioning here that does with twins showed a higher AG than does with a single fetus, which might be due to the larger volume covered by twins in the uterus. Therefore, AG was considered to be a potential indicator for deciding litter sizes among the pregnant does under the current study, and was in accordance with reports by Mellado *et al.* (2004) who conducted a study for the prediction of goat litter size using body measurements.

The present results also showed an increase in UC during late gestation and also provided significance for differentiating between pregnant and non-pregnant does at 132 and 140 days. (Linzell, 1966; Fleet *et al.*, 1975; Zahraddeen *et al.*, 2008; James and Osinowo, 2021). Davis (2017) also reported that larger litter size in goats led to a 20–25% increase in mammary development at the end of gestation. These findings indicated that UC alone could be useful for detecting advanced gestation, especially near to kidding, and also may not be useful for differentiating twin litter size.

LDA was performed to find out the potential for morphological and physiological parameters, along with age and weight at service, to discriminate pregnant does bearing single and twin fetuses at different gestation times. The present results indicated the greater accuracy for the prediction of prenatal litter size based on various parameters. The sensitivity and specificity for developed discriminant function were also at satisfactory levels in the present study.

Mellado *et al.* (2004) performed discriminant analysis to predict litter size based on linear measurements, such as live weight, abdominal circumference and vulva–cervix distance, and reported an ~60% accuracy for predicting twin-bearing goats correctly based on abdominal circumference or its combination with other traits. Our study suggested a greater accuracy for predicting litter size among pregnant does based on morphological and physiological parameters compared with reports by Mellado *et al.* (2004). Pan

https://doi.org/10.1017/S0967199423000035 Published online by Cambridge University Press

et al. (2015) used a discriminant function for deciding litter size in Black Bengal goats and suggested that heart girth, punch girth, BW, and distance between the trochanter major and pelvic triangle area could be the predictive indices for larger litter size.

The use of a discriminant function for classifying the genetic resources of goats into distinct categories based on morphology characteristics has been reported by Yakubu *et al.* (2011), Rodero *et al.* (2015), Hilal *et al.* (2016) and Melesse *et al.* (2022). However, the use of morphological and physiological parameters for predicting the litter size in goats is very rare. However, it has been suggested that validation of the present findings needs to be done using a greater number of morphological and physiological parameters in increased sample sizes of pregnant goats in order to predict litter size in advance.

In conclusion, the present study focused on the evaluation of important physiological and morphological parameters between pregnant and non-pregnant does to set some criteria for deciding prenatal litter size. Although physiological parameters were not influenced by doe categories, morphological parameters such as abdominal girth and udder circumference were significantly higher in does bearing twins followed by does bearing a single fetus and non-pregnant does. The results of a discriminant function indicated that future pregnant does may be identified in advance for single or twin litter size with greater accuracy.

Data availability statement. Data are available on request due to privacy/ethical restrictions.

Acknowledgements. We duly acknowledge the facilities provided by the Director of Research, LUVAS, Hisar, India and used to conduct this research. We also acknowledge funding obtained from the HSCSIT, Science and Technology Department (HSCSIT/R&D/2021/538), Haryana, India.

Animal welfare statement. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as original research data were collected from the Goat Breeding Farm, Department of Animal Genetics and Breeding, LUVAS, Hisar (India).

Funding. Funding was received from the HSCSIT, Science and Technology Department, Haryana, India with grant no. HSCSIT/R&D/2021/538.

Conflicts of interest. The authors declare that there is no conflict of interest regarding the publication of this paper.

Consent for publication. The authors give their consent for publication.

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RESEARCH & DEVELOPMENT PROJECT

Study of plant/candidate feed supplement to reduce methane production without affecting productivity in buffaloes

SUBMITTED BY

Dr. Jyotsana Madan

To

HARYANA STATE COUNCIL OF SCIENCE, INNOVATION AND TECHNOLOGY, PANCHKULA

HSCSIT Reference No. HSCSIT/R&D/2021/541 Dated: 05.03.2021

Project Duration: 3-years

RESEARCH PROJECT TITLE:

Study of plant/candidate feed supplement to reduce methane production without affecting productivity in buffaloes

By

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Project Summary

Ruminants are one of the major sources of methane production with a contribution of nearly 84Tg global annual enteric methane emission. Several mitigation options and strategies have been explored, which involve intervention at the animal level like change in dietary composition, antibiotics and other feed additives, genetic manipulation in rumen ecosystem leading to modulation of rumen fermentation pattern and inhibition of methanogenicarchaea. Several plant secondary metabolites have been identified to have potential for methane inhibition in the rumen. Management practices and genetic selection have also been proved very effective in reducing methane emission by increasing feed efficiency and production in animals. Present study not only aims to screen targeted plant extract and molecules from the limited scale to larger scale in-vitro trial with respect to change in fermentation pattern and gas production but also in relation to different rumen bacterial expression profiles. The study further aims to select most potent as feed supplement to improve rumen fermentation without affecting productivity in dairy animals.

The studies on plant feed additives *on in vitro* and *in vivo*rumen fermentation and bacterial profile can strengthen our ability to either select, or adapt, or rumen microbial contributions in methane production in buffalo calves. Reducing methane production is important for the efficient conversion of feedstuffs to improve health, productivity and environment stability.

Objectives

1. *In-vitro* analysis of plant extract/and/or candidate molecule in solo or in combination on rumen fermentation parameters and bacterial population.
2. Effect of supplementation of best selected plant additive on rumen fermentation and growth in growing buffalo calves.
3. Effect of supplementation of best selected plant extractand/or candidate moleculeon milk production and milk composition parameters in dairy animals.

Workplace/methodology:Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences, LUVAS, Hisar.

Objective1: The *in- vitro*rumen fermentation studies wereconducted by using different levels/combinations of natural plant feed additives like, babool, *E. officinalis*, *Menthe piperata*, citrus fruit peelat 6 and 24 hour after incubation for the rumen fermentation parameters(Total gas production, Ammonia nitrogen (NH_3-N), Total nitrogen, pH, Total volatile fatty acids.Expression of various bacteria vizCellulolytic, total bacteria,total methanogens, Methanobrevibacter bacteriawere studied by Real Time PCR.

The levels and combinations of different feed additives were screened on the basis of *in-vitro* results, which were tested in *in-vivo* in animal model.In-vitro study was carried out by using 10% aqueous and ether extract of *Aeglemarmelos* leaves, *Menthapiperata*(mint) leaves, *E.officinalis*fruit powder, citrus fruit peelwere used. Aqueous and ethanolic extract of plant (10%) were prepared by incubating at 39^0 C on a rotary shaker and filtering through whatmanNo.1 filter paper. The *in vitro* trial were conducted by taking different concentrations of plant extracts @ 0.2ml, 0.5ml, 1ml and 2 ml, with substrate media containing rumen fluid. After incubation rumen fermentation parameters were analysed in samples. Total gas production was measured by syringe method, pH and temperature by pH meter and thermometer, ammonia- nitrogen by Conway disc method, total nitrogen by Micro kjeldahl method, Total volatile fatty acids were measured by Markham distillation apparatus method.

Thirty five mg (35 mg) of solid after centrifugation of 2 ml of incubated media @9000 g for 5 min was collected from each vial separately for DNA extraction. In the homogenised sample, DNA was extracted with QiAmp DNA kit. Presence of bacterial DNA in samples was confirmed, as DNA bands were observed in agar gel electrophoresis. Primer of total bacteria, total methanogen, cellulolytic bacteria (*Fibrobacter succinogenus*) and 16sRNA were standardized for PCR before going for qPCR (Fig 1and 2, table 1 and 2). The condition of qPCR is given in table 3.

Table1: Reaction Mixture for PCR

Sr. No.	Reaction Components	Volume (µl)
1.	Nuclease free water (NFW)	12.15
2.	Master Mix	10.45
3.	Forward Primer	00.20
4.	Reverse Primer	00.20
5.	gDNA template	02.00
	Total	25.00

Table 2: PCRprogramme for amplification of Methanogenic, Total bacteria, *Fibrobactersuccinogenus* and 16S RNA

Sr. No.	Steps	Temperature	Time
1	Initial Denaturation	95°C	6min
2	35 cycles of		
	a) Denaturation	95°C	45 sec
	b) Annealing	58°C	55 sec
	c) Primer extension	72°C	1 min
3	Final extension	72°C	10 min

Table 3: Reaction programme for all the genes was as follows: Thermal Profile for real time PCR

Segment	Cycle	Incubation temperature (°C)	Incubation time
1	1	50	2min
2	1	95	10min
3	40	95	30sec
		T_m of respective gene (Table 3.5)	30sec
		72	30sec
4	1	95	1min
		55	30sec
		95	30sec

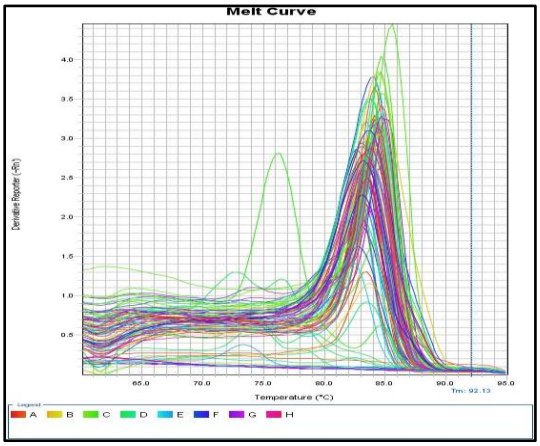


Figure1a: Melting Curve analysis

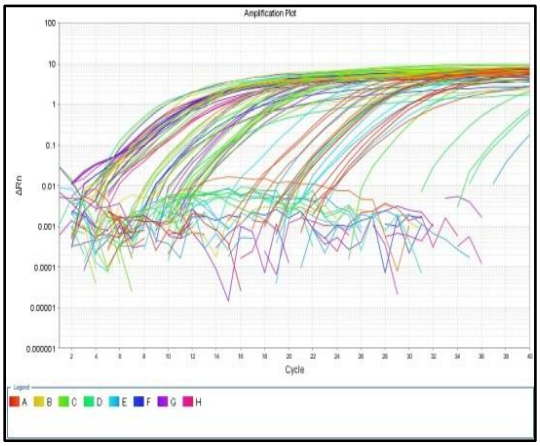
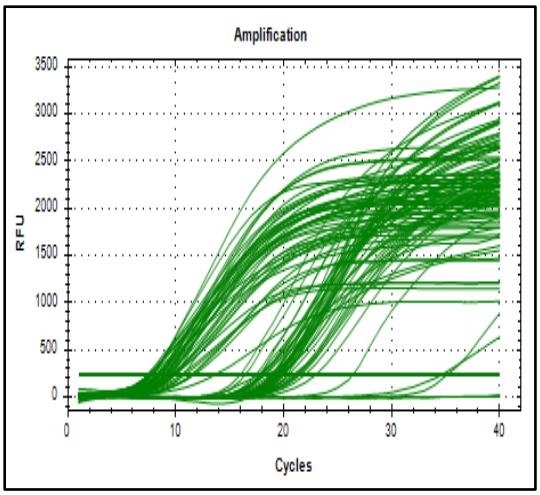


Fig 1b: Amplication curve



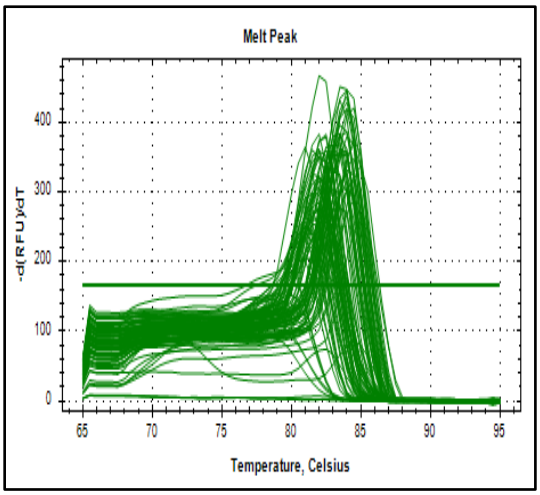


Fig. 2 a Amplification plot ***Fig. 2b*Melting Curve**

Results: The results of *in vitro* rumen fermentation parameters in rumen samples after supplementing different plant extracts have been presented below.

The results (table 4) revealed a significant ($p<0.05$) decrease in total gas production (ml) in T2@0.5 (45.0± 7.03) and T3 (1ml)(95.0±7.07) groups at 24 hour. A non significant difference in total nitrogen and ammonia nitrogen concentration was observed in all treatment groups as compared to control at different time interval. TVFA concentration increased in T3 and T4 groups but non significantly in samples treated with *E. officinalis* (EE).

A significant ($p<0.05$) decrease in total gas production(ml) in all treatment groups at 24 hr post incubation in samples treated with *E. officinalis* (Aq) extract. The results presented in table 5(*E. officinalis*,aq) revealed a significant ($p<0.05$) increment in total gas production(ml) in T1, T4 groups as compared to control at 24 hr post incubation. A significant decrease in gas (ml) was observed in T2 and T3 groups as compared to control at 24 hour. A significant ($p<0.05$) decrease in pH in T1, T2, T3 and T4 groups was observed as compared to control at 24 hour post incubation. Total nitrogen (mg/dl) concentration increased significantly ($p<0.05$) in all treatment groups as compared to control at 6 hour time period and non significantly at 24 hour time period. TVFA (meq/l) concentration increased in all treatment groups as compared to control although non significantly at 6 and 24 hour post incubation. A significant ($p<0.05$) decrease in pH in T1, T2, T3 and T4 groups was observed at 24 hour post incubation. Total nitrogen (mg/dl) concentration increased significantly ($p<0.05$) in all treatment groups at 6 hour time period.

A significant ($p<0.05$) decrease in pH in all groups as compared to control at both 6 hour and 24 hour post incubation in samples treated with *M.piperata* (EE)extract (Table 6). An increasing trend in TVFA concentration observed in all treatment groups as compared to control although non significantly. Ammonia nitrogen concentration decreased significantly ($p<0.05$) in T1 and T3 treatments as compared to control at 24 hour time period.A significant decrease ($p<0.05$) in gas production was observed in T1, T2 and T3 groups as compared to control at 24 hour(table 6)

A significant ($p<0.05$) decrease in pH in all treatment groups was observed as compared to control at 6 hour post incubation after supplementing *Menthapiperata* (Aq)extract(Table 7). Total volatile fatty acids (TVFA) production increased significantly ($p<0.05$) in all treatment groups as compared to control at 6 hour post incubation. A decreasing trend in gas production was observed in T1, T2 and T3 groups (Table 7).The results revealed a non significant difference in rumen fermentation parameters in samples treated with *E. officinalis* and *M. piperata* combination aq extract and ethanolic extract except a decreasing gas production and increased TVFA production was observed(Table 8 and 9). The results of samples treated with babool aqueous and ethanolic extract revealed a significant increase in TVFA production at 6 hour although non significantly at 24 hour (Table 10 and 11). Gas production was decreased in samples treated with aq extract of babool at 24 hour post incubation at the rate of 1 ml but non significant in ethanolic extract treated samples.

The results presented in table citrus fruit peelextract (Table 12)aqueous) revealed a significant ($p<0.05$) decrease in pH in all treatment groups as compared to control at 24 hour time period. Gas production decreased although non significantly in all treatment groups as compared to control at 24 hour. A non significant difference was observed in TVFA concentration and total nitrogen concentration at different time interval among all groups. Ammonia nitrogen decreased significantly($p<0.05$) in T1 as compared to control at 24 hour while non significantly in T2, T4 groups at 24 hour as compared to control. The results presented in table citrus fruit peelextract (Table 13) (EE) revealed a significant ($p<0.05$) decrease in pH in all treatment groups as compared to control at 6 hour and 24 hour post incubation. A non significant difference was observed in gas production, TVFA concentration and total nitrogen concentration at different time interval among all groups.

Rumen Bacterial Profile

Methanogenic Bacteria profile expression decreased in samples treated with aqueous extract (*E. officinalis, M. piperata, E. officinalis+M. Piperata,* Citrus fruit peel, and the trend persist till 24 hours. The same trend was observed in ethanolic extract and comparable at 24 hour.

The expression of Bacteroides bacteria were increased in samples treated with aqueous Extract (*E. officinalis, M. piperata, E. officinalis+M. Piperata,* Citrus fruit peel) and the increase was persistent till 24 hour except citrus and babool. In samples treated with ethanolic extract the expression increased at 24 hour in citrus fruit peel and *Menthapiperata*supplemented, whereas almost similar concentration was observed in others as compared to control. Regarding cellulolytic bacteria the expression profile of *Fibrobactersuccinogenus*was enhanced in samples treated with aqueous extract(*E. officinalis, M. piperata, E. officinalis+M. Piperata*, Citrus peel) at 6 hour and significantly increase in *E.officinalis + M. piperata, Emblica, Menthapiperata* at 24 hours whereas in samples treated with ethanolic extract a similar expression was observed at 6 hour except citrus fruit peel which showed increase in bacteria number at 24 hour.

Table4: *In vitro* Rumen fermentation parameters in *Emblicaofficinalis* (EE) extract

Parameters	Time(hour)	Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	PValue
Temp.(^{o}c)	6 hour	30.5±3.53	31.55±.07	32.20±.56	32.40±.14	33.70±.14	.458
	24 hour	31.25±1.34	31.20±.28	32.15±.49	32.05±.07	32.200±.14	.426
Gas(ml)	6 hour	12.5±3.53	7.50±3.53	12.50±3.23	7.50±.07	10.00±00	.665
	24 hour	122.5^{a}±38.8	135.00^{a} ±7.07	45.00^{b} ±7.03	95.00^{c} ±7.07	117.50^{a} ±3.53	.002
pH	6 hour	7.77±0.10	7.77±.03	7.70±.14	7.70±.00	7.45±.21	.124
	24 hour	5.55±.07	5.55±.63	5.65±.07	5.75±.07	5.95±.07	.510
Ammonia N (mg/dl)	6 hour	11.20±5.93	13.30±.98	14.00±1.97	16.80±3.95	10.60±11.03	.839
	24 hour	20.30±.98	21.70±.98	22.40±1.97	19.10±2.96	19.60±1.0	.346
Total Nitrogen (mg/dl)	6 hour	153.50±14.84	128.50±9.19	157.50±34.64	151.25±12.34	125.95±3.32	.372
	24 hour	127.85±2.33	153.50±24.74	148.00±18.38	145.50±7.77	135.75±1.06	.473
TVFA(meq/l)	6 hour	30.50±.70	27.00±1.41	28.00±8.48	29.00±1.41	27.00±1.41	.879
	24 hour	61.75±4.59	64.50±13.44	57.00±4.24	78.50±9.19	67.50±.70	.158

Table 5: *In vitro* Rumen fermentation parameters in *Emblicaofficinalis* (Aq) extract

Parameters	Time (hour)	Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{0}c)	6 hour	30.50±3.53	33.25±0.07	32.7±1.27	32.95±0.21	33.20±0.28	.515
	24 hour	31.25±1.34	33.30±0.28	32.70±0.28	32.20±0.56	31.55±0.07	.127
Gas(ml)	6 hour	12.50±3.53	12.50±3.53	10.00±7.07	17.50±3.53	15.00±0.0	.515
	24 hour	192.50^{a} ±18.09	257.50^{c}±3.53	105.0^{b}±7.07	101.50^{b}±10.6	185.00^{a}±7.07	.003
pH	6 hour	7.77±0.10	7.75±0.07	7.75±0.07	7.50±0.0	7.75±0.07	.302
	24 hour	5.55^{a} ±0.07	5.32^{b}±0.03	5.10^{c}±0.00	5.32^{b}±0.03	5.30^{b}±0.14	.016
Ammonia N (mg/dl)	6 hour	11.20±5.94	11.15±0.07	11.85±0.91	12.30±0.14	14.10±0.14	.090
	24 hour	20.30±0.98	18.75±1.20	20.80±0.84	16.10±2.96	21.00±1.97	.160
Total Nitrogen (mg/dl)	6 hour	103.50^{a}±14.84	124.60^{b}±1.97	124.95^{b} ±1.48	135.10^{c} ±3.11	128.25^{b}±2.75	.009
	24 hour	117.85±2.33	124.35±2.61	135.45±3.36	124.60±1.97	133.10±9.75	.227
TVFA (meq/l)	6 hour	30.50±0.70	32.50±3.53	38.25±2.47	39.75±1.35	25.75±3.18	.208
	24 hour	61.75±4.59	61.85±3.18	65.00±7.07	78.00±2.82	71.00±8.48	.116

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 6:*In vitro* Rumen fermentation parameters in *Menthapiperata*(EE) extract at different concentration

Parameters		Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{o}C).	6 hour	30.50± 3.53	30.40± 0.14	31.10± 0.42	30.85± 0.49	31.45± 0.91	.962
	24 hour	31.25^{b} ± 1.34	26.75^{a}± 0.49	27.60^{a} ± 0.42	28.90^{b} ± 0.56	29.05^{b} ± 0.77	.016
Gas(ml)	6 hour	12.50± 3.53	11.00± 1.41	20.00± 7.07	19.00± 8.48	17.50± 10.60	.653
	24 hour	122.50^{a} ± 38.89	16.50^{c}± 2.12	67.50^{b} ± 10.60	95.00^{b} ± 17.07	137.50^{a} ± 31.81	.018
pH	6 hour	7.77^{a}± 0.10	7.45^{a}± 0.21	6.75^{b} ± 0.21	6.60^{b} ± 0.14	6.80^{b} ± 0.14	.003
	24 hour	5.55^{a}± 0.07	6.00^{b} ± 0.14	5.70^{b} ± 0.14	5.80^{b} ± 0.14	5.35^{a}± 0.21	.044
Ammonia nitrogen(mg/dl)	6 hour	11.20± 5.93	16.10± 2.96	16.20± 4.38	13.25± 1.34	12.00± 5.93	.720
	24 hour	20.30± 0.98	15.15± 1.62	19.25± 3.18	17.50± 2.96	20.70± 0.42	.194
Total Nitrogen(mg/dl)	6 hour	153.50± 14.84	99.80± 25.17	113.85± 12.23	110.00± 7.07	101.85± 7.28	.070
	24 hour	127.85± 2.33	86.65± 9.40	106.35± 44.61	132.15± 18.17	123.00± 5.65	.346
TVFA(meq/l)	6 hour	30.50± 0.70	31.25± 2.47	37.25± 6.71	34.50± 6.36	37.00± 7.07	.627
	24 hour	61.75± 4.59	67.00± 16.97	68.25± 2.47	65.25± 3.188	62.50± 17.67	.968

Table 7: *In vitro* Rumen fermentation parameters in *Menthapiperta* (Aq) extract at different concentration

Parameter	Period(hrs)	Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{o}C)	6 hour	30.50±3.53	31.05±0.35	30.45±0.21	31.85±0.21	31.20±0.84	.901
	24 hour	31.25±1.34	26.95±0.49	25.80±0.14	28.70±0.56	27.90±0.84	.007
Gas(ml)	6 hour	12.50±3.53	17.50±10.60	21.50±9.19	31.50±16.26	15.00±14.14	.564
	24 hour	122.50±38.89	61.50±16.26	68.50±33.23	91.50±23.33	140.00±42.42	.205
pH	6 hour	7.77^{c} ± 0.1	6.70^{b} ±0.56	6.80^{b} ±0.14	6.10^{a} ±0.14	6.30^{a}±0.28	.015
	24 hour	5.55±0.07	5.60±0.14	5.45±0.21	5.65±0.21	5.55±0.35	.911
Ammonia nitrogen(mg/dl)	6 hour	11.20±5.93	15.80±0.56	21.00±5.93	19.10±2.68	22.60±7.63	.327
	24 hour	20.30±0.98	22.50±0.98	20.65±4.45	15.30±5.79	21.00±4.94	.513
Total Nitrogen(mg/dl)	6 hour	153.50± 14.84	159.60±1.97	123.50±3.53	126.00±29.69	115.90±29.13	.243
	24 hour	127.85±2.33	148.05±20.29	134.25±8.13	109.15±5.86	157.75±20.85	.092
TVFA(meq/l)	6 hour	30.50^{a} ±0.70	66.25^{b} ±2.47	75.75^{c}±6.01	67.50^{b}±2.12	68.00^{b} ±7.07	.001
	24 hour	61.75±4.59	61.25±8.83	84.50±7.77	70.00± 21.21	85.75±4.59	.199

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 8: *In vitro* Rumen fermentation parameters in *E officinalis+M.piperata* (aq) extract at different concentration

Parameters	Period(hrs)	Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{o}C)	6 hour	31.70± 1.55	30.70± 2.26	31.70±0.00	31.90± 0.56	32.20± 0.14	0.8
	24 hour	31.50± 0.28	30.40± 0.14	30.30±0.28	30.90± 0.14	30.70± 0.42	0.038
Gas(ml)	6 hour	30.00± 0.0	35.00± 35.35	85.00±21.21	50.00± 28.28	70.00± 0.0	0.157
	24 hour	52.50^{b} ± 3.53	95.00^{a} ± 25.35	76.00^{c} ± 0.70	37.50^{b} ± 10.6	32.50^{b} ± 3.53	0.051
pH	6 hour	6.30^{a}± 0.28	6.55^{b} ± 0.07	6.55^{b} ±0.07	6.50± 0.0	6.70^{b} ± 0.84	0.05
	24 hour	6.60^{b} ± 0.14	5.40^{a} ± 0.14	5.40^{a}±0.14	5.35^{a} ± 0.07	5.35^{a} ± 0.07	0.049
Ammonia nitrogen(mg/dl)	6 hour	5.30± 3.53	11.90± 4.94	15.40±1.97	14.70± 0.98	12.60± 5.93	0.222
	24 hour	9.80± 3.53	17.50± 4.94	13.30±1.97	16.80± 0.98	22.40± 5.93	0.734
Total Nitrogen(mg/dl)	6 hour	100.50± 5.93	85.75± 14.84	72.30±4.94	91.95± 9.89	63.80± 7.91	0.957
	24 hour	82.20± 10.60	90.15± 12.12	87.85±8.62	92.60± 0.49	86.45± 8.9	0.172
TVFA(meq/l)	6 hour	41.75± 5.51	50.00± 0.21	58.50±5.44	33.00± 1.27	31.75± 0.49	0.765
	24 hour	50.00^{b} ± 10.25	105.00^{a} ± 31.11	101.00^{a}± 41.71	68.75^{b} ± 1.41	115.50^{a} ± 1.76	0.018

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 9: *In vitro* Rumen fermentation parameters in *E officinalis+M.piperata* (EE) extract at different concentration

Parameters		Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp. (^{o}C)	6 hour	30.95±0.49	31.40±0.28	31.65±0.21	32.85±0.21	31.55± 1.06	0.114
	24 hour	29.85±2.61	30.90±0.28	29.85±0.91	28.75±0.63	29.40± 0.42	0.605
Gas(ml)	6 hour	35.00±7.07	62.50±24.74	70.00±42.42	12.50±3.53	40.00±42.42	0.403
	24 hour	125.00±10.6	30.00±21.21	160.00±14.14	150.00± 21.21	42.50±17.67	0.133
pH	6 hour	6.55±0.35	6.50±0.14	6.35±0.07	6.40±0.14	6.35± 0.07	0.757
	24 hour	6.10±0.84	5.45±0.21	5.50±0.0	5.35±0.07	5.45± 0.07	0.423
Ammonia nitrogen(mg/dl)	6 hour	9.10±08.9	14.00±1.97	13.00±7.35	11.90±6.92	12.60± 3.95	0.944
	24 hour	14.70^{b} ±0.98	16.10^{b} ±0.98	16.30^{b} ±0.70	11.90^{a}±2.96	23.20^{c}± 2.82	0.017
Total Nitrogen(mg/dl)	6 hour	117.00±12.72	136.00±10.60	106.65±3.32	96.25±19.44	158.30±35.49	0.119
	24 hour	88.15^{b} ±13.93	127.75^{a}±7.42	76.85^{b} ±7.7	94.95^{b} ±7.28	92.15^{b}± 8.27	0.019
TVFA(meq/l)	6 hour	40.00±12.72	30.75±2.47	30.50±2.12	27.50±3.53	29.00± 1.41	0.388
	24 hour	75.00±14.14	49.75±27.93	68.75±5.3	82.25± 14.49	76.50± 12.02	0.43

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 10: *In vitro* Rumen fermentation parameters in babool (aq) extract at different concentration

Parameters		Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp. .(^{o}C)	6 hour	27.10±0.28	29.25±1.20	31.20±1.55	29.70±0.56	29.45±0.21	0.054
	24 hour	31.90±0.70	28.55±0.07	27.75±1.06	28.45±0.21	26.90±0.84	0.005
Gas	6 hour	26.50±9.19	22.50±3.53	15.00±14.14	27.50±17.67	25.00±28.23	0.94
	24 hour	170.0^{a} ±14.14	68.50^{c}±9.19	135.00^{a}±14.14	92.50^{b} ±10.60	100.0^{b} ±14.14	0.031
pH	6 hour	6.30±0.42	7.40±0.14	7.20±0.42	6.90±0.14	7.05±0.21	0.083
	24 hour	5.55±0.35	5.60±0.14	5.65±0.07	5.60±0.14	5.80±0.14	0.747
Ammonia nitrogen(mg/dl)	6 hour	9.80±3.95	10.50±2.96	11.20±3.95	7.00±5.93	12.75±2.19	0.703
	24 hour	19.10±2.68	17.50±4.94	18.90±6.92	22.20±1.69	11.90±4.94	0.373
Total Nitrogen	6 hour	109.00±5.65	135.80±20.08	127.00±8.48	130.35±8.98	143.90±24.18	0.345
	24 hour	115.50±4.94	138.00±5.65	127.50±21.92	151.90±16.82	134.90±9.75	0.238
TVFA	6 hour	33.50±2.12	34.65±1.20	60.75±14.49	27.25±0.35	37.25±6.71	0.034
	24 hour	69.50±3.53	58.75±8.83	83.50±7.77	79.75±3.18	81.50±10.6	0.083

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 11: *In vitro* Rumen fermentation parameters in babool (EE) extract at different concentration

Parameters		Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{o}C)	6 hour	27.10±0.28	26.05±0.35	24.95±0.91	28.10±0.28	28.85±0.21	0.003
	24 hour	31.90±0.7	28.75±0.35	27.60±01.55	28.90±0.28	28.50±00.70	0.026
Gas	6 hour	24.00±12.72	47.50±8.89	20.00±14.14	30.00±21.21	17.50±10.6	0.686
	24 hour	170.00±14.14	65.50±4.94	105.00±77.78	87.50±31.81	77.50±24.74	0.212
pH	6 hour	6.80±1.13	6.50±0.28	6.90±0.28	6.80±0.42	6.55±0.07	0.935
	24 hour	6.35^{c} ±0.77	4.70^{a} ±0.14	5.35^{b} ±0.07	5.7^{b} ±0.07	5.42^{b} ±0.03	0.042
Ammonia nitrogen(mg/dl)	6 hour	9.80^{b} ±3.95	13.10^{b} ±4.66	17.25^{a} ±1.62	22.75^{c} ±2.47	8.40^{b} ±1.97	0.029
	24 hour	19.1^{b} ±2.68	16.30^{b} ±1.27	19.60^{b} ±1.97	26.50^{a} ±2.12	18.20^{b} ±0.0	0.018
Total Nitrogen	6 hour	115.50±5.65	120.00±14	128.10±4.52	126.00±24.25	129.00±16.26	0.262
	24 hour	33.50±4.94	44.75±35.35	34.25±2.68	28.75±9.89	68.75±4.24	0.905
TVFA	6 hour	78.50^{b} ±2.12	68.50^{b} ±10.96	51.25^{c} ±5.30	85.00^{a} ±1.76	78.25^{b} ±1.76	0.005
	24 hour	27.10±16.26	26.05±8.48	24.95±1.76	28.10±4.24	28.85±5.3	0.065

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 12: *In vitro* rumen fermentation parameters at different concentrations of citrus fruit peel extract (Aq) supplementation

Parameters	Time(hour)	Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{0}c)	6 hour	33.45 ±0.15	30.5 ±1.6	31.15 ±0.75	32.2 ±0.4	32.9 ±0.3	0.208
	24 hour	27.4^{b} ±0.30	30.75^{a} ± 0.55	29.7^{b} ±0.40	30.9^{a} ±0.40	30.0^{a} ±0.20	0.007
Gas(ml)	6 hour	22.5 ±17.50	31.5 ±3.50	42.5 ±2.50	67.5 ±7.50	50 ±30	0.423
	24 hour	187.5 ±12.5	130 ±30.0	112.5 ±37.5	165 ±35	187.5±12.5	0.328
pH	6 hour	7.2 ±0.10	6.9 ±0.30	7 ±0.10	7.15 ±0.15	7.15 ±0.15	0.734
	24 hour	6.35^{b} ±0.15	5.9^{a} ±0.10	5.95^{a} ±0.05	5.75^{c}±0.05	6.05^{a}±0.05	0.035
Ammonia nitrogen (mg/dl)	6 hour	16.1 ±0.70	14.7 ±0.70	13.3 ±0.70	14.7 ±2.10	15.4 ±1.40	0.631
	24 hour	25.2^{b} ±0.20	19.6^{a} ±1.40	23.1^{b} ±0.70	27.3^{c} ±0.70	25.9^{b} ±2.10	0.037
Total Nitrogen (mg/dl)	6 hour	101.2 ±17.10	63.7 ±1.40	47.95±24.15	102.55±3.15	85.25±8.55	0.132
	24 hour	95.15 ±25.85	84 ±7.70	84.35±22.05	89.6 ±18.9	97.85 ± 25.75	0.983
TVFA (meq/l)	6 hour	36.5 ±5.50	38.25 ±1.25	37.75 ±1.75	39.5 ±1.50	37.0 ±4.10	0.473
	24 hour	81 ±16.0	83.5 ±6.0	88.75 ±1.25	90.75 ±1.25	99 ±1.5	0.126

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 13: *In vitro* rumen fermentation parameters at different concentrations of citrus fruit peel extract (EE) supplementation

Parameters	Time (hrs)	Control	0.2 ml(T1)	0.5ml(T2)	1 ml(T3)	2ml(T4)	P Value
Temp.(^{0}c)	6 hour	33.45 ±0.15	33.85 ±0.25	32.95 ±1.25	32.25 ±1.05	34.35 ±0.25	0.424
	24 hour	27.40 ^{b} ±0.30	33.20 ^{a} ±0.40	33.60 ^{a} ±1.50	33.90 ^{a} ±1.60	34.55 ^{a} ± 0.25	0.02
Gas(ml)	6 hour	22.5 ±17.50	47.50 ±27.50	57.50 ±22.50	62.50 ±27.50	65.00 ±25.0	0.735
	24 hour	187.5 ±12.50	95.00 ±15.0	97.50 ±72.5	152.5 ±32.5	87.50 ±22.5	0.371
pH	6 hour	7.2 ^{b} ±0.1	6.00^a ±0.1	5.85 ^{a} ±0.05	6.10 ^{a} ±0 .10	6.10 ^{a} ±0.20	0.008
	24 hour	6.35 ^{b} ±0.15	5.20 ^{c} ±0.10	5.10 ^{c} ±0.20	5.50 ^{a} ±0.20	5.28 ^{a} ±0.08	0.011
Ammonia nitrogen (mg/dl)	6 hour	16.1 ±0.70	15.40 ±1.40	12.00 ±0.60	16.10 ±0.70	11.90 ±2.10	0.127
	24 hour	25.20 ±0.0	23.35 ±1.85	28.00 ±2.80	28.90 ±3.30	28.70 ±2.10	0.439
Total Nitrogen(mg/dl)	6 hour	101.20 ±17.1	67.20 ±7.7	72.95 ±10.65	72.80 ±11.9	64.65 ±8.65	0.311
	24 hour	95.15 ±25.85	80.85 ±12.95	84.70 ±15.4	85.00 ±14.3	75.25 ±7.35	0.928
TVFA(meq/l)	6 hour	31.25 ±0.25	34.50 ±1.50	30.75 ±1.25	26.50 ±1.50	32.75 ±2.75	0.114
	24 hour	81.0 ±16.0	89.25 ±0.75	99.50 ±4.50	99.25 ± 10.75	89.00 ±5.0	0.606

Objective2: Effect of supplementation of best selected plant extract on rumen fermentation and growth in growing buffalo calves

Results: Based on *in-vitro* results, *E. officinalis* fruit powder was selected and supplemented in the diet of fistulated buffalo calves. Two male fistulated buffalo calves were taken for feeding trial. The animals were housed in open shed having proper arrangement of individual feeding and watering. The ration was provided to individual animal according to their body weight (ICAR, 2013). The concentrate mixture comprised of maize 30%, groundnut cake 15%, mustard cake 20%, wheat bran 32%, mineral mixture 2% and common salt 1% containing 17.58% CP and 70% TDN + 10-12 kg green fodder + wheat straw *ad lib.* The ration was supplemented with 30 gm of *E. officinalis* powder. The feeding was done for a period of three months. Rumen fluid samples were taken at 0 hour pre-feeding, at 0 day, 30 day, 60 day, 90 day for two consecutive days in air tight flask and brought to laboratory for further processing. pH, temperature were measured in strained rumen liquor immediately. Rumen sample were processed and preserved for further analysis of ammonia nitrogen, total nitrogen and TVFA as per standard protocol.

A significant decrease in ruminal fluid pH was observed (6.7±0.2 (0 day) to6.10±0.00 (30 day) ($p<0.05$). TVFA production increased (122.5±7.5(0 day) significantly post feeding at 60 day(172.5±14.3) and 90 day post feeding. Temperature decreased significantly among groups at all time intervals. Total nitrogen production increased (88.9±4.9) significantly at 30 day.

Growth and haemato-biochemical studies in buffalo calves

Selection, distribution and feeding of growing animals

Twelve male buffalo calves (12-14 months old) were selected from the herd of Department of Livestock Production and Management and were divided into two groups (Control and Treatment) of six animals in each group. The animals were housed in open shed having proper arrangement of individual feeding and watering. The calves were fed the ration as per ICAR (2013).

Control group (T-1): Concentrate mixture + green fodder + wheat straw *ad lib*

Treatment Group (T-2): Diet same as in control + 30 gm of *Emblicaofficinalis* powder.

Body growth parameters:

Body growth parameters include:

1. Abdominal girth: The circumference of the body over the flank just in front of the udder.
2. Heart girth: Circumference of the body over the chest of the animal just behind the elbow (Plate- 3).
3. Body length: The distance from point of shoulder to the point of pin bone.
4. Height of animal: The distances from the floor to the highest point of wither.
5. Body weight: was taken at digital weighing balance.

Body growth parameter of buffalo calves of two groups (Control and Treatment) recorded twice at the beginning and at the end of the trial. Average body weights of buffalo calves were 156.83±9.78 kg (control) and 159.5±4.36 kg (treatment) at the beginning of feeding trial. Final body weights (kg) were 176.17±12.71 (control) and 172.83±8.69 (treatment) and there was no significant variation found between control and treatment groups during the trial period

Blood Sampling: During 90 days of experimental period, blood samples were collected thrice, one at the beginning, in the middle and at the end of trial, from all the animals by jugular puncture in two separate heparinized vials, mixed well by rotating tubes between palms to ensure proper mixing of blood and anticoagulant and brought to the laboratory after placing in ice box. One set of the samples were centrifuged at 3000 rpm 15 minutes to separate the plasma. The plasma samples were stored at -20°C for further analysis. Second set of blood sample was used for hematological analysis immediately.

Hematobiochemical parameters

Analysis of hematological parameters Total erythrocyte count(TEC), Hemoglobin (Hb), Total leucocyte count(TLC), Packed cell volume (PCV), Mean corpuscular volume(MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Total granulocytes (GRA), Lymphocyte (LYM) and Monocytes (MON)) was done by using fully automated hematology analyzer (MS4Se®)

The results(Table 14) of biochemical parameters revealed a significant ($p < 0.05$) difference in ALT concentration between treatment and control group at 90 days. Phosphorus concentration increased significantly at 90 days (7.19±0.99) as compared to 0 day (6.14±0.44) ($p < 0.05$) in control group. Triglyceride concentration increased significantly ($p < 0.05$) in control group at 45 day (26.33±2.69) and 90 day (25.3±2.4) as compared to 0 day (14.67±2.81) while it decreased

significantly(19.33±2.16) at 90 day in treatment group as compared to 0 day(32.66±7.65).A significant increase in cholesterol concentration was observed at 45 day and 90 day as compare to 0 day in both treatment and control group. Low density lipoprotein (LDL) concentration increased significantly ($p < 0.05$) at 45 day and 90 day as compare to 0 day in both control and treatment group. Total protein concentration increased significantly ($p < 0.05$) in treatment group at 45day and 90 day. The albumin concentration was 1.53±0.05 at 0 day and 2.43±0.04 at 90 day in treatment group indicating a significant increase ($p < 0.05$) post feeding *E. officinalis* powder. Total protein concentration decreased significantly in control group at 45 day and 90 day as compared to 0 day while albumin concentration increased significantly in control group (1.59±0.08 (0 day) to 2.41±0.12 (90 day).

The results of haematological parameters(table 15) revealed a significant ($p < 0.05$) increase in TEC concentration at 45 day(6.7± 0.1) and at 90 day (7.21 ± 0.18) after feeding *Emblicaofficinalis* as compared to 0 day (4.80 ± 0.60) in treatment group. A significant ($p < 0.05$) increase in haemoglobin concentration was observed at 45 day (10.84 ± 0.29) and 90 day(11.68 ± 0.31) as compared to 0 day(7.80 ± 1.03) and also there was significant ($p< 0.05$) difference between treatment and control group at 0 day. A significant ($p < 0.05$) decrease in TLC concentration was observed at 45 day (9.08 ± 0.31) and 90 day (8.43 ± 0.57) as compared to 0 day(11.57 ± 0.79). A significant ($p < 0.05$) increase in PCV concentration was observed after feeding *E. officinalis* powder as compared to 0 day(21.37 ± 2.50) and also there was significant ($p < 0.05$) difference between treatment and control group at 0 day and 45 day. A significant ($p < 0.05$) decrease in lymphocyte and monocyte count was observed at 45 day and 90 day as compared to 0 day while a significant ($p < 0.05$) increase in granulocyte concentration was observed at 45 day (32.64±2.64) and 90 day (33.78 ± 2.72) as compared to 0 day(29.07 ± 3.51).

Table 14: Effect of feeding *Emblicaofficinalis*powder on biochemical parameters in buffalo calves

Parameters	Group	Days		
		0 day	45 day	90 day
Alanine aminotransferase(U/L)	Treatment	51.78±11.09	48.88±13.77	63.08^{b}±5.09
	Control	45.5±5.76	65.02±13.35	48.73^{a}±3.7
Aspartate aminotransferase(U/L)	Treatment	131.32±8.02	119.75±12.15	130.95±4.24
	Control	122.27±11.96	147.13±31.32	126.35±8.86
Phosphorus(mg/dl)	Treatment	6.1±0.35	6.68±0.54	6.67±0.63
	Control	6.14^{A} ±0.44	6.56^{AB} ±0.54	7.19^{B}±0.99
BUN(mg/dl)	Treatment	29.03^{b} ±4.15	36.53 ±5.25	29.35±1.14
	Control	15.28^{a}±4.6	35.72±6.26	30.18±3.1
Glucose(mg/dl)	Treatment	54.5±5.93	74.9±6.35	79.3±2.3
	Control	65.58±3.98	68.52±5.46	80.5±1.3
Triglyceride(mg/dl)	Treatment	32.67^{b}±7.65	23.00±2.91	19.33 ±2.16
	Control	$14.67^{A,a}$±2.81	26.33^{B}±2.69	25.33^{B} ±2.4
Cholesterol(mg/dl)	Treatment	62.5^{A}±4.6	77.67^{B}±4.18	78.17^{B}±1.74
	Control	53.17^{A}±3.61	71.50^{B}±4.85	75.33^{C}±2.03
HDL-C(mg/dl)	Treatment	43.15±2.9	44.15±3.37	46.78±3.26
	Control	34.33±3.82	43.83±4.11	37.75±2.65
LDL(mg/dl)	Treatment	13.17^{A}±2.31	22.94^{B}±4.55	21.81^{B}±2.46
	Control	11.42^{A}±1.87	22.57^{B}±1.5	19.3^{B}±0.89
Albumin(g/dl)	Treatment	1.53^{A} ±0.05	1.87^{B} ±0.08	2.43^{C} ±0.04
	Control	1.59^{A} ±0.08	1.91^{B} ±0.06	2.41^{C} ±0.12
Total protein(g/dl)	Treatment	$6.72^{A,b}$ ±0.38	7.19^{B} ±0.38	$7.17^{B,a}$ ±0.72
	Control	$7.38^{A,a}$±0.53	6.99^{B}±0.42	$5.65^{C,b}$±0.35

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 15: Effect of feeding *Emblicaofficinalis*powder on haematological parameters in buffalo calves

Parameters	Group	Days		
		0 day	45 day	90 day
TEC(M/mm^3)	Treatment	$4.80^{A,a} \pm 0.60$	$6.70^{B} \pm 0.10$	$7.21^{C} \pm 0.18$
	Control	$7.87^{b} \pm 0.48$	6.87 ± 0.38	7.81 ± 0.49
Haemoglobin(g/dl)	Treatment	$7.80^{A,a} \pm 1.03$	$10.84^{B} \pm 0.29$	$11.68^{B} \pm 0.31$
	Control	$12.97^{b} \pm 0.90$	11.71 ± 0.64	12.37 ± 0.66
TLC(m/mm$^{3)}$	Treatment	$11.57^{B,b} \pm 0.79$	$9.08^{A} \pm 0.31$	$8.43^{A} \pm 0.57$
	Control	$8.00^{a} \pm 1.09$	8.79 ± 0.54	8.37 ± 0.76
PCV(%)	Treatment	$21.37^{A,a} \pm 2.50$	$32.1^{B,a} \pm 0.65$	$33.70^{B} \pm 1.33$
	Control	$36.83^{b} \pm 1.68$	$36.01^{b} \pm 1.55$	39.00 ± 2.12
MCV(fl)	Treatment	$44.98^{a} \pm 1.08$	48.01 ± 1.34	46.75 ± 0.92
	Control	$48.35^{b} \pm 0.95$	53.27 ± 3.79	50.22 ± 2.13
MCH(pg)	Treatment	16.17 ± 0.20	16.2 ± 0.42	16.17 ± 0.36
	Control	15.85 ± 0.17	17.39 ± 1.48	15.85 ± 0.28
MCHC(g/dl)	Treatment	$36.12^{b} \pm 1.02$	33.88 ± 1.37	34.75 ± 0.89
	Control	$32.83^{a} \pm 0.42$	32.54 ± 1.12	31.32 ± 1.42
Lymphocytes (%)	Treatment	$67.68^{B} \pm 3.38$	$64.43^{A} \pm 2.6$	$63.65^{A} \pm 2.74$
	Control	58.40 ± 5.47	59.03 ± 3.94	59.62 ± 3.87
Monocytes (%)	Treatment	$3.25^{B} \pm 0.32$	$2.93^{A} \pm 0.23$	$2.57^{A} \pm 0.26$
	Control	2.95 ± 0.31	2.87 ± 0.22	2.82 ± 0.15
Granulocytes (%)	Treatment	$29.07^{A} \pm 3.51$	$32.64^{B} \pm 2.64$	$33.78^{B} \pm 2.72$
	Control	38.65 ± 5.72	38.1 ± 4.02	37.57 ± 3.97

[a, b] Mean values bearing different superscripts between groups varies significantly ($p<0.05$)

[A, B, C] Mean values bearing different superscripts between days varies significantly ($p<0.05$)

Objective3:.Effect of supplementation of best selected plant combinationfor three monthsin lactating animals on milk yield and milk composition.

Prior approval was taken to conduct the present investigation by the Institutional Animal Ethics Committee. The work was divided into two phases:

A. Rumen fermentation *in-vivo* study: Study of selected plant feed blend for evaluation of rumen fermentation parameters in fistulated buffalo calves.

B. Milk profile and haemato-biochemical studies:Study of selected plant feed blend for evaluation of milk yield, milk composition, somatic cell count in milk and haemato-biochemical parameters in lactating Murrah buffaloes.

A. Rumen fermentation *in-vivo* study

Asparagus racemosus(Shatavari root), ***Emblicaofficinalis*** dried fruit (Amla) ground to pass through 1 mm sieve and powder was kept in dry place to avoid contact with moisture. The AOAC (2005) protocol was followed to analyze representative feed samples for proximate principles, specifically total ash, ether extract, crude protein, crude fiber, dry matter, and nitrogen-free extract. The feeding trial was conducted on three male fistulated buffalo calves. The animals were kept in an open shed with appropriate set up for watering and feeding each animal individually. All fistulated animals were provided a maintenance ration having 40% concentrate and 60% roughage as per ICAR (2013). Roughage consisted of wheat straw and green fodder.The concentrate mixture containing 17.58% CP and 70% TDN comprised of maize 30%, groundnut cake 15%, mustard cake 20%, wheat bran 32%, mineral mixture 2% and common salt 1% containing + 10-12 kg green fodder + wheat straw *ad lib.* The ration was supplemented with total 75gm of plant blend (50gm *E. officinalis* powder + 25gm of *A. racemosus*powder). The feeding was done for a period of two months.

The rumen liquor (RL) samples were collected before feeding and watering from three rumen fistulated male buffalo calves at 0 hour pre-feeding at 0 day, 30 days and 60 days for two consecutive days in air tight flask.A homogeneous and representative sample was collected at varied depths from different parts of the rumen,strained through four layered muslin cloth into a thermos flask which was previously flushed with carbon dioxide to maintain anaerobic condition and immediately brought to laboratory for further processing. Temperature, pH were measured in strained rumen liquor immediately. Strained rumen liquor (SRL) samples

were processed and preserved for further analysis of total nitrogen, ammonia nitrogen and TVFA as per standard protocol.

A significant ($p \leq 0.05$) decrease in ruminal fluid pH was observed at 30 days (6.54±0.03) and 60 days (6.44±0.13) after feeding plant blend. TVFA concentration increased significantly ($p \leq 0.05$) at 30 days (72.50± 5.77) and at 60 days (86.67±2.47) post feeding plant blend. A non significant difference in temperature was observed at 30 days (38.33±0.08) and at 60 days (37.510±0.70) post feeding. A non significant difference was observed in ammonia nitrogen concentration at different time intervals after feeding the blend as compared to before feeding (0 day). Total nitrogen (mg/dl) concentration increased significantly ($p \leq 0.05$) at 30 days (56.41±0.50) and at 60 days (59.90±1.29) after feeding with *A. racemosus*and *E. officinalis*.

B Milk profile and haemato-biochemical studies

Twenty -four lactating Murrah buffaloes of early and mid lactation stages were selected and divided into control and treatment groups having six in each following a completely randomized design (CRD) from the herd of buffalo farm of the Department of Livestock Production Management of College of Veterinary Sciences, LalaLajpatRai University of Sciences, Hisar. All the experimental Murrah buffaloes used in the present study were kept under a loose housing system.Buffaloes were fed ration composed of concentrate mixture and roughage individually according to their nutrient requirement (ICAR 2013). The study was conducted for a period of 3 months, individually for animals taken in early, mid lactation.The grouping of animal as follows:

Early lactation stage and Mid lactation stage

Control group (n=6)=Standard ration concentrate fed as per ICAR (2013) feeding standards.

Treatment group (n=6) = Standard ration concentrate fed along with 150gram of plant blend (100gram of Amla+50gram of Shatavari).

Milk production and composition

During the trial, buffaloes were manually milked twice a day at 4:00 A.M. and 4:00 P.M. A digital weighing balance was used to record the milk yield, and the weekly average was computed.Milk samples for composition analysis were taken at weekly intervals until the end of the experiment. The samples from each experimental animals were taken during the morning and evening milking and the same day processing with the help of Milko-Scan autoanalyzer was done and the average of

both was calculated for estimation of daily milk composition. Milkoscan machine was used at a temperature range of 5-35 degrees Celsius and homogenous samples at a temperature range of 5-40 degrees Celsius were used. Milkoscan Mars is CE-labelled& in compliance with Association Of Analytical Chemists (AOAC) and International Dairy Federation (IDF) & works on FTIR technology. The respective data were obtained from the Milkoscan for milk composition parameters and **Somatic cell count (*10^3cells/ml) of milk** by Lactoscan somatic cell counter 6010 at fortnightly interval.

Haemato-Biochemical parameters

From all the twenty-four Murrah buffaloes, belonging to the early and mid lactation group, blood samples were collected thrice at 0^{th}, 45^{th}, 90^{th} day of experiment, aseptically during early morning hours before watering and feeding of buffaloes by jugular vein puncture.The blood sample from EDTA coated vial was used for haemoglobin (Hb), total erythrocyte count (TEC), total leucocyte count (TLC), lymphocytes, monocytes, granulocyte, mean corpuscular haemoglobin (MCH), and mean corpuscular volume (MCV) analysis by using fully automated haematology analyzer (MS4Se®). The sera samples were analyzed for estimation of blood biochemical parameters (glucose (mg/dl), total protein (g/dl), albumin (g/dl), blood urea nitrogen (BUN) (mg/dl), triglyceride (mg/dl), cholesterol (mg/dl), calcium(mg/dl), phosphorus(mg/dl), aspartate aminotransferase (AST) (IU/L) and alanine aminotransferase (ALT) (IU/L)) using Automated Random Access Clinical Chemistry Analyser (EM Destiny 200)^{TM}, Erba Diagnostics Mannheim GmbH) placed at Department of Veterinary Physiology and Biochemistry, LUVAS, Hisar

Feeding of *A. racemosus* and *E. officinalis*powder affects the nutrient metabolism, haematological parameters indicated by a significant increase in Hb concentration in early lactation stage at 45 and 90 days. Haematological parameters (Table 16 a) indicated a significant increase in Hb concentration in mid lactation stage at 45 days. Regarding biochemical parameters(Table 16 b) glucose concentration increased significantly ($p<0.05$) in treatment group of early and mid lactation at 45 days and 90 days. Saponin supplementation has been shown to lower rumen ammonia-nitrogen concentration and form protein complexes that protect proteins from degradation in the rumen, ultimately increasing the duodenal flow of microbial nitrogen. Saponins in Shatavari may enhance propionate production, which is generally regarded as a key substrate for gluconeogenesis leading to better blood

glucose levels. Adding saponin to the diet significantly enhanced the ruminalVFA profile and increased propionate levels. Total protein concentration increased significantly ($p < 0.05$) in treatment group of early lactation at 45 days. The level of lipid peroxidation is reduced in mid lactation animals due to one of the most potent sources of vitamin C, amla which is rich in amino acids, tannins and flavanoids that are known to protect the body against free radicals.

Table 16a: Effect of *A. racemosus*and*E. officinalis*feeding on haematological parameters of Murrah buffaloes under different lactation stages

Stage of lactation		**Early**			**Mid**		
Parameter	**Days**	**Control**	**Treatment**	**p-value**	**Control**	**Treatment**	**p-value**
Haemoglobin (g/dl)	0	10.25±0.31	10.40±0.40	0.78	10.79±0.55	11.22±0.28	0.58
	45	10.48^{a}±0.27	11.29^{b}±0.23	0.04	11.02^{a}±0.20	11.84^{b}±0.20	0.02
	90	10.79^{a}±0.32	11.80^{b}±0.31	0.05	11.10±0.23	11.71±0.34	0.16
TEC (million/mm^3)	**0**	7.35±0.21	6.95±0.31	0.31	6.48±0.12	6.87±0.24	0.19
	45	6.50±0.15	6.73±0.34	0.55	6.42±0.20	6.75±0.16	0.23
	90	7.19±0.20	7.63±0.12	0.51	6.80±0.16	7.68±0.21	0.30
MCH (pg)	0	17.77±0.96	16.33±0.76	0.27	16.17±0.54	15.33±1.09	0.51
	45	15.83±1.14	16.67±0.71	0.55	17.08±0.70	16.33±0.71	0.17
	90	17.33±1.05	16.67±0.61	0.60	16.92±1.12	17.33±1.20	0.62
MCV (fl)	0	40.47±1.85	42.33±1.61	0.46	42.72±2.32	41.77±1.04	0.72
	45	41.83±1.35	42.33±1.26	0.79	43.93±1.42	44.86±1.21	0.34
	90	42.50±2.19	41.67±0.99	0.50	43.38±1.32	42.22±1.66	0.30
TLC (10^3/mm^3)	**0**	8.78±0.33	9.61±0.37	0.13	10.70±0.70	10.89±0.32	0.81
	45	9.21±0.57	9.13±0.94	0.95	10.89±0.46	11.65±0.30	0.20
	90	9.40±0.35	10.89±0.74	0.10	11.28±0.53	11.34±0.58	0.94
Granulocytes (%)	**0**	54.88±3.50	56.98±5.21	0.74	60.07±2.55	58.22±8.62	0.84
	45	56.72±2.63	58.37±3.40	0.71	58.49±3.49	55.81±4.07	0.63
	90	53.93±2.92	55.37±2.81	0.73	56.95±2.75	58.53±5.99	0.82
Lymphocytes (%)	**0**	42.18±2.21	39.65±3.25	0.53	42.38±26.88	38.38±3.53	0.31
	45	35.87±2.34	39.20±2.32	0.39	36.65±3.09	38.28±1.81	0.66
	90	38.38±2.62	41.67±2.71	0.40	36.23±2.96	40.88±3.29	0.30
Monocytes (%)	**0**	2.23±0.30	2.18±0.14	0.88	2.42±0.23	2.32±0.34	0.82
	45	2.48±0.20	2.33±0.14	0.55	2.30±0.24	2.13±0.53	0.78
	90	2.25±0.28	2.02±0.23	0.53	2.55±0.56	1.83±0.23	0.26

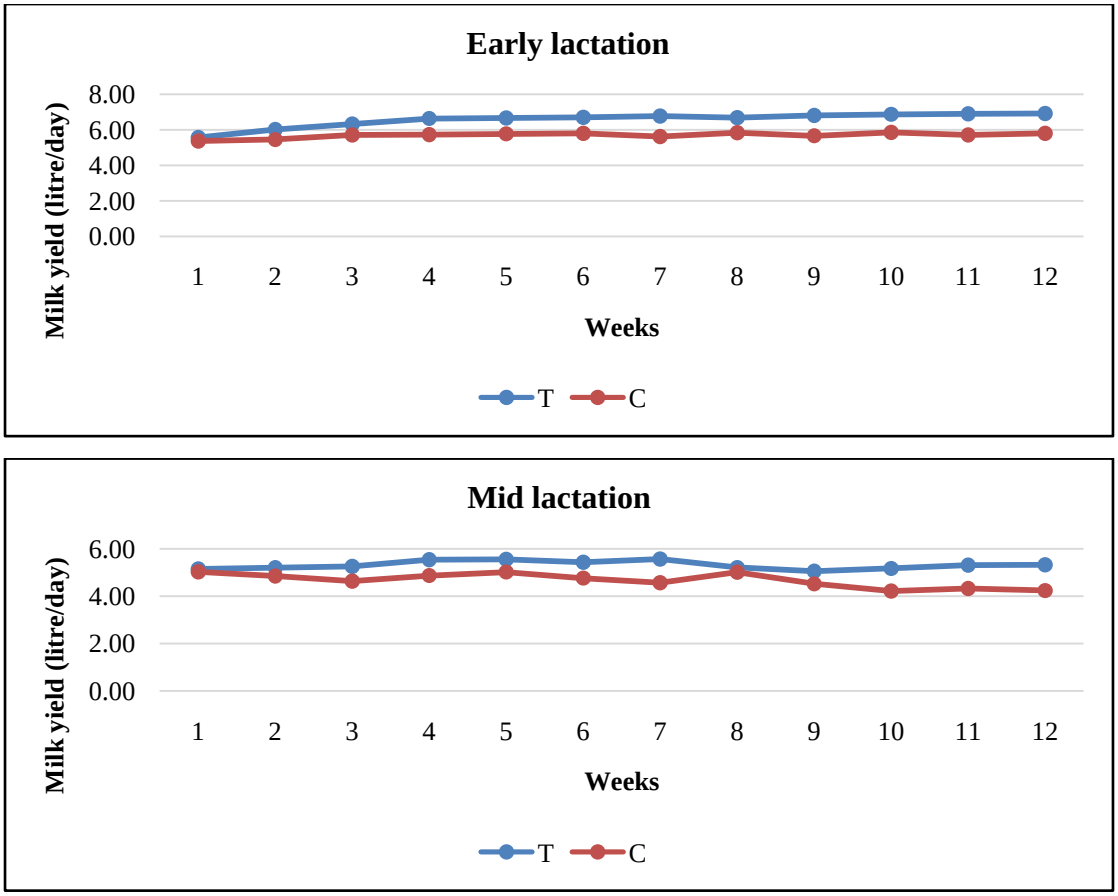


Fig. A Effect of *A. racemosus*and *E.officinalis*feeding on milk yield of Murrah buffaloes under different lactation stages

The weekly average milk yield as shown in Fig A in treatment group was 6.92 litre which was significantly ($P<0.05$) more as compared to the control group(5.79 litre) in early lactation stage. The weekly average milk yield in treatment group was 5.33 litre which was significantly ($P<0.05$) more as compared to control group(4.23 litre) in mid lactation stage. The average weekly milk fat content of buffalo in control and treatment group was 7.62% and 8.24% respectively in the early lactation stage and the fat content in control and treatment group was 7.69% and 7.91% respectively in mid lactation stage. The results indicated significant($p<0.05$) increase in milk fat % in early lactation stage and whereas no significant effect in mid lactation stage. The average weekly milk protein percentage indicated significant ($p<0.01$) increase in milk protein percentage in early lactation stage and no significant effect in mid lactation stage. The average weekly milk lactose percentage in the buffalo milk indicated significant ($p<0.01$) increase in milk lactose % in early lactation stage and mid lactation stage. The average weekly solid not fat (SNF) percent in the early lactation stage and in mid lactation stage increased significantly ($p<0.01$). Regarding

the average milk total solids percentage in buffalo milk the results indicated non-significant effect on milk solids % in early lactation stage and mid lactation stage.

The fortnightly average somatic cell count of milk in early and mid lactation stage results indicated significant decrease($p<0.01$) in treatment group showing better mammary health due to herbal mixture supplementation.The shatavari supplementation reduced number of somatic cell counts in milk, indicating low level of intramammary infection due to immuno stimulant and immuno modulatory properties of shatavari and amla. Feeding of *A. racemosus and E. officinalis* powder is is beneficial for improving production performance by increasing milk yield, milk composition as well as having a positive impact on mammary health by lowering somatic cell count without any adverse effect on haemato-biochemical parameters.

Table 16b: Effect of *A. racemosus*and*E.officinalis*feeding on biochemical parametersof Murrah buffaloes under different lactation stages

	Stage of lactation	Early			Mid		
Parameter	**Days**	**Control**	**Treatment**	**p-value**	**Control**	**Treatment**	**p-value**
Glucose (mg/dl)	0	50.62±5.74	53.23±1.89	0.64	44.12±2.82	48.76±2.82	0.25
	45	50.78±2.50	59.53±3.28	0.06	46.93^{a}±2.73	53.70^{b}±1.21	0.05
	90	49.32^{a}±3.12	62.80^{b}±3.48	0.01	46.83^{a}±2.45	55.65^{b}±2.48	0.05
Triglyceride (mg/dl)	0	15.83±1.40	12.57±0.87	0.07	15.33±1.71	14.16±2.24	0.69
	45	18.83±2.36	13.76±1.18	0.08	13.23±1.24	13.50±0.96	0.87
	90	16.16±2.46	15.99±2.14	0.95	14.50±1.09	13.83±1.64	0.47
Cholesterol (mg/dl)	0	121.83^{a}±2.87	135.66^{b}±3.63	0.01	110.50±6.18	103.16±13.43	0.63
	45	126.67±4.22	107.5±16.79	0.29	101.83±10.10	108.83±10.70	0.64
	90	110.17±9.99	108.5±10.67	0.91	108.17±9.19	101.33±12.12	0.66
Total protein (g/dl)	0	6.13±0.28	6.30±0.20	0.64	6.58±0.36	7.10±1.26	0.70
	45	6.10^{a}±0.27	6.80^{b}±0.17	0.05	6.55±0.22	7.57±1.21	0.43
	90	6.12±0.43	6.98±0.18	0.09	6.48±0.64	7.76±0.65	0.19
Albumin (g/dl)	0	2.76±0.27	2.32±0.06	0.14	2.12^{a}±0.07	2.32^{b}±0.06	0.03
	45	2.41±0.14	2.19±0.25	0.47	2.41±0.14	2.19±0.25	0.47
	90	2.54±0.37	2.30±0.11	0.71	2.54±0.37	2.39±0.11	0.71
Blood Urea (mg/dl)	0	24.55±1.78	28.13±1.77	0.18	26.29±2.74	27.8±3.02	0.72
	45	28.63±1.74	29.8±1.69	0.64	26.48±2.04	26.58±1.70	0.97
	90	28.87±1.80	24.33±0.51	0.13	27.28±2.13	29.90±2.23	0.42
Calcium (mg/dl)	0	11.65±0.69	11.28 ±0.40	0.65	11.06±0.29	11.32±0.34	0.59
	45	11.30±0.47	11.60±0.42	0.64	11.24±0.30	11.48±0.18	0.50
	90	11.47±0.46	11.33±0.31	0.81	11.17±033	11.23±0.40	0.92
Phosphorus (mg/dl)	0	5.88±0.20	5.78±0.20	0.75	6.13±0.20	6.27±0.10	0.56
	45	5.93±0.22	5.85±0.22	0.62	5.51±0.31	5.71±0.44	0.71
	90	5.79±0.53	5.86±0.26	0.81	5.92±0.55	5.21±0.43	0.34
Alanine aminotransferase (IU/L)	0	35.10±5.14	31.10±0.5	0.52	22.83±3.53	33.45±4.28	0.08
	45	34.00±4.93	36.9±3.87	0.65	26.3±2.92	30.83±4.22	0.40
	90	31.93±3.73	28.16±3.50	0.42	31.16±2.65	27.07±4.91	0.47

Lipid peroxidation nmol	0	360±11.06	335±21.03	0.61	320±15.04	348±23.05	0.73
	90	340±10.55	280±22.05	0.58	360^{a}±21.06	241^{b}±24.03	0.05

Mean values bearing different superscripts varies significantly ($p<0.05$)

In another experiment **,***Emblicaofficinalis* dried fruit (Amla), *Asparagus racemosus* (Shatavari root) and *Menthapiperata* (Mentha leaves) ground to pass through 1 mm sieve and powder was kept in dry place to avoid contact with moisture.The feeding trial was conducted on three male fistulated buffalo calves for a period of twomonths after 14 days of adaptation period. The animals were kept in an open shed withappropriate set up for watering and feeding each animal individually. All fistulated animalswere provided a maintenance ration having 40% concentrate and 60% roughage as per ICAR(2013). Roughage consisted of wheat straw and green fodder. The concentrate mixture containing 17.58% CP and 70% TDN comprised of maize 30%, groundnut cake 15%, mustard cake 20%, wheat bran 32%, mineral mixture 2% and common salt 1% containing + 10-12 kg green fodder + wheat straw ad lib. The ration was supplemented with total 100gm of plant blend (50gm E. officinalis powder + 25gm of A.racemosus powder + 25gm of M. piperata powder). The feeding was done for a period of twomonths. Before feeding and watering, the rumen liquor (RL) samples were collected fromthree rumen fistulated male buffalo calves at 0 hour pre-feeding at 0 day, 30 days and 60 daysfor two consecutive days in air tight flask. Strained rumen liquor (SRL) samples were processed and preserved for further analysis of total nitrogen, ammonia nitrogen and TVFA as per standard protocol.

b. Milk profile and haemato-biochemical studies

Twelve lactating Murrah buffaloes of early lactation stages were selected and divided into control and treatment groups having six in each following a completely randomized design (CRD) from the herd of buffalo farm of the Department of Livestock ProductionManagement of College of Veterinary Sciences, LalaLajpatRai University of Sciences,Hisar. All the experimental Murrah buffaloes of early lactation stage used in the present study were kept under a loose housing system. Control group (n=6) = Standard ration concentrate fed as per ICAR (2013) feeding standards.

Treatment group (n=6) = Standard ration concentrate fed along with 200gram of plant blendmixture. The study was conducted for a period of 3 months, individually for animals taken inearly lactation.

Milk Yield and composition: During the trial, buffaloes were manually milked twice a day at 4:00 A.M. and 4:00P.M. A digital weighing balance was used to record the milk yield, and the weekly averagewas computed and milk yield and milk

composition parameters were analysed as already mentioned above by satandard protocol.

Rumen Fermentation Parameters :The results of rumen fermentation after supplementation*Emblicaofficinalis,,Asparagus racemosus* and *Menthapiperata* powder in diet of fistulated buffalo calveshas been presented in table 17. Ammonia nitrogen is decreased at 30 days(8.80±0.611) and 60 days (8.9±0.430) after feeding of plant based mixture. A nonsignificant difference was observed in total nitrogen at 30 days (73.0±2.35) and 60days (76.0±1.18) after feeding plant mixture.A non significant difference in pH was observed on 30 days (6.43±0.080) and at 60days (6.31 ± 0.047).A non significant difference was observed in total volatile fatty acids at 30 days (92.8±3.99) and at 60 days (99.5 ±5.72) after feeding plant mixture powder.

Table 17: Effect of *E. officinalis, A. racemosus and M. piperata* feeding on rumen fermentation parameters of buffalo calves

		Days		
Parameter	**0 day**	**30 days**	**60 days**	**p-value**
pH	6.46±0.055	6.43±0.080	6.31 ±0.047	0.240
Temperature (ºC)	35.9±0.258	35.8±0.412	35.3± 0.484	0.420
Total volatile Fatty Acids (meq/L)	82.1 ±2.86^{a}	92.8±3.99^{b}	99.5±5.72^{c}	0.04
Ammonia Nitrogen (mg/dl)	9.63±0.568^{c}	8.80±0.611^{a}	8.9±0.430^{b}	0.028
Total Nitrogen (mg/dl)	72.3±1.47	73.0±2.35	76.0 ±1.18	0.300

Mean values bearing different superscripts varies significantly ($p<0.05$)

Table 18: Effect of *E. officinalis, A. racemosus and M. piperata*feeding on milk yield ofMurrah buffaloes under early lactation stage

GroupFortnight	Control	Treatment	p - value
1	4.75±0.23	4.95±0.23	0.551
2	4.85±0.23	5.05±0.23	0.551
3	4.95±0.23	5.35±0.21	0.502
4	4.89±0.21	5.25±0.20	0.351
5	4.85±0.23	5.35±0.22	0.312
6	5.05±0.23	5.45±0.23	0.245

Milk yield and Milk composition

The observations on fortnightly interval milk yield(Table 18) and weekly milk composition were recorded for both (control and treatment) groups during the experimental period of 90 days. At fortnightly intervals, the milk composition was assessed in terms of milk protein, fat, lactose, total solids and solid not fat. The somatic cell count of milk was determined at fortnightly intervals. The values of milk yield ranged from 4.75±0.23 to 5.05±0.23 in control group and from 4.95±0.23

to 5.45±0.23 in treatment group of early lactation stage animals. The results revealed increase in milk yield after feeding plant blend from 3rd fortnight onwards in treatment group and it continued to increase upto 6th fortnight in treatment group as compared to control group. Thus, it can be inferred that the supplementation of plant blend had a promising effect on milk yield. The average fortnighlty milk fat content of buffalo in treatment group was 8.75% which was significantly increased as compared to control group (7.25%) in the early lactation stage. The result revealed a significant increase in milk protein% from 2nd to 3rd fortnight and at 5th fortnight intervals after feeding plant blend in treatment group animals as compared to control group in early lactation stage animals. A non-significant difference has been observedin the milk protein% in the treatment group as compared to control over 4 th and 6 th fortnight intervals after feeding with *E. officinalis*, *A. racemosus* and *M. piperata* blend. The average fortnightly solid not fat (SNF) percent in the early lactation stage increased significantly. Regarding the average milk total solids percentage in buffalo milk the results indicated significant increased in milk solids % in early lactation. The fortnightly average somatic cell count of milk in early stage results indicated non-significant although decrease in treatment group showing better mammary health due to herbal mixture supplementation. Supplementation reduced number of somatic cell counts in milk, indicating low level of intramammary infection due to immuno stimulant and immuno modulatory properties of plant blend mixture. Saponin supplementation has been shown to lower rumen ammonia-N concentration and form protein complexes that protect proteins from degradation in the rumen, ultimately increasing the duodenal flow of microbial nitrogen. Likewise, low levels of tannins can enhance the undegradability of protein in the rumen and improve the efficiency of microbial protein production and its absorption, making more feed protein available after the rumen for production purposes. This could help sustain higher milk protein levels in lactating animals.

Haemato biochemical parameters:

Haemato-biochemical parameters (Hb, TEC, TLC, MCV, MCH, lymphocytes, monocytes, granulocytes, glucose, total protein, albumin, blood urea nitrogen, triglyceride, cholesterol, phosphorus, calcium, AST and ALT) were also analyzed in serum samples at 0, 45 and 90 days. The results (mean ± S.E.) of haematological parameters in two groups of Murrah buffaloes at different intervals have been presented in table 19 .The haemoglobin values (g/dl) ranged from 9.52±0.32 to

9.77±0.18 in control group and from 10.01±0.33 to 11.1±0.33 in treatment group in early lactation stage animals. The results revealed a significant ($p<0.05$)increase in haemoglobin concentration at 45 days and 90 days after feeding the plant blend in treatment group in early lactation as compared to control group. Regarding biochemical parameters glucose concentration was non-significantly although increased ($p<0.05$) in treatment group of early lactation stage at 45 day and 90 day.

Table 19: Effect of *E. officinalis, A. racemosusand M. piperata*feeding on haematologicalparameters of Murrah buffaloes under early lactation stages

Parameters	Days	Control	Treatment	p - value
Haemoglobin (g/dl)	0	9.52 ± 0.32^{A}	10.01 ± 0.33^{B}	0.07
	45	9.72 ± 0.32^{A}	11.1 ± 0.33^{B}	0.007
	90	9.77 ± 0.18^{A}	10.93 ± 0.71^{B}	0.04
TEC (million/mm3)	0	4.40± 0.11	4.66± 0.37	0.586
	45	4.42± 0.12	5.74± 0.32	0.371
	90	4.54 ±0.19	4.58 ±0.34	0.927
MCH (pg)	0	14.22 ±1.97	12.38 ±6.35	0.788
	45	14.7 ±1.63	16.84± 3.39	0.65
	90	13.73± 1.59	16.84 ±3.39	0.095
MCV (fl)	0	36..88± 1.45	36.01± 1.27	0.426
	45	39.86± 1.25	39.48 ±1.34	0.844
	90	36.95± 0.56	38.04 ±1.98	0.359
TLC (10^3/mm3)	0	10.78 ±0.16	10.61± 0.42	0.722
	45	11.58 ±0.31	10.72 ±0.42	0.127
	90	11.34 ±0.58	11.48 ±0.56	0.861
Granulocyte (%)	0	63.13 ± 5.9	63.9 ±5.81	0.944
	45	63.74 ±3.52	58.68 ±3.54	0.335
	90	58.35 ±3.92	63.65 ±4.24	0.483
Lymphocytes (%)	0	26.92 ± 0.87	27.43 ± 4.27	0.36
	45	30.93 ±1.19	36.87 ±2.78	0.078
	90	37.93 ±0.8	36.19 ±3.09	0.270
Monocytes (%)	0	2.37 ± 0.08	2.55 ± 0.26	0.10
	45	2.13 ±0.54	2.67 ±0.29	0.404
	90	2.77±0.21	2.62±0.54	0.171

Mean values bearing different superscripts varies significantly ($p<0.05$)

The shatavari saponins may enhance propionate production, which is generally regarded as a key substrate for gluconeogenesis leading to better blood glucose levels. Adding saponin to the diet significantly enhanced the ruminalVFA profile and increased propionate levels. Total protein concentration increased significantly (p0.05) in treatment group of early lactation at 45 day. The level of lipid peroxidation

is reduced in mid lactation animals due to one of the most potent sources of vitamin C, amla which is rich in amino acids tannins and flavinoids that are known to protect the body against free radicals. Therefore, use of amla powder as an antioxidant can be of practical importance to ameliorate the adverse effect of in buffaloes.

Considering all together, feeding of *E. officinalis, A. racemosus* and *M. piperata* powder is likely to affect the rumen fermentation and is beneficial for improving production performance by increasing milk yield, milk composition as well as having a positive impact on mammary health by lowering somatic cell count without any adverse effect on haemato-biochemical parameters.

RESEARCH & DEVELOPMENT PROJECT

"Expression profiling of microRNA in blood samples from various stages of cancer patients as novel diagnostic and prognostic biomarker for early detection of cancer"

SUBMITTED BY

Dr. Sanjay Kumar

To

HARYANA STATE COUNCIL OF SCIENCE, INNOVATION AND TECHNOLOGY, PANCHKULA

HSCSIT Reference No. HSCSIT/R&D/2021/543 Dated: 05.03.2021

Project Duration: 3-years

Progress report

1) Title of the project
   "Expression profiling of microRNA in blood samples from various stages of cancer patients as novel diagnostic and prognostic biomarker for early detection of cancer"
2) Date of start of project
   5th March 2021
3) Name of principal investigator and address
   Dr. Sanjay Kumar
   Additional Professor
   Room no. 415, B-block, 3rd floor, Department of Biochemistry, Medical College AIIMS Bathinda, Mandi, Dabwali Road, 151001
4) Name of co-investigators (if any)
   Dr. Vivek Kaushal, Sr. Professor & Head, Department of Radiation Oncology, PGIMS, Rohtak
   Dr. Manjit Kaur, Additional Professor & Head, Department of Pathology, AIIMS Bathinda.
5) Place of work
   Department of Biochemistry, 3rd floor, Medical College, AIIMS Bathinda
6) Objectives
   - Collection of blood samples from Breast cancer patients of different stages/grades
   - Isolation of circulating miRNA from serum samples
   - cDNA synthesis of isolated miRNA.
   - miRNA expression profiling via qRT-PCR (Real-Time PCR) to validate the identified target microRNA in breast cancer patients.
7)
8) Time schedule of project indicating year-wise activities and financial requirements.

   1st year
   - Recruitment of JRF
   - Procurement of consumables.
   - Standardizing protocol design for experiments.
   - Institutional Ethical Committee approved.
   - Biosafety Committee approval.
   - Collection of serum samples from Breast cancer patients and healthy individuals.

   2nd year
   - miRNA Isolation from Breast Cancer Patients and Controls
   - c-DNA synthesis
   - Bioinformatic analysis
   - Primer designing
   - qRT-PCR for gene expression analysis and Target miRNA shortlisting
   - Review writing

3rd year

Sample collection followed by miRNAs isolation.

- Comprehensive miRNA profiling
- Validation by tumor sample via qPCR
- Statistical analysis using SPSS to evaluate differences in miRNA expression between cancer patients and healthy individuals.
- To check the sensitivity, specificity, and dynamic range of miRNA quantification in peripheral blood.

9) a) Summary of research work done so far viz-a-viz time schedule

First Year: April 2021 to March 2022

- A questionnaire was designed before collecting samples from breast cancer patients and healthy volunteers. Parameters like sociodemographic profile (name, age, weight, gender, etc.), baseline demographics (Smoker, Former smoker, Alcohol, Tobacco), Staging of cancer, Body Mass Index, Reproductive history, Nutrition status, Family history of cancer, Chemotherapy status, etc. have been taken into consideration in questionnaire form.
- Ethical committee approval.
- Biosafety Committee approval
- Junior Research Fellow position recruitment.
- Purchasing of consumables
- Sample collection started after ethical approval; serum being separated..
- Literature writing is underway in conjunction with protocol standardization.

Second Year: April 2022 to March 2023

- Purchasing of consumables
- Literature on tissue-specific miRNAs were explored (precipitation method) to determine the best approach for isolating miRNAs with the highest yield.
- Sample collection
- Isolation of miRNA from collected serum samples.
- Technique and kits for isolating miRNA were standardized, and experiments were designed accordingly.
- Standardization of the cDNA Synthesis for miRNA enrichment according to experimental design.
- Primer Designing
- The Manuscript writing for review and research article is in progress.

Third Year: April 2023 to March 2024

- Purchasing of consumables
- Continuous sample collection
- Isolation of miRNA from collected serum samples followed by cDNA synthesis.
- Bioinformatic analysis
- Protocol Standardization of Designed Primer
- qRT-PCR of isolated miRNAs
- The Manuscript writing for review and research article is in progress.

Fourth Year: April 2024 to April 2025

- Purchasing of consumables
- Continuous sample collection followed by isolation of miRNA from collected serum samples and then cDNA synthesis.
- qRT-PCR of isolated miRNAs (miR-210, miR-155, miR-21)
- The Manuscript writing for the research article is under progress.
- **COMMUNICATED REVIEW IN ELSEVIER JOURNAL MOLECULAR ASPECTS OF MEDICINE (IF-10.1)**

b) Expected date of completion of the project

22nd April 2025

10) Report of work done so far (please indicate time versus activity schedule)

Activity / Year	First Year	Second Year	Third Year	Fourth Year
Statistical analysis				
qRT-PCR				
c-DNA synthesis				
miRNAs Isolation				
Protocol standardization				
Bioinformatics Analysis				
Sample collection				
Ethical Committee approval				
Manuscript writing and communication				
Purchasing of consumables				
Literature Search				
JRF Recruitment				

11) Staff position

Junior Research Fellow position was sanctioned and recruited.

12) Details of head wise & year wise expenditure

Head	**1st Year**	**2nd Year**	**3rd Year**	**Total (INR)**
Salaries/Wages	4,03,000			4,03,000
Consumable	5,00,000	4,00,000	2,65,000	11,65,000
Travel	20,000	20,000	15,000	55,000
Other Costs/ Contingencies	22,000	20,000	20,000	62,000
Overheads	5,000	5,000	5,000	15,000
Total	9,50,000	4,45,000	3,05,000	**17,00,000**

13) List of equipment purchased (if any)

None

a) Name of equipment
b) Date of receipt
c) Date of installation

14) Difficulties/problems faced in implementation of project and suggestions for remedies.

Due to the COVID-19 pandemic, we were unable to collect samples from patients as the OPD was closed. Even, the PhD registration process was delayed. The purchasing of consumables was also delayed, as the isolation kits, reagents, and chemicals used in the experiments have a short shelf life, and sufficient samples were not available to initiate the experiments.

15) List of publications indicating grant no. of HSCSIT along with reprints related to the project.

The Manuscript is currently under review in **Molecular Aspects of Medicine Journal (IF – 10.1),** and the research article is in progress.

Molecular Aspects of Medicine

Unravelling the Mystery of Hypoxia-induced miRNAs in Breast Cancer: A Molecular Cross-Talk Driving Tumour Progression and Therapy Resistance

--Manuscript Draft--

Manuscript Number:	MAM-D-25-00151
Article Type:	VSI: Cancer and diabetes: Review Articles
Keywords:	Hypoxia; miRNAs; Breast Cancer; Invasion & Metastasis; Angiogenesis; Drug Resistance
Corresponding Author:	Sanjay Kumar, Ph.D. All India Institute of Medical Sciences Bhatinda Bathinda, Punjab INDIA
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	Sapna Marcus, M.D.
	Mahendra Singh, M.D.
	Sharanjot Kaur, Ph.D.
	Nikhil Garg, M.D.
	Akhilesh Pathak, M.D.
	Balachandar Vellingir, Ph.D
Manuscript Region of Origin:	Asia Pacific
Abstract:	Breast cancer (BC) is estimated to be around 2.3 million new cases and 685,000 deaths in 2020 resulting it into the most frequent cancer globally and ranks fifth among mortalities. Numerous studies have established the link between BC and microRNAs

16) Any other relevant information.

(i) Result

- **Clinical information for breast cancer patients**

Clinicopathological factors		Screening Phase (n=65) No.	%	Testing Phase (n=80) No.	%	Validation Phase (n=30) No.	%
Age	>50 (greater)	49	75.3%	53	66.2%	11	36.6%
	<50 (less)	16	24.7%	27	33.8%	3	1%
Menopausal status	Premenopausal	10	15.3%	18	22.5%	2	6.6%
	Postmenopausal	55	84.7%	62	77.5%	12	40%
ER Status	Positive	41	63.6%	57	71.25%	11	36.6%
	Negative	24	36.4%	23	28.75%	3	1%
PR Status	Positive	42	64.6%	57	71.25%	11	36.6%
	Negative	23	35.4%	23	28.75%	3	1%
HER Status	Positive	13	20%	10	12.5%	2	6.67%
	Negative	52	80%	70	87.5%	12	40%
Pathological Subtypes	Luminal A	22	33.8%	31	38.75%	5	16.6%
	Luminal B	20	30.7%	26	32.5%	5	16.6%
	HER 2	13	20%	10	12.5%	2	6.67%
	TNBC	10	15.38%	13	16.25%	1	3.3%
Tumor	0-2 cm	12	18.46%	23	28.75%	-----	----------
	2-5 cm	48	73.84%	48	60%	-- 9	30%
	≥ 5cm	5	7.60%	9	11.25%	2	6.67%

Lymph Node Metastasis	**N0**	**7**	**10.76%**	**3**	**3.75%**	**-----**	**-------**
	N1	**18**	**27.69%**	**28**	**35%**	**3**	**10%**
	N2	**37**	**56.9%**	**49**	**61.25%**	**8**	**26.67%**
	N3	**3**	**4.6%**	**----------**	**---------**	**-----**	**---------**
TNM Stage	**Stage I**	**8**	**12.3%**	**23**	**28.75%**	**-----**	**------**
	Stage II	**39**	**60%**	**47**	**58.75%**	**9**	**30%**
	Stage III	**15**	**23.07%**	**10**	**12.5%**	**2**	**6.67%**
	Stage IV	**3**	**4.6%**	**-------**	**------**	**-----**	**---------**
Histological Grade	**Grade 1**	**9**	**13.8%**	**9**	**11.25%**	**2**	**6.67%**
	Grade 2	**40**	**61.5%**	**52**	**65%**	**12**	**40%**
	Grade 3	**16**	**24.6%**	**19**	**23.75%**	**-----**	**-------**

Table 1: Clinicopathological characteristics of breast cancer patients

- **Dysregulated miRNA in Breast Cancer – miRNet 2.0**

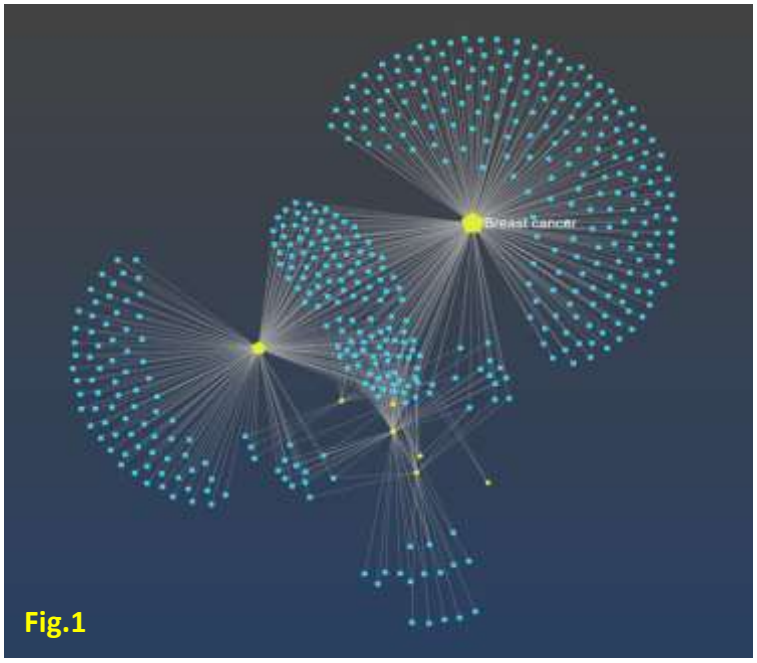


Fig. 1 Construction and analysis of the miRNA target networks for Breast cancer disease model. The interactome of miRNA (blue in color) targeted in Breast cancer disease (Hereditary BC, TNBC, Early-stage BC, Breast carcinoma represented in yellow) using miRNet 2.0 bioinformatics software. The resulting processes found common cluster of miRNAs.

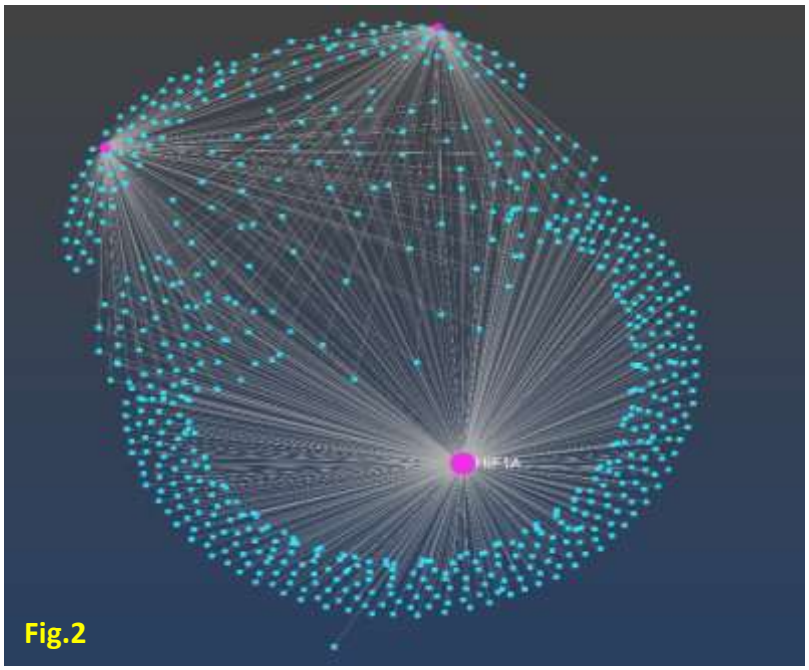
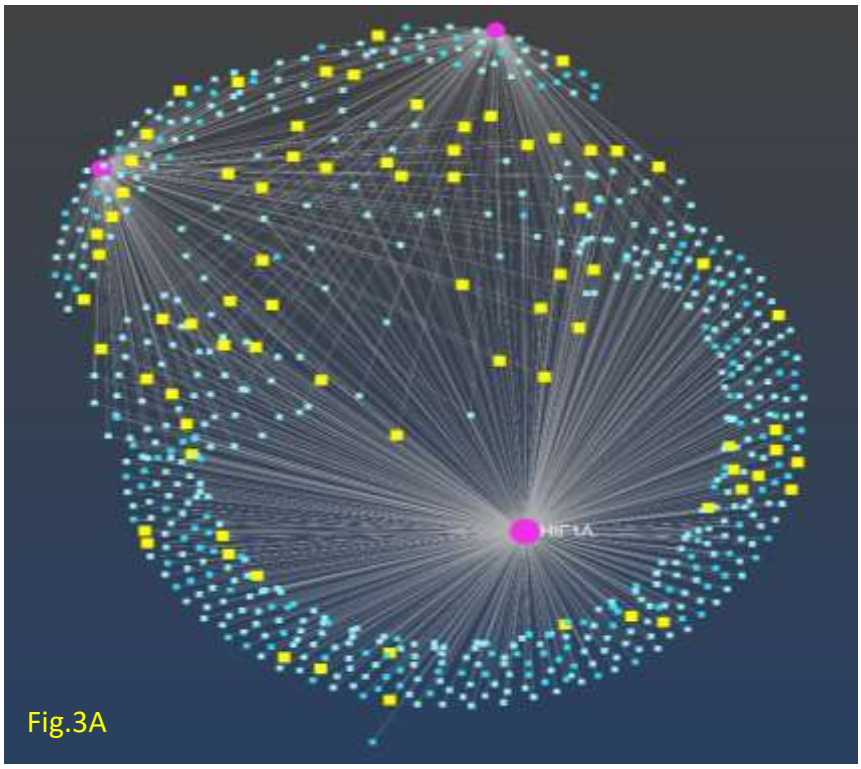


Fig. 2 The network of the miRNA- targeted genes (HIF-1α, BRCA 1, BRCA 2) using miRNet 2.0 bioinformatics software. The interactome results in a common cluster of miRNAs reported in different disease and tissue. miRNA are represented blue in color and targeted genes are in pink



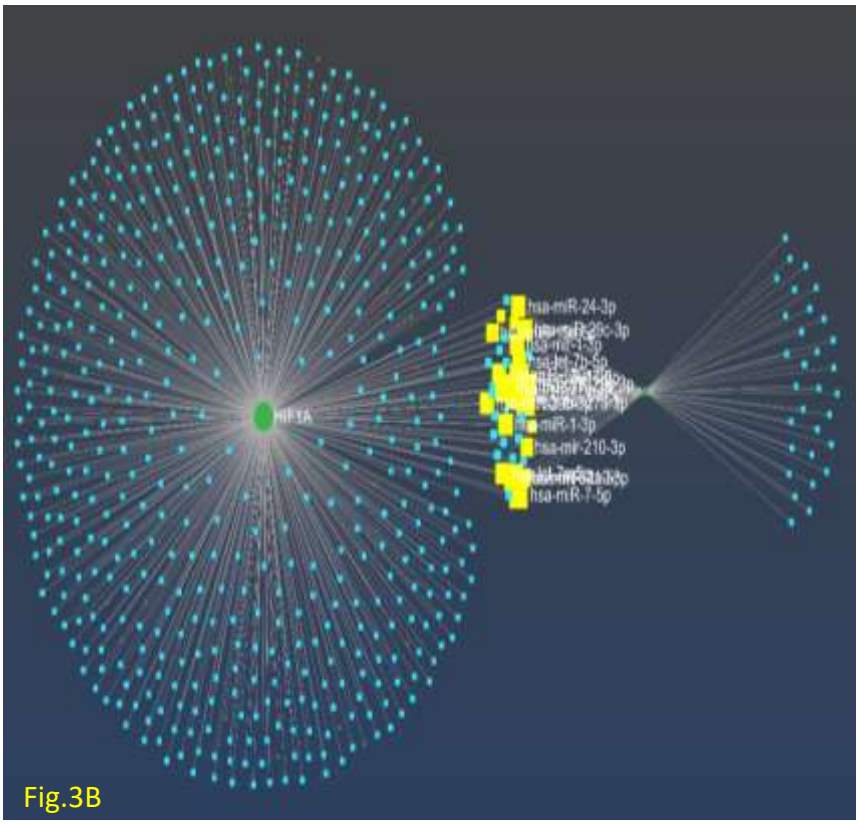


Fig. 3 The gene-target miRNA network using miRNet 2.0 Bioinformatics software.

A. highlighting oncomiRNAs regulated by HIF-1α, BRCA1 and BRCA2 in breast cancer.

B. highlighting oncomiRNAs regulated by HIF-1α and HIF-2α. in breast cancer

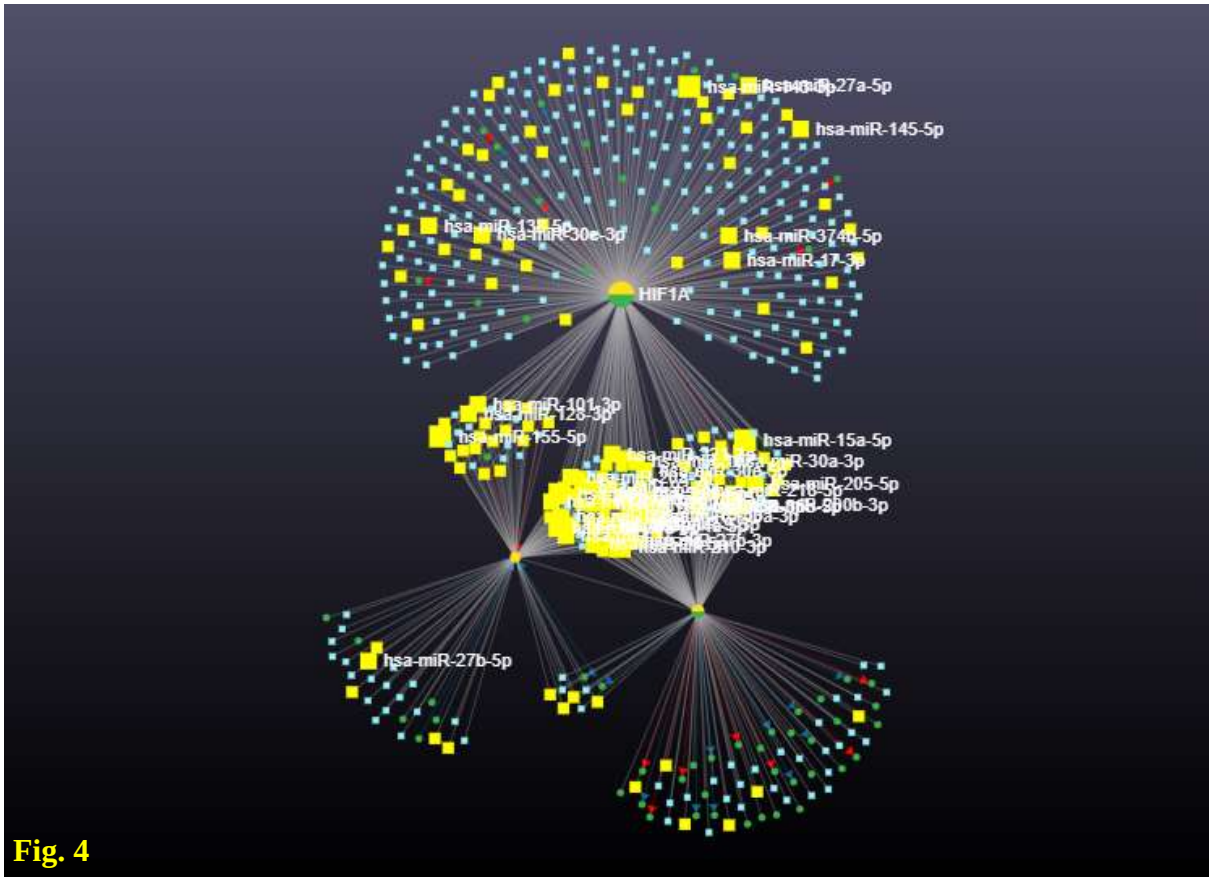


Fig. 4. This network shows the interaction between HIF 1 alpha and HIF 2 alpha genes regulating miRNAs. only the Yellow highlighted miRNAs are oncomiRs involved in Breast cancer

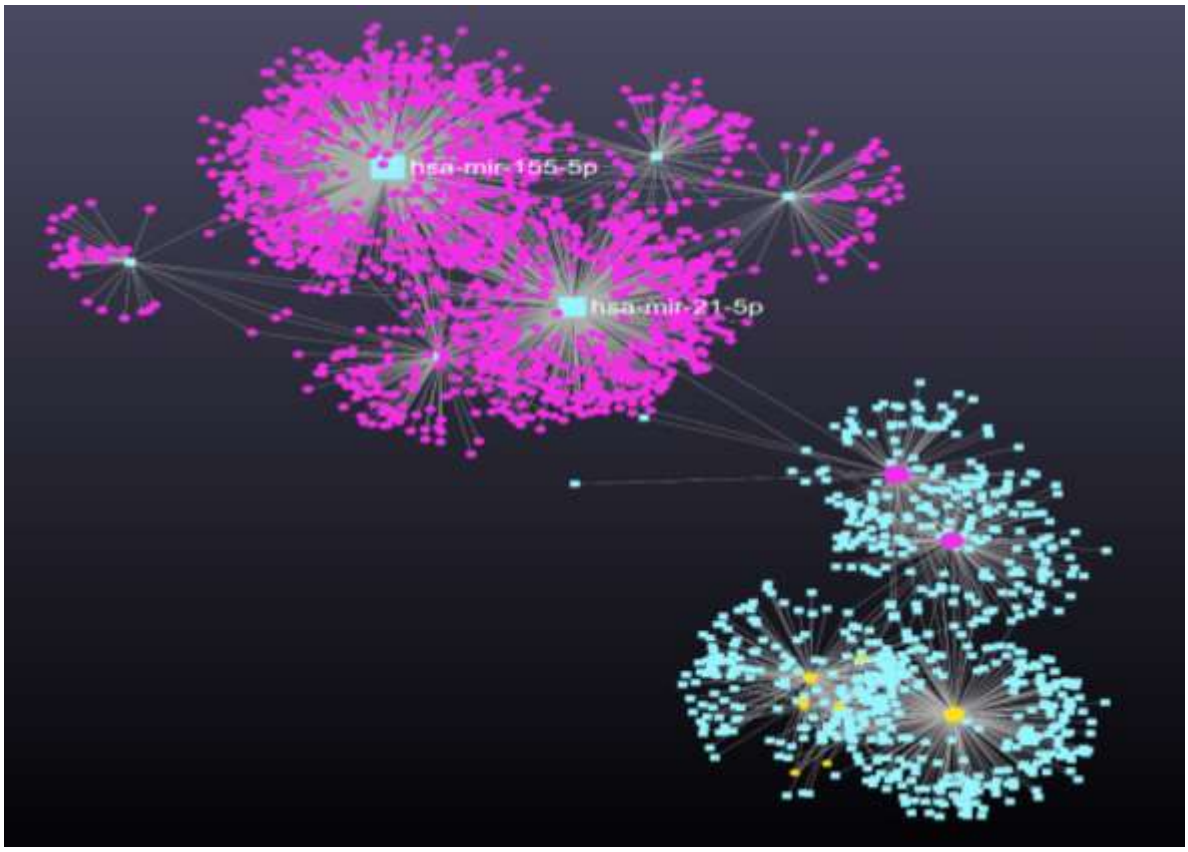


Fig. 5 The hypoxamiR-gene target (directly) network highlighted in sub-set. Pink highlights miR-155-5p regulated genes and blue depicts miR-210-3p & miR-21-3p-regulated genes.

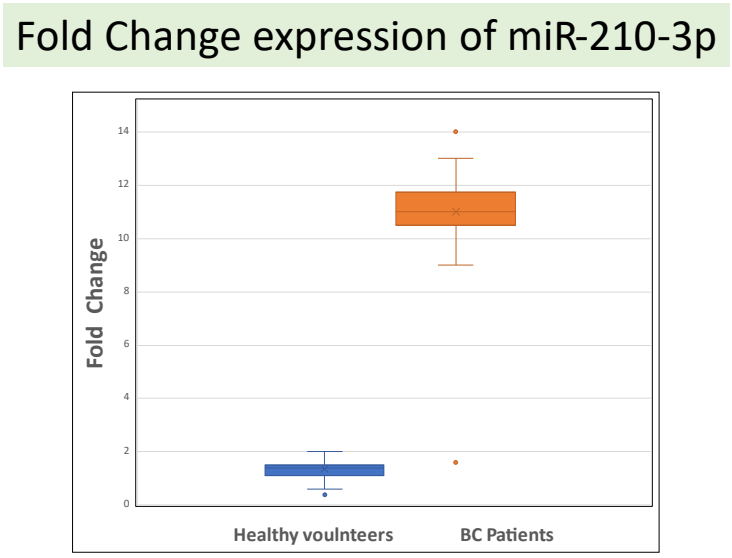


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Fig. 6 Box plots of the circulating miR -210-3p expression levels in BC patients compared to healthy volunteers . Circulating miR-210-3p significantly upregulates (*P*=0.002) . The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively.

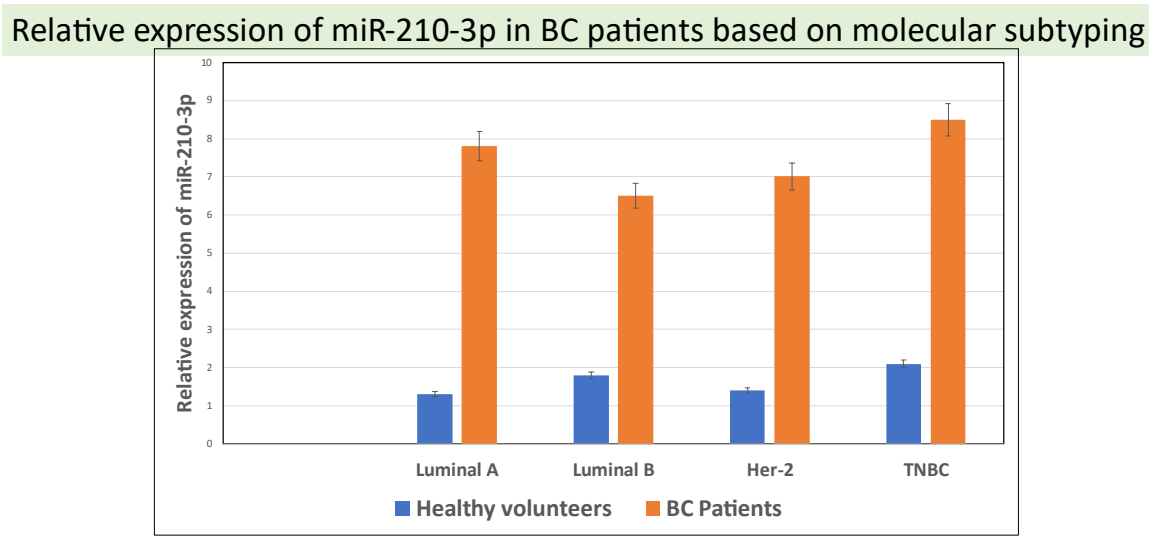


Fig. 7 Relative expression of miR-210-3p according to breast cancer subtype. Each bar represents relative miR-210-3p expression in comparison to healthy volunteers in subtypes of breast cancer: A, Luminal (estrogen receptor [ER]+, progesterone receptor [PR]+ and human epidermal growth receptor [HER-2]-). B, HER-2 (ER-, PR-, and HER-2+). C, Triple-negative breast cancer (ER-, PR-, and HER-2-)

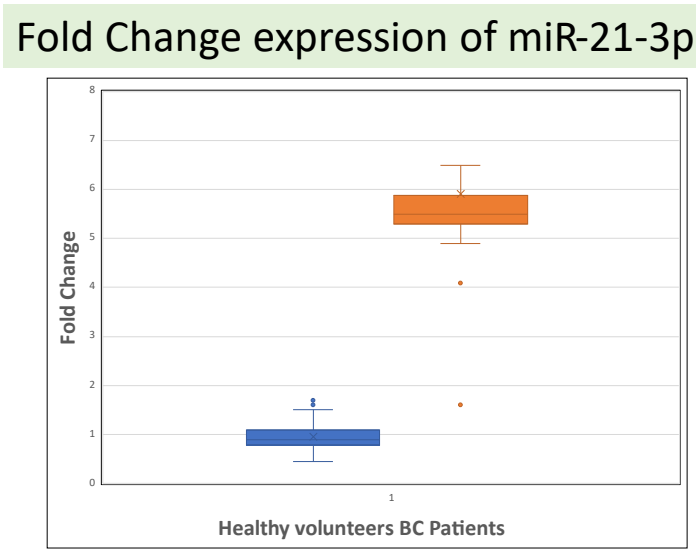


Fig. 8 Box plots of the circulating miR-21-3p expression levels in BC patients compared to healthy volunteers. Circulating miR-21-3p significantly upregulates (*P*=0.001) . The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively.

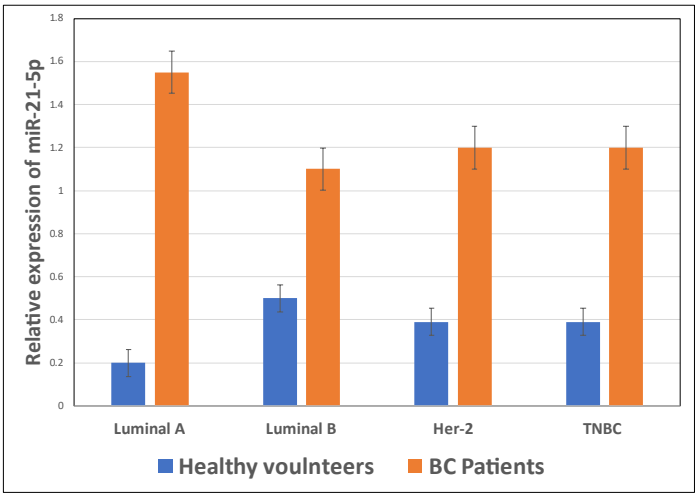


Fig. 9 Relative expression of miR-21-3p according to breast cancer subtype. Each bar represents relative miR-210-3p expression in comparison to healthy volunteers in subtypes of breast cancer: A, Luminal (estrogen receptor [ER]+, progesterone receptor [PR]+ and human epidermal growth receptor [HER -2]-). B, HER-2 (ER-, PR-, and HER-2+). C, Triple-negative breast cancer (ER -, PR-, and HER-2-)

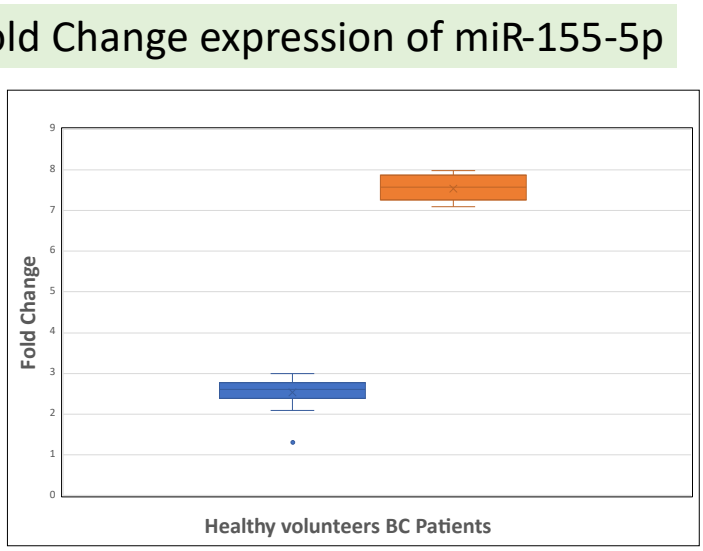


Fig. 10 Box plots of the circulating miR-155-5p expression levels in BC patients compared to healthy volunteers. Circulating miR-155-5p significantly upregulates (*P*=0.006). The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively.

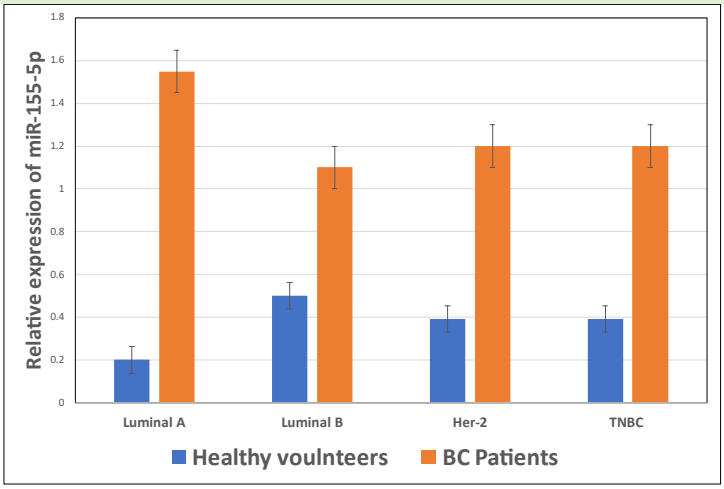
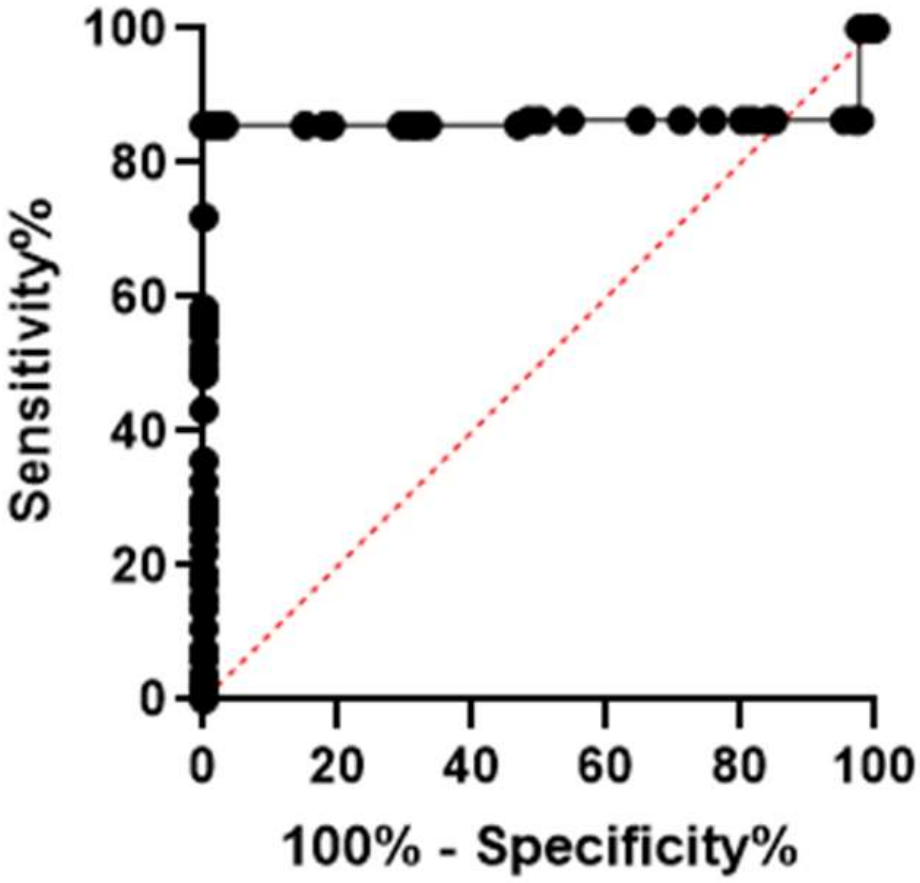


Fig. 11 Relative expression of miR-155-5p according to breast cancer subtype. Each bar represents relative miR-210-3p expression in comparison to healthy volunteers in subtypes of breast cancer: A, Luminal (estrogen receptor [ER]+, progesterone receptor [PR]+ and human epidermal growth receptor [HER -2]-). B, HER-2 (ER-, PR-, and HER-2+). C, Triple-negative breast cancer (ER -, PR-, and HER-2-)



Area under the ROC curve	
Area	0.8631
Std. Error	0.02935
95% confidence interval	0.8056 to 0.9206
P value	<0.0001

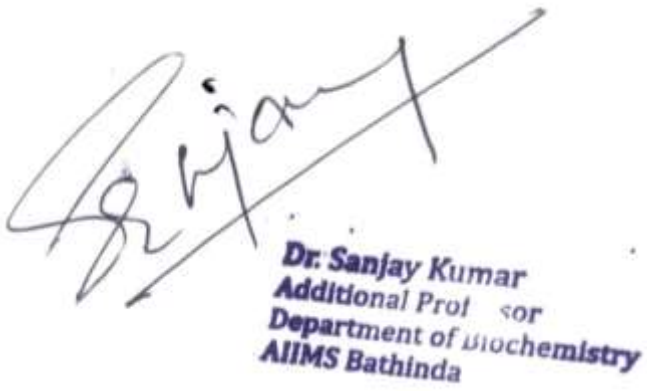
Fig. 12 Receiver Operating characteristic (ROC) curve analysis of the panel of miRNAs with Area, Standard Error, 95% confidence interval and p value.

(ii) <u>Submitted Data in National and International Conferences:-</u>

Type of Abstract Submission	Abstract Title	Abstract no.	Conference Name	
Poster Presentation	Expression Analysis of Hypoxia-induced MicroRNA of Breast Cancer Patients as Novel Diagnostic and Prognostic Biomarker for Early Detection of Cancer	EACR25-3010	**European Association for Cancer Research**	Selected
Oral Presentation	Expression Profiling of Hypoxia-regulated MicroRNA in Breast Cancer Patients at Various Stages as Novel Diagnostic and Prognostic Biomarker for Early Detection of Cancer	9906-Abstract	**Cancer R&D-2025 Boston**	Selected
Poster Presentation	Expression Profiling of Hypoxia-induced MicroRNA from Various Stages of Breast Cancer Patients as Novel Diagnostic Biomarker for Early Detection of Cancer		**3rd International Conference on Cancer and Breast Cancer Therapy in Dubai, UAE.**	Selected
Conference (Selected Abstract)	-	-	**Selection for the Young Travel Scientist Award and the Clinical Multi-Omics (Proteomics and Metabolomics) & Big Data Analytics workshop-APT 2025 in IIT Mumbai**	Selected

(iii)<u>Enrolled 3 PhD scholars</u>

(iv) <u>Trained 12 students</u>



Date: - 18.06.2025

Place: - Department of Biochemistry, AIIMS Bathinda

Signature of PI