Vol. 78 No.2, 2025
Impact Factor:0.2

ISSN 0019-5146 (Print)
ISSN 2454-2172 (Online)

INDIAN JOURNAL of DAIRY SCIENCE

an IDA publication

Regd. No. 15665/68
Covered by Clarivate Analytics Services: Emerging Sources Citation Index
https://mjl.clarivate.com/search-results

INDIAN JOURNAL OF DAIRY SCIENCE **MARCH-APRIL VOL.78, NO.2, 2025**

Contents

ISSN 0019-5146 (Print)
ISSN 2454-2172 (Online)

CENTRAL OFFICE : Indian Dairy Association, IDA House, Sector IV, R.K. Puram, New Delhi-110022. Phones: 011-26170781, 26165237, 26165355. Email: idahq@rediffmail.com /www.indiandairyassociation.org

ZONAL BRANCHES & CHAPTERS : **South Zone:** Dr. Satish Kulkarni, Chairman, IDA House, NDRI Campus, Adugodi, Bangalore-560 030. Ph.: 080-25710661 Fax: 080-25710161. **West Zone:** Dr.J.B. Prajapati, Chairman; A-501, Dynasty Business Park, Andheri-Kurla Road, Andheri (East), Mumbai 400059 Email: chairman@idawz.org / secretary@idawz.org Ph.: 91 22 49784009 **North Zone :** Dr. Rahul Saxena, Chairman; c/o IDA House, Sector IV, R.K. Puram, New Delhi - 110 022 Phones: 011-26170781, 26165355. **East Zone:** Shri Sudhir Kumar Singh, Chairman, c/o NDDB, Block-DK, Sector-II, Salt Lake City, Kolkata-700 091 Phones: 033-23591884-7. **Gujarat State Chapter:** Dr. Amit Moolchand Vyas, Chairman; c/o SMC College of Dairy Science, AAU Campus, Anand-388110 Gujarat. Email: idagscac@gmail.com **Kerala State Chapter:** Dr. S.N. Rajakumar, Chairman; c/o Prof. and Head, KVASU Dairy Plant, Mannuthy, E mail: idakeralachapter@gmail.com **Rajasthan State Chapter:** Chairman; 418-419, Fourth Floor, Sunny Mart, New Atish Market, Jaipur-302 020 E-mail: idarajchapter@yahoo.com **Punjab State Chapter:** Dr. Inderjit Singh, Chairman, H.No. 1620, Sector-80, SAS Nagar, Mohali-140 308 (Punjab) Email: secretaryidapb2023@gmail.com **Bihar State Chapter:** Shri D.K. Srivastava, Chairman; c/o Former Managing Director, Mithila Milk Union; House No. 16 Mangalam Enclave, Baily Road, Near Saguna SBI, Patna-801503 (Bihar). E-mail: idabihar2019@gmail.com **Haryana State Chapter:** Dr. S.K. Kanawjia, Chairman; c/o D.T. Division, NDRI, Karnal-132 001 (Haryana). Ph.: 09896782850 Email: skkanawjia@rediffmail.com. **Tamil Nadu State Chapter:** Shri Kanna K.S., Chairman; c/o Department of Dairy Science, Madras Veterinary College, Vepery, Chennai-600007. **Andhra Pradesh Local Chapter:** Prof. Ravi Kumar Sreebhashyam, Chairman; c/o College of Dairy Technology, Sri Venkateshwara Veterinary University, Thirupathi - 517502 Email: idaap2020@gmail.com **Eastern UP Local Chapter:** Dr. Arvind, Chairman; Assistant Professor, Department of Dairy Science & Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221005 Ph.: 7007314450 Email: arvind1@bhu.ac.in **Western UP Local Chapter:** Dr. Ashok Kumar Tripathi, Chairman; c/o Flat no. 1003/8, Zen Spire, Ramprastha Greens, Vaishali, Ghaziabad-201010 (UP). Email: ttreddy@arvinddairy.com **Jharkhand Local Chapter:** Shri Pavan Kumar Marwah, Chairman; c/o Jharkhand Milk Federation, FTC Complex, Dhurwa Sector-2, Ranchi, Jharkhand-834004 Email: jharkhandida@gmail.com **Telangana Local Chapter:** Shri Rajeshwar Rao Chalimeda, Chairman; c/o Dodla Dairy Ltd Corporate Office, # 8-2-293/82/A, 270/Q, Road No 10-C, Jubilee Hills, Hyderabad – 500 033 Telangana. **Karnataka State Chapter:** Shri N.B. Marathe, Chairman; c/o Dairy Science College, Mahagaon Cross, Kalaburagi-585316. Mobile: 9483124271 Email: ida.kar.chapter@gmail.com

---

Printed and published by Shri Hariom Gulati and edited by Dr. (Mrs.) Bimlesh Mann on behalf of the Indian Dairy Association and printed at National Printers, B-56, Naraina Industrial Area, Phase II, New Delhi and published at IDA House, Sector-IV, R.K. Puram, New Delhi-110022.

Indian J Dairy Sci 78(2): 103-113
https://doi.org/10.33785/IJDS.2025.v78i02.001

RESEARCH ARTICLE

Storage related changes in Pizza cheese as affected using Saccharomyces *boulardii* adjunct culture

Ankit Bihola[1](✉), Atanu H. Jana[2], Satish C. Parmar[3], Bhargav Rajani[4] and Shaikh Adil[5]

Received: 10 January 2024 / Accepted: 21 November 2024 / Published online: 23 April 2025

Abstract: The investigation examined the storage related changes in Pizza cheeses for a period of 28 days at 4°C. The physicochemical properties [i.e. moisture, titratable acidity (TA), pH, soluble nitrogen (SN), total volatile fatty acids (TVFA)] were markedly affected by temperature (T), period (P) and their interaction T×P. The TA, SN and TVFA exhibited marked increases with the advancement of the storage period. The sensory scores differed markedly for cheeses amongst each other with regard to T and P, except for stringiness. The stringiness score was markedly affected by T, P and their interaction T×P. Microbial analysis revealed the absence of coliforms in fresh as well as stored cheeses. The *S. boulardii* and LAB counts were markedly affected by T, P and their interaction T×P. The *S. boulardii* and LAB counts of all the cheeses tended to show a gradual decrease during the storage period. Cheese CBHM was associated with a higher *S. boulardii* count and elicited the desired baking and sensory characteristics when judged as topping on pizza pie. Since storage beyond 14 days led to a reasonable decline in the count of *S. boulardii*, only 2 weeks of refrigerated storage is advocated to reap the health benefits accrued from the consumption of such cheese containing probiotic microbe.

Keywords: Pizza cheese, *Saccharomyces boulardii*, Cheddaring, Homogenized milk, Refrigerated storage

[1]Dairy Technology Division, ICAR-National Dairy Research Institute, Karnal, Haryana
[2]SMC College of Dairy Science, Kamdhenu University, Anand, Gujarat
[3]Dairy Chemistry Department, SMC College of Dairy Science, Kamdhenu University, Anand, Gujarat
[3]Banas Dairy, Palanpur, Gujarat
[4]Dairy Technology Department, Parul Institute of Technology, Parul University, Vadodara, Gujarat

(✉)E-mail: ankitbihola2111@gmail.com

Abbreviations

CBHM – Cheese from 'milk blend' comprising of homogenized and unhomogenized milks (1:1, w/w)
CFU – Colony Forming Unit
CUM (C1) – Cheese from unhomogenized milk
CUMY (C2) – Cheese from unhomogenized milk with *S. boulardii* (yeast) culture
FCRD – Factorial Completely Randomized Design
FDM – Fat-on-Dry Matter
IMCU – International Milk Clotting Units
LAB – Lactic Acid Bacteria
P – Period
SC – Starter Culture
SN – Soluble nitrogen
T – Treatment
T × P – Interaction of Treatment with Period
TA – Titratable Acidity
TVFA – Total Volatile Fatty Acids

Introduction

Mozzarella cheese (particularly the Pizza cheese type, also known as low-moisture part-skim Mozzarella) is the preferred choice with regard to its application as a topping on pizza pie (Bihola et al. 2024a). Historically, the production of Mozzarella cheese utilized the traditional technique employing "Starter Culture" (SC). Various researchers have prepared Mozzarella/Pizza cheese employing the SC technique utilizing adjunct cultures such as *Lactobacillus acidophilus* and *Lacticaseibacillus rhamnosus* (Ortakci et al. 2012; Cuffia *et al.* 2017; Akarca and Yildirim 2022). There is lack of information on the manufacture of Pizza cheese using *Saccharomyces boulardii* (a proven probiotic yeast) as an adjunct culture. *Saccharomyces boulardii is a* well-known thermotolerant probiotic yeast that grows well at 37°C (Bihola et al. 2024c, Bihola et al. 2024d).

Clinical trials by Unique Biotech revealed that *S. boulardii* (unique 28 strain) could relieve the symptoms of diarrhoea and other intestinal problems, including traveller's diarrhoea and Irritable Bowel Syndrome with constipation and even prevented gastrointestinal infections (Unique Biotech 2023). *S. boulardii*

has found application in ice cream as well as in cultured dairy products such as yoghurt, kefir, buttermilk and cheese. When consumed at the recommended dose (minimum 10^7 CFU/g or mL), *S. boulardii* had a positive impact on the health of host. Improvement in the gut flora, immunological modulation, avoidance of enteric infections, diarrhoea and inflammatory bowel disease are some of the advantages gained when consuming food products containing *S. boulardii* (Staniszewski and Kordowska-Wiater 2021, Bihola et al. 2024b).

S. boulardii showed antidiarrheal activity by restoring the normal balance of microorganisms in the intestines. Such organism inhibited bacterial toxins from binding with the intestinal cells and neutralizing them prior to its absorption. *S. boulardii* improved the digestive enzyme activity and strengthened the integrity of epithelial cells of the intestine, preventing infection from spreading to other parts of the body. The immune system gets augmented in the presence of *S. boulardii* in the digestive tract. Such effect took place through stimulation of T-cells and macrophages as well as greater killer cell activity (Unique Biotech 2023).

Hence, the investigation was carried out to study the storage related changes in Pizza cheeses prepared using *S. boulardii* as an adjunct starter through technological interventions in order to obtain the desired viable counts. This research generated helpful data for cheesemakers who intend to produce Pizza cheese made using lactic acid bacteria (LAB) along with *S. boulardii* as an adjunct culture.

Materials and Methods

Materials

Chilled, mixed (i.e. buffalo and cow) milk was purchased from Vidya Dairy, Anand. The milk was separated at Anubhav Dairy, Anand to produce skim milk and cream. The cheese milk was standardized employing freshly separated skim milk. A fungal rennet from *Rhizomucor miehei* (strength of 2400 IMCU/g) was obtained from M/s. Caglificio Clerici, Cadorago, Italy and used as the milk coagulant. *Saccharomyces boulardii* unique 28, sourced from M/s. Unique Biotech, Hyderabad was used as an adjunct culture in Pizza cheese making. Starter cultures (i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) were obtained from M/s. DSM, Netherlands. Calcium chloride, dihydrate was purchased from M/s. Loba Chemie Pvt. Ltd., Mumbai and added to the cheese milk. Tata brand vacuum-evaporated common salt (NaCl) was used for salting the cheese.

Equipment

Kenstar 3D Power OM-34ECR baking oven was used to bake the cheese-topped pizza pie. An Infrared Thermometer (model No. GIS-500, Bosch, Bengaluru) was used to note the temperature of the plasticized cheese mass during cheese making.

Pizza Cheese Making

Pizza cheese was prepared from a 'milk blend' [i.e. unhomogenized and homogenized (1.96 and 0.98 MPa pressure, 65ºC temperature) milks - 1:1, w/w; 3.2% milk fat] following the SC method of Patel (2022) using *S. boulardii* as an adjunct culture (cheese designated as CBHM). CBHM was prepared by inoculating cheese milk with *S. boulardii* culture adjunct at a level of 3.5 g/100 kg milk and keeping salt (NaCl) levels at 1.75% by weight of the cheese curd during dry salting (Bihola et al. 2024c).

Control Pizza cheeses, with and without inclusion of *S. boulardii* adjunct culture, were prepared from standardized (3.2% milk fat), pasteurized (72°C/15 s) mixed milk as per the process of Patel et al. (1986) and Rajani et al. (2024a) employing the SC method (moulding water temperature was 95±3°C).

The *S. boulardii* (unique 28) adjunct culture was incorporated into cheese milk during Pizza cheese making [in control (C2) and experimental] and after preincubation of such culture in a sufficient quantity of milk (i.e. ~ 500 mL) at 25°C for 6 h in a thermostatically controlled incubator (Bihola et al. 2024b, Bihola et al. 2024c).

Analyses

The moisture content and titratable acidity (TA) of Pizza cheeses were determined employing standard procedures (AOAC 2023). The pH of cheese slurry made in distilled water was measured using a digital pH meter (M/s. Mettler Toledo AG, Schwerzenbach). The soluble nitrogen content of fresh and stored cheese samples was determined using the procedure of Mamo (2017). Total volatile fatty acids (TVFA) were measured using the procedure of Kosikowski and Dahlberg (1946). The LAB and *S. boulardii* counts of Pizza cheese were determined using the methods described by ISO (1998) and Niamah et al. (2017) respectively. The coliform count of the cheese samples was determined (BIS 1964).

Refrigerated storage of Pizza cheese

Pizza cheeses were vacuum-packed in polyethylene bags (~ 80.0 µm thick) and stored in a refrigerator maintained at 7±1°C until they were organoleptically acceptable (for up to 21 days). The storage stability of Pizza cheeses was evaluated in terms of the changes occurring in their physico-chemical properties (i.e. moisture, TA, pH, SN and TVFA); sensory evaluation of cheese as pizza topping (at 7^{th}, 14^{th} and 21^{st} day of storage) and microbial count [i.e. Lactic Acid Bacteria count (LAB), *S. boulardii* count] during refrigerated storage. Analyses were performed every 7 days for 28 days of refrigerated storage. Coliform count was

performed for freshly prepared cheese (i.e. 0 day) and cheese stored for 21 days only.

Statistical Analysis

A factorial completely randomized design (FCRD) was applied to statistically evaluate the findings obtained in the investigation. The averages of the results of the investigation of duplicate samples of pizza cheese, obtained in four separate replications for three treatments were examined by statistical examination employing FCRD (Steel and Torrie 1980).

Results and Discussion

Temperature of Plasticized Cheese Mass

It is obvious that the cheese produced from milk blend (unhomogenized: homogenized, 1:1 w/w), the homogenized milk portion possesses a greater amount of protein adsorbed onto the increased fat surface area. Additionally, the pH of curd at stretching (i.e. whey acidity at stretching stage of curd was 0.44% LA) was considerably lower than for control cheese (Jana and Upadhyay, 1993). Both these factors led to cheese curds getting plasticized at a much lower temperature for cheese CBHM.

Based on the plasticizing conditions adopted in preparing control cheeses (i.e. C1, C2) and cheese CBHM, the temperature of the plasticized cheese mass was 63.6°C and 59.5°C respectively. The plasticizing conditions for the cheeses, in the same order as specified above, were 93.5°C temperature with contact period with cheese curd of 4.5 min. (for both control cheeses) and 79.0°C with contact period of 2.5 min. respectively (Bihola et al. 2024b, Bihola et al. 2024c, Bihola et al. 2024c).

Changes Occurring in Pizza Cheeses During Refrigerated Storage

Fresh mozzarella cheese is not considered suitable for its application on pizza because it melts into a tough, rubbery and grainy consistency, exhibiting limited stretch (Jana and Mandal 2011). The refrigerated storage of Pizza cheese, over a period of approximately 2 weeks, is reported to bring about desirable changes (i.e. mellowness, melt and stretch), as well as simultaneous unwanted changes (i.e. shred, fat leakage and sliminess), most of which are dictated by proteolytic activity. Enzymes from milk, starter culture, non-starter flora and rennet all contribute to the proteolytic changes in pizza cheese during refrigerated storage (Jana and Tagalpallewar 2017). Storage changes can have a significant impact on the survival of desired microbes (such as LAB and probiotics) and the end-use applications of cheese, such as its application as a pizza topping.

The end use application of Pizza cheese (fresh or stored) on pizza pie was conducted only from the 7th day until two more times, at an interval of one week (i.e. till 21st day of storage). This was followed deliberately, since freshly prepared Pizza cheese does not behave satisfactorily when used as a topping on pizza pie (Jana and Mandal 2011). Refrigerated storage of the cheese beyond 21 days posed problem related to ease of shredding; in some instances, an unpleasant flavour was perceived and subsequent storage sometimes showed visible mold on cheese surface. The pertinent results related to the changes in the quality characteristics of Pizza cheeses are described in this research investigation.

Physicochemical Changes in Cheese

Moisture

The moisture content of the cheeses gradually decreased throughout the storage period of 28 days. The changes in the moisture content of cheeses were significantly ($p<0.05$) affected by the treatment (T), storage period (P) and their interaction i.e. T x P. All the three cheeses differed markedly ($p<0.05$) from each other. Cheeses CBHM and C1 were associated with the maximum and minimum moisture content respectively. During storage, the first significant ($p<0.05$) drop in the moisture content of cheeses was noted on the 14th day; a marked decline took place again on the 28th day of refrigerated storage (Table 1).

Moisture loss during refrigerated storage of Mozzarella cheese is a usual feature. A reduction in the moisture content (loss of 4.2%) of Mozzarella cheese has been reported during refrigerated storage (7°C) of one month (Felfoul et al. 2018). Rajani et al. (2021b) also noted a gradual decline in the moisture content of Pizza cheeses during refrigerated storage (7°C); the moisture content of cheeses was 51.75, 51.39 and 51.05% at storage periods of 0, 2 and 4 weeks, respectively.

Titratable Acidity

There was a progressive increase in the titratable acidity (TA) of all the cheeses during storage. The changes in the TA of cheeses were markedly affected by T, P and interaction T x P. All the cheeses differed markedly ($p<0.05$) from each other; maximum (i.e. 0.67% LA) and minimum (i.e. 0.48% LA) values were associated with cheeses CBHM and C1 respectively. There was a linear increase in the TA of cheeses during the advancement of storage; such a marked ($p<0.05$) increase in the TA of cheeses was first noted on the 7th day and subsequently on the 14th and 28th days of storage (Table 1).

The TA of Mozzarella cheeses prepared employing SC method was 0.78 and 0.90% LA at storage (4°C) periods of 2 and 4 weeks respectively; freshly prepared cheese had 0.68% LA (Ahmed et al. 2011). Abd El-Gawad et al. (2012) reported a progressive rise in the TA values of Mozzarella cheeses made using SC method from homogenized (2.45 MPa) and unhomogenized milks (3.0% fat for both) during storage at 4°C. The TA value (as % LA) of homogenized milk cheese was 0.67 and 0.76 when fresh and at 4

weeks of storage respectively; the TA value of cheese from unhomogenized milk was 0.64 and 0.72 respectively at the same period stated above.

pH

There was a progressive decrease in the pH of all the cheeses throughout storage (i.e. up to 28th the day). The changes in the pH of cheeses differed significantly ($p<0.05$) with respect to T, P and interaction T x P. There was a marked drop in the pH of cheeses, at each 7 days interval of storage, up to 28th day. The pH of all the cheeses differed significantly ($p<0.05$) from each other; maximum and minimum pH was associated with cheeses C1 (i.e. 5.26) and CBHM (i.e. 4.97) respectively (Table 1).

Several researchers have reported a steady decline in the pH of Mozzarella cheeses during their refrigerated storage of one month (Felfoul et al. 2018). During ageing, the cheese undergoes several types of microbiological and biochemical changes, involving proteolysis, lipolysis with simultaneous conversion of remaining

Table 1: Changes in the physico-chemical properties of Pizza cheeses during refrigerated storage (4°C)

Cheeses	Storage Period (Days)					Mean (Treatment)
	0	7	14	21	28	
			Moisture (%)			
CUM (C1)	48.88^{f}	48.61^{h}	48.41^{i}	48.22^{jk}	48.13^{k}	48.45^{c}
CUMY (C2)	49.03^{e}	48.77^{g}	48.56^{h}	48.37^{i}	48.24^{j}	48.60^{b}
CBHM	52.30^{a}	52.10^{b}	51.95^{c}	51.86^{c}	51.80^{d}	52.00^{a}
Mean (Period)	50.07^{a}	49.82^{b}	49.64^{c}	49.48^{d}	49.39^{e}	---
Source of Variation	Treatment (T)		Period (P)			T x P
SEm±	0.018		0.023			0.039
			Titratable Acidity (% LA)			
CUM (C1)	0.46^{j}	0.47^{ij}	0.48^{i}	0.49^{h}	0.50^{h}	0.48^{c}
CUMY (C2)	0.56^{g}	0.57^{g}	0.57^{g}	0.59^{f}	0.61^{e}	0.58^{b}
CBHM	0.62^{e}	0.65^{d}	0.67^{c}	0.70^{b}	0.72^{a}	0.67^{a}
Mean (Period)	0.55^{d}	0.56^{d}	0.58^{c}	0.59^{bc}	0.61^{a}	---
Source of Variation	Treatment (T)		Period (P)			T x P
SEm±	0.003		0.004			0.007
			pH			
CUM (C1)	5.31^{a}	5.27^{b}	5.26^{b}	5.23^{c}	5.21^{d}	5.26^{a}
CUMY (C2)	5.24^{c}	5.20^{d}	5.16^{e}	5.08^{g}	4.94^{i}	5.12^{b}
CBHM	5.13^{f}	5.05^{h}	4.95^{i}	4.90^{j}	4.83^{k}	4.97^{c}
Mean (Period)	5.23^{a}	5.18^{b}	5.12^{c}	5.07^{d}	5.00^{e}	---
Source of Variation	Treatment (T)		Period (P)			T x P
SEm±	0.003		0.004			0.006
			Soluble Nitrogen (%)			
CUM (C1)	0.208^{j}	0.224^{i}	0.240^{h}	0.253^{g}	0.261^{f}	0.237^{c}
CUMY (C2)	0.237^{h}	0.250^{g}	0.273^{e}	0.295^{d}	0.330^{c}	0.277^{b}
CBHM	0.268^{e}	0.300^{d}	0.332^{c}	0.362^{b}	0.392^{a}	0.331^{a}
Mean (Period)	0.238^{e}	0.258^{d}	0.282^{c}	0.304^{b}	0.328^{a}	---
Source of Variation	Treatment (T)		Period (P)			T x P
SEm±	0.001		0.001			0.002
			Total Volatile Fatty Acids (ml of 0.1 N NaOH/100 g cheese)			
CUM (C1)	2.29^{k}	2.91^{j}	3.78^{hi}	5.81^{f}	9.96^{c}	4.95^{c}
CUMY (C2)	2.92^{j}	3.62^{i}	4.83^{g}	7.55^{e}	12.69^{b}	6.32^{b}
CBHM	3.16^{j}	4.07^{h}	5.46^{f}	8.47^{d}	14.09^{a}	7.05^{a}
Mean (Period)	2.79^{e}	3.54^{d}	4.69^{c}	7.28^{b}	12.25^{a}	---
Source of Variation	Treatment (T)		Period (P)			T x P
SEm±	0.063		0.081			0.140

CUM (C1) – Cheese from unhomogenized milk; CUMY (C2) – Cheese from unhomogenized milk with *S. boulardii* culture; CBHM – Cheese from 'milk blend' comprising of homogenized and unhomogenized milks (1:1, w/w); Figures placed after ± indicates standard deviation, the values indicated row and column wise having differing superscripted alphabets differs significantly ($p<0.05$) from each other; n=4

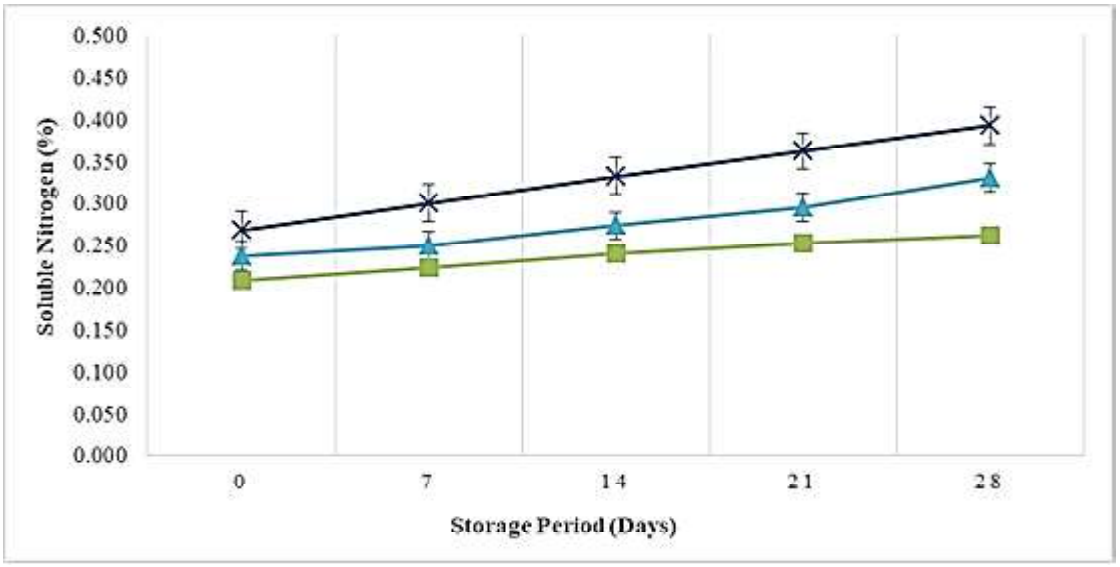


Fig. 1 Changes in the soluble nitrogen content of Pizza cheeses during storage

lactose to lactate and citrate. Residual lactose is quickly transformed into lactate during the initial phases of maturation. Lactate is a necessary precursor for a number of reactions that reduce the pH of cheese, comprising of racemization, oxidation, and microbial metabolism (Mc Sweeney and Fox 2004).

The pH of Pizza cheeses prepared employing SC method was 4.80 and 4.00 as noted during 2 and 4 weeks of refrigerated (4°C) storage respectively; freshly prepared cheese had pH of 5.30 (Ahmed et al. 2011). Likewise, the pH of freshly prepared Pizza cheese made using SC method was 5.30; the values were 4.90 and 4.60 respectively as noted at 2 and 4 weeks of storage (4°C) (Abd El-Gawad et al. 2012).

Soluble nitrogen

The proteolytic and peptidolytic activities of starter bacteria have been held responsible as key factors in determining proteolytic breakdown during Pizza cheese ripening; plasmin has also been implicated. There is a consequential release of amino groups as a result of proteolysis. As a result of proteolytic breakdown, the soluble nitrogen (SN) content of cheese tended to increase linearly with the age of the product (Costabel et al. 2007). Such proteolytic changes occurring in Pizza cheeses during refrigerated storage improves the water binding capacity of casein and brings about desirable or undesirable changes in the functionality (melt, flow and stretch) of the product (Jana and Mandal 2011). Hence, it was necessary to monitor the changes in SN during the refrigerated storage of Pizza cheeses.

The SN content of all the cheeses increased markedly throughout the storage period; such an increase in SN was found to be significant ($p<0.05$) at each 7 days interval of storage, until the 28th day (Figure 1). The changes noted in the SN content of all three cheeses were significantly ($p<0.05$) influenced by T, P and the interaction T x P. The experimental cheese CBHM had a markedly higher SN content (i.e. 0.331%) compared to that of the other two cheeses; control cheese C1 had the least SN value (i.e. 0.237%). The minimum and maximum values of SN were noted for fresh control cheese C1 (0.208%) and 28 days-old experimental cheese CBHM (0.392%) respectively (Table 1).

The SN content (at pH 4.6, expressed as % of total nitrogen) of Fior di Latte (similar to high moisture Mozzarella) cheese prepared employing the SC method using adjunct cultures (i.e. *L. rhamnosus* GG, *L. acidophilus* LA5 and their combination) showed an increase during refrigerated storage (4°C) of up to 15 days. The SN content of cheeses containing *L. rhamnosus* GG exhibited an increase from 2.47% when fresh to 3.68% as noted on the 15th day of refrigerated storage (Cuffia *et al.* 2019).

Abd El-Gawad et al. (2012) also noted an increase in the SN content during storage (4°C) of Mozzarella cheeses for up to 4 weeks; the cheeses were prepared using SC method from homogenized (2.45 MPa) and unhomogenized milks. The values of SN reported were 0.166 and 0.162% for freshly prepared cheeses from homogenized and unhomogenized milks respectively; the pertinent values noted on the 28th day of storage were 0.325 and 0.274% respectively. The SN content of Pizza cheese made using thermophilic SC increased from 0.173 to 0.228% over a span of 28 days; storage was at 7°C (Rajani et al. 2021b).

Total Volatile Fatty Acids

The determination of free volatile fatty acids (VFAs) is of interest with respect to the lipolytic changes taking place in cheese during ageing and the resultant flavour profile of aged cheese. These VFAs are components of taste and flavour, and their concentration gives an indication of the metabolic reactions that took place during cheese ageing Hence, the TVFA content of cheese is used to monitor the lipolytic changes occurring in the product during ageing (Collins et al. 2003).

The TVFA content of Pizza cheeses was significantly (P<0.05) affected by T, P and the interaction T x P. Such an increase in the TVFA of Pizza cheeses was found to be statistically significant (p<0.05) at each 7 days interval of storage, up to 28th day (Figure 2). All the cheeses differed markedly (p<0.05) from each other with regard to the TVFA content; the maximum (7.05) and minimum (4.95) values (expressed as ml of 0.1 N NaOH/100 g cheese) were associated with cheeses CBHM and C1 respectively (Table 1).

The linear increase in the TVFA content of Mozzarella cheese during refrigerated storage is documented in the literature. Ahmed et al. (2011) reported TVFA values (ml 0.1 N NaOH/100 g cheese) of 6.46 and 12.55 for Mozzarella cheeses prepared using SC Method stored for 2 and 4 weeks respectively; freshly prepared cheese had TVFA value of 3.10. The TVFA content of Mozzarella cheese, made utilizing *L. rhamnosus* and *L. paracasei* as an adjunct culture, increased from 2.0 to 13.0 mg/kg during refrigerated (4 °C) storage of 20 days (Huang et al. 2022).

Changes in the sensory scores of cheeses as Pizza topping

The sensory scores of Pizza cheeses, judged as pizza topping, are depicted in Table 2. The pertinent findings related to the sensory scores of Pizza cheeses are discussed in the following paragraphs.

Appearance

There was an increase in the appearance score of all the cheeses in a span of 14 days (i.e. 7th to 21st day of storage). The changes in the appearance scores of cheeses were significantly (p<0.05) affected by T and P; the interaction T x P remained unaffected. The data shown in Table 2 revealed that the appearance score of the cheeses was in the order: C1 > C2 > CBHM; such difference in the appearance score was found to be significant (p<0.05). The overall improvement in the appearance score of cheeses, judged as pizza topping, could be attributed to the improvement in the melting property (uniform melt, devoid of any unmelted shred particles) and improved glossy appeal of the cheese as a result of ageing. The improvement in glossiness of melted cheese might be attributed to the increased water binding capacity of the cheese proteins as a consequence of ageing (Jana and Tagalpallewar 2017).

Flavour

The changes in the flavour scores of cheeses were significantly (p<0.05) affected by T and P; interaction T x P remained unaffected. An increase in the flavour scores of all the cheeses was noted during the initial 7 days (i.e. 7th to 14th day) of storage. The mean values of flavour scores (out of 10.0) for cheeses C1, C2 and CBHM were 8.12, 7.93 and 7.47 respectively. The difference in the flavour scores of the three cheeses was found to be significant (p<0.05); the maximum score was associated with cheese C1. Initial 7 days of storage (from the 7th day onward) exhibited a significant (p<0.05) increase in the flavour scores of the cheeses, judged on pizza pie. However, subsequent storage for a week (i.e. 7 days) led to a significant (p<0.05) decline in the flavour score of cheeses (Table 2).

An improvement in the flavour score of Mozzarella/Pizza cheeses prepared using SC method, especially those prepared using SC, up to a certain period of refrigerated storage has been reported in the literature (Rajani 2021; Patel 2022).

Melting

The changes in the melting scores of Pizza cheeses, judged as pizza topping, were significantly (p<0.05) affected by T and P; the interaction T x P remained unaffected. The superiority in the melting scores of Pizza cheese C2 over cheeses C1 and CBHM was in consonance with the increasing trend noted for Schreiber meltability values of Pizza cheeses (Table 2). Cheese C2 had a markedly (p<0.05) higher melting score compared to the remaining two cheeses; CBHM had the least melting score. The melting scores of C1 and CBHM were also significantly (p<0.05) different; C1 had superior score (Table 2).

Stringiness

The changes in the stringiness score of cheeses, assessed on pizza pie, were significantly (p<0.05) affected by T, P and their interaction T x P. Cheese C1 was associated with the highest stringiness score; such score was significantly (p<0.05) superior when compared with the scores of the other two cheeses. Cheese CBHM had the least stringiness score; such score was significantly (p<0.05) lower than the score allotted to cheese C2. There was a linear decrease in the stringiness scores of all the cheeses with the advancement of the storage period; such change was significant (p<0.05) at each 7 days interval of storage, until the 21st day (Table 2).

Such a marked decrease in the stringiness score of cheeses upon refrigerated storage was due to a decrease in the stretch character of the product upon ageing; attributed to the proteolytic changes. With regard to interaction T x P, the highest and least scores for stringiness (out of 10.00) were noted for 7 days old cheese C2 (i.e. score of 8.30) and 21 days old cheese CBHM (i.e. score of 7.24) respectively (Table 2).

Mozzarella cheese made utilizing SC method is associated with poor stretch and melt when freshly prepared; such properties improve during the initial stage of ageing and become optimal within 2 to 3 weeks of refrigerated storage. Nevertheless, such functional traits tend to deteriorate upon further storage/ageing (Rajani 2021; Patel 2022).

Chewiness

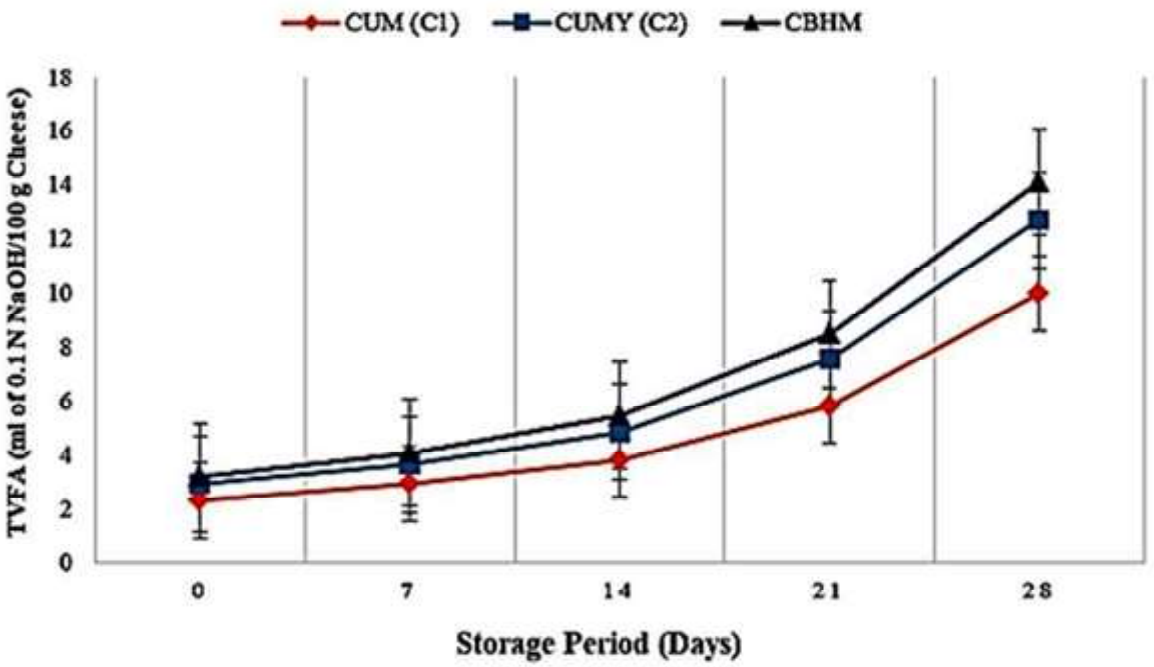


Fig. 2 Changes in the total volatile fatty acids content of Pizza cheeses during storage

Table 2 Changes in the sensory scores of Pizza cheeses, evaluated as a pizza topping, during refrigerated storage (4°C)

Cheeses	Storage Period (Days)			Mean (Treatment)
	7	14	21	
	Appearance Scores (out of 10.00)			
CUM (C1)	8.30	8.66	8.98	8.64[a]
CUMY (C2)	8.28	8.52	8.65	8.49[b]
CBHM	7.81	8.30	8.61	8.24[c]
Mean (Period)	8.13[c]	8.49[b]	8.74[a]	---
Source of Variation	Treatment (T)	Period (P)	T x P	
SEm±	0.061	0.061	0.0106	
	Flavour Scores (out of 10.00)			
CUM (C1)	8.16	8.34	7.87	8.12[a]
CUMY (C2)	7.91	8.10	7.78	7.93[b]
CBHM	7.45	7.78	7.18	7.47[c]
Mean (Period)	7.84[b]	8.07[a]	7.61[c]	---
Source of Variation	Treatment (T)	Period (P)	T x P	
SEm±	0.028	0.028	0.048	
	Melting Scores (out of 10.00)			
CUM (C1)	7.66	8.01	8.32	8.00[b]
CUMY (C2)	8.15	8.26	8.55	8.32[a]
CBHM	7.48	7.74	8.05	7.75[c]
Mean (Period)	7.76[c]	8.00[b]	8.30[a]	---
Source of Variation	Treatment (T)	Period (P)	T x P	
SEm±	0.028	0.028	0.048	
	Stringiness Scores (out of 10.00)			
CUM (C1)	8.22[a]	8.07[b]	7.52[e]	7.94[a]
CUMY (C2)	8.30[a]	7.94[c]	7.26[f]	7.83[b]
CBHM	8.00[bc]	7.63[d]	7.24[g]	7.62[c]
Mean (Period)	8.18[a]	7.88[b]	7.34[c]	---

It is important to note that Pizza cheese with moderate chewiness, tends to obtain a higher score than its counterpart cheese exhibiting greater chewiness. The changes in the chewiness scores of cheeses, assessed on pizza pie, were significantly ($p<0.05$) affected by T and P; the interaction T x P remained unaffected. Cheese CBHM had significantly ($p<0.05$) superior

	Chewiness Scores (out of 10.00)			
CUM (C1)	7.85	8.10	8.30	8.08[b]
CUMY (C2)	7.88	8.22	8.48	8.19[b]
CBHM	8.30	8.65	8.82	8.59[a]
Mean (Period)	8.00[c]	8.30[b]	8.53[a]	---
Source of Variation	Treatment (T)	Period (P)	T x P	
SEm±	0.041	0.041	0.072	
	Total Sensory Scores (out of 50.00)			
CUM (C1)	40.19	41.18	40.99	40.78[a]
CUMY (C2)	40.52	41.06	40.71	40.76[a]
CBHM	39.04	39.77	39.90	39.68[b]
Mean (Period)	39.92[b]	40.10[a]	40.54[a]	---
Source of Variation	Treatment (T)	Period (P)	T x P	
SEm±	0.112	0.112	0.194	

CUM (C1) – Cheese from unhomogenized milk; CUMY (C2) – Cheese from unhomogenized milk with *S. boulardii* culture; CBHM – Cheese from 'milk blend' comprising of homogenized and unhomogenized milks (1:1, w/w); Figures placed after ± indicates standard deviation, the values indicated row and column wise having differing superscripted alphabets differs significantly ($p<0.05$) from each other; n=4

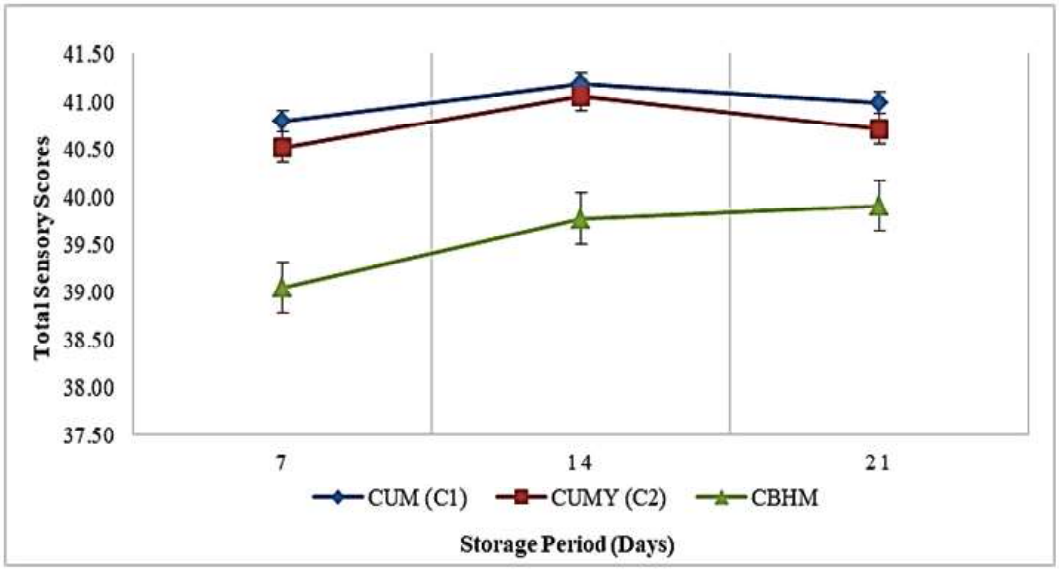


Fig. 3 Changes in the total sensory scores of Pizza cheeses as pizza topping as affected by storage

(i.e. 8.59 out of 10.00) chewiness score (i.e. since cheese had moderate chewiness) when compared to the scores associated with the other two cheeses; the chewiness scores of the latter two control cheeses (i.e. C1, C2) were at par with each other. The chewiness scores of all the cheeses improved with advancement in storage; such improvement was noticed at each 7 days interval of storage, until the 21st day (Table 2). The mellowing of Mozzarella cheese (i.e. decrease in chewiness) during its refrigerated storage is documented in the literature (Rajani 2021).

Total sensory score

The changes in the total sensory scores of cheeses, judged as pizza topping, were significantly ($p<0.05$) affected by T and P; the interaction T x P remained unaffected. Both the control cheeses (i.e. C1, C2) had total scores that were at par with each other (Figure 3). Cheese CBHM had the least total score (i.e. 39.68 out of 50.0). During storage, the total sensory scores of cheeses remained fairly constant up to the 14th day (sensory evaluation started on the 7th day). Further storage (i.e. up to 21st day) did not influence the total sensory score of the resultant cheeses markedly (Table 2). The incorporation of *S. boulardii* as an adjunct culture in experimental cheeses (i.e. CBHM and C2) led to greater flavour impairment during storage when compared to control cheese (i.e. cheese C1) containing only yogurt starter.

The appearance, flavour, body and texture and overall acceptability score (each attribute out of 5.00) of Mozzarella cheese prepared using SC, assessed on baked pizza pie, were 5.00, 4.90, 4.60 and 4.70 respectively for freshly prepared products. The aforementioned pertinent scores were 2.80, 1.30, 1.30 and 1.00 as noted on the 10th day of refrigerated (7°C) storage (Singh and Goyal 2010).

Changes in the Microbial Count of Cheeses as Influenced by Storage

The microbial count of cheeses is depicted in Table 3. The relevant findings relating to such aspects are discussed herein. Based on the plasticizing conditions adopted in preparing cheeses CUM (C1), CUMY (C2) and CBHM, the temperatures of the cheese mass during plasticizing were 63.6°C (for both C1 and C2) and 59.5°C respectively. The plasticizing conditions for the cheeses, in the same order as specified above, were 93.5°C for 4.5 min. (for C1, C2) and 79°C for 2.5 min. respectively.

***S. boulardii* count**

The changes in the *S. boulardii* count of cheeses were significantly ($p<0.05$) affected by T, P and the interaction T x P. The experimental cheese CBHM had significantly ($p<0.05$) higher *S. boulardii* count compared to its counterpart control cheese (i.e. C2). This implied that the less severe plasticizing conditions employed during stretching of the cheese curd (79°C for 2.5 min.) and the lower pH (5.13) of cheese CBHM led to greater survivability of the inoculated *S. boulardii*, even during storage (Table 3, Figure 4). The salting rate was, however, kept constant at 1.75% by weight of cheese curd (plasticizing in hot moulding water followed) in both the cheese making protocols.

A progressive but significant ($p<0.05$) decline in the count of *S. boulardii* for all the cheeses was noted during refrigerated storage of up to 21 days; the changes in the *S. boulardii* count were marked ($p<0.05$) at each 7 days interval of storage, up to 21st day. In context to the interaction T x P, the highest and least counts of *S. boulardii* were noted in fresh cheese CBHM (i.e. 6.08 $\log_{10}$ cfu/g) and 21 days aged control cheese CUMY (i.e. C2) (i.e. 2.46 $\log_{10}$ cfu/g) respectively (Table 3).

The *Lactobacillus rhamnosus GG (an adjunct culture)* count of Fior di Latte cheese was 7.76 $\log_{10}$ cfu/g when freshly prepared; the count decreased to 7.55 $\log_{10}$ cfu/g on the 15th day of refrigerated (4°C) storage. A viability loss of 0.21 $\log_{10}$ cfu/g was observed for *L. rhamnosus* GG after 15 days of vacuum storage at 4°C. As per their findings, adjusting the technological variables [i.e. acidification of the curd to pH 5.25 and keeping plasticizing conditions (81°C with contact period of 10 min.)] in the manufacture of Fior di Latte cheese enabled the *L. rhamnosus GG population to exceed* 7.5 $\log_{10}$ cfu/g in the resultant cheese, till it's use-by-date (Cuffia et al. 2017). Such count satisfied the requirement of probiotic food in such cheese.

Akarca and Yildirim (2022) prepared Pizza cheeses from cow and buffalo milks utilizing the *L. acidophilus* adjunct culture. The probiotic bacterial count increased during storage (initial count was 5.0 $\log_{10}$ cfu/g). However, in the case of *Bifidobacterium lactis subsp. animalis,* the count showed a rise during the initial 14 days of refrigerated storage and subsequent storage led to a decrease in their count. Cow milk Pizza cheese tended to show higher count of probiotic bacteria (i.e. 5.00 $\log_{10}$ cfu/g) compared to the counterpart buffalo product (i.e. 4.71 $\log_{10}$ cfu/g).

The probiotic *L. paracasei* ssp. *paracasei* LBC-1 count of freshly prepared Mozzarella cheese was 8.73 $\log_{10}$ cfu/g; the count declined to 8.40 $\log_{10}$ cfu/g on the 42nd day of refrigerated (4°C) storage (Ortakci et al. 2012).

Table 3 *S. boulardii* and LAB count of Pizza cheeses during refrigerated storage (4°C)

Cheeses	Storage Period (Days)				Mean (Treatment)
	0	7	14	21	
	S. boulardii Count ($\log_{10}$ cfu/g)				
CUMY (C2)	4.31^{d}	3.96^{e}	3.23^{f}	2.46^{g}	3.48^{b}
CBHM	6.08^{a}	5.70^{b}	5.22^{c}	3.96^{e}	5.24^{a}
Mean (Period)	5.20^{a}	4.83^{b}	4.23^{c}	3.21^{d}	---
Source of Variation	Treatment (T)		Period (P)		T x P
SEm±	0.008		0.011		0.016
	LAB Count ($\log_{10}$ cfu/g)				
CUM (C1)	7.90^{e}	7.84^{fg}	7.80^{gh}	7.68^{i}	7.81^{b}
CUMY (C2)	7.96^{d}	7.88^{ef}	7.80^{gh}	7.70^{i}	7.83^{b}
CBHM	9.16^{a}	9.06^{b}	8.94^{c}	7.78^{h}	8.74^{a}
Mean (Period)	8.34^{a}	8.26^{b}	8.18^{c}	7.72^{d}	---
Source of Variation	Treatment (T)		Period (P)		T x P
SEm±	0.008		0.010		0.017

CUM (C1) – Cheese from unhomogenized milk; CUMY (C2) – Cheese from unhomogenized milk with *S. boulardii* culture; CBHM – Cheese from 'milk blend' comprising of homogenized and unhomogenized milks (1:1, w/w); Figures placed after ± indicates standard deviation, the values indicated row and column wise having differing superscripted alphabets differs significantly ($p<0.05$) from each other; n=4

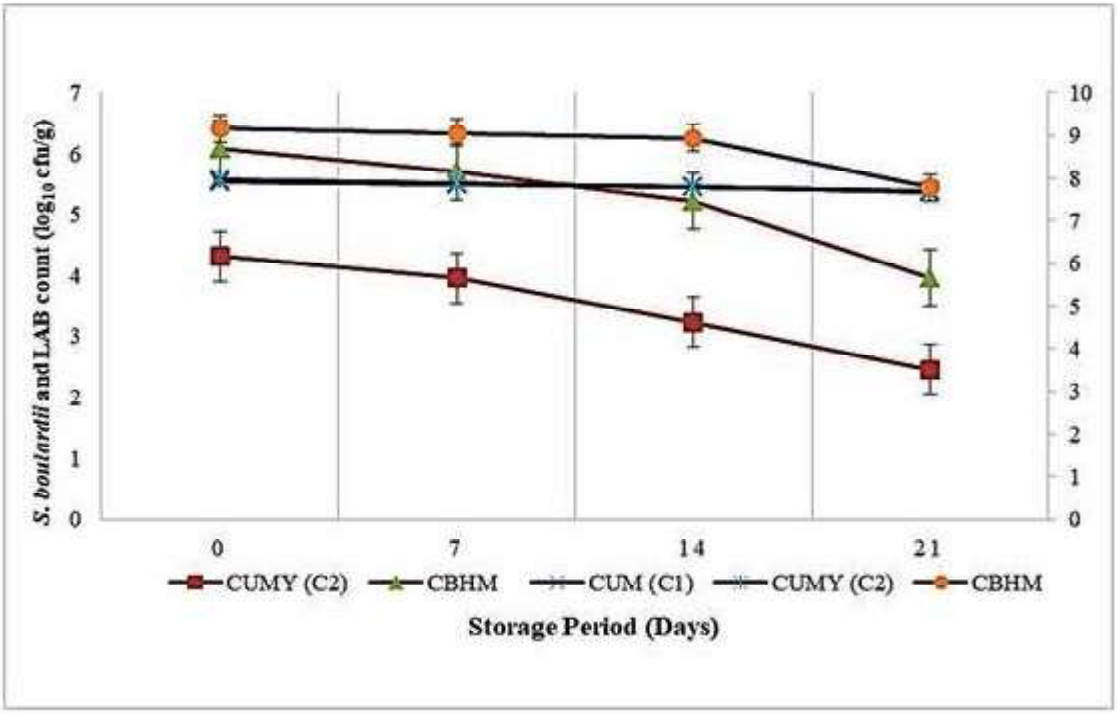


Fig. 4 *S. boulardii* and LAB count of Pizza cheeses during storage

LAB count

The changes in the LAB count of cheeses were significantly ($p<0.05$) affected by T, P, and interaction T x P. The experimental cheese CBHM had significantly ($p<0.05$) higher LAB count when compared with the count of other two control cheeses. The two control cheeses had LAB counts that were statistically at par ($p>0.05$) with each other. It is clearly evident that the use of adjunct *S. boulardii* culture helped in boosting the LAB count of Pizza cheese (Table 3). In addition to the probiotic count, the large number of LAB cells in fermented dairy products (including cheese) is beneficial to the well-being of humans (Staniszewski and Kordowska-Wiater 2021).

A progressive decrease in the LAB count of all the three cheeses was observed during storage for up to 21 days. Such a decrease in LAB count was significant ($p<0.05$) at each 7 days interval of storage, until the 21st day. The highest and least counts of LAB were noted for freshly prepared CBHM cheese (i.e. 9.16 $\log_{10}$ cfu/g) and 21 days aged C1 cheese (i.e. 7.68 $\log_{10}$ cfu/g) respectively (Table 3, Figure 4).

The LAB count of control and probiotic Pizza cheese containing *L. rhamnosus GG* was 9.23 and 9.38 $\log_{10}$ cfu/g respectively when freshly prepared; the respective counts were 9.14 and 9.37 $\log_{10}$ cfu/g on the 15th day of refrigerated (4°C) storage. Earlier during the manufacturing stage of Pizza cheese, post-plasticizing of cheese curd with hot water (82°C) for 10 min. contact period, the LAB count of both cheeses got reduced by 0.16 $\log_{10}$ cfu/g (Cuffia et al. 2017).

Coliform count

Coliform bacteria were absent in the freshly prepared and stored (up to 21st day) Pizza cheeses; control as well as in experimental cheese. This implied that the manufacturing protocol, handling of the product and packaging of cheeses were performed under strict hygienic conditions. Rajani (2021) did not detect any coliforms either in fresh cheese or in aged (21 days at 7°C temperature) Pizza cheeses made using SC method.

Conclusion

For the production of health-promoting Pizza cheese featuring the presence of *S. boulardii* as an adjunct starter, it is recommended that cheese makers follow the standardized cheesemaking process indicated for product CBHM. Cheese CBHM prepared using SC method bearing *S. boulardii* as an adjunct culture was preferred over cheese CUMY containing the adjunct culture in the same amount for being associated with higher count of both *S. boulardii* and LAB. The moisture content of cheese CBHM complied with the FSSR specification as well as performed satisfactorily in sensory aspects for their end-use application as a topping on pizza pie. The incorporation of *S. boulardii* as an adjunct starter led to an increased LAB count in the resultant Pizza cheese. Cheese CBHM registered counts of *S. boulardii* and LAB that was higher by 20.20% and 5.49% respectively when compared to the count associated with control cheese C2. Since refrigerated storage beyond 14 days resulted in perceptible decline in the count of *S. boulardii*, only 2 weeks of refrigerated storage has been recommended to the cheesemakers

in order to reap the health benefits associated with consumption of such cheese containing probiotic microbes.

Acknowledgements

The authors are highly thankful to Dr. N. Jayanthi, Head – Scientific Affairs, Unique Biotech, Hyderabad and Mr. Pravin Singh, Key Account Manager, DSM Food Specialties Ltd., Anand for providing '*Saccharomyces boulardii unique 28 strain*' and 'Delvo DSL Direct Set Lyophilized Starter Cultures RST-776' for the present research work.

References

Abd El-Gawad MA, Ahmed NS, El-Abd MM, El-Rafee SA (2012) Effect of homogenization on the properties and microstructure of Mozzarella cheese from buffalo milk. Acta Sci Pol Technol Aliment 11(2):121-135

Ahmed NS, El-Gawad MA, El-Abd MM, Abd-Rabou NS (2011) Properties of buffalo Mozzarella cheese as affected by type of coagulant. Acta Sci Pol Technol Aliment 10(3):339-357

Akarca G, Yildirim G (2022) Effects of the probiotic bacteria on the quality properties of Mozzarella cheese produced from different milk. J Food Sci Technol 59(9):3408-3418

AOAC (2023) Official Methods of Analysis of AOAC International. Oxford University Press, UK

Bihola A, Adil S, Kumar D (2024a) Mozzarella mastery: Exploring the factors influencing stretching characteristics. Indian Food Ind Mag 6(2):19–26

Bihola A, Jana AH, Parmar SC, Adil S (2024b) Functionality of pizza cheese as affected using *Saccharomyces boulardii* adjunct culture during refrigerated storage. Asian J Dairy Food Res (In press)

Bihola A, Jana AH, Parmar SC, Shaikh A (2024c) Feasibility study of utilizing *Saccharomyces boulardii* as an adjunct culture in Mozzarella-type cheese and its quality characterization. *Discov Food* 4:105

Bihola A, Sharma H, Chaudhary MB, Bumbadiya MR, Kumar D, Shaikh A (2024d) Recent developments in cheese technologies. Food Rev Int 1–35

BIS (1964) Specification for cheese BIS 2802. Bureau of Indian Standards, Manak Bhavan, New Delhi, pp14-15

Collins YF, Mc Sweeney PL, Wilkinson MG (2003) Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int Dairy J 13(11):841-866

Costabel L, Pauletti MS, Hynes E (2007) Proteolysis in Mozzarella cheeses manufactured by different industrial processes. J Dairy Sci 90 (5):2103-2112

Cuffia F, George G, Godoy L, Vinderola G, Reinheimer J, Burns P (2019). *In vivo* study of the immunomodulatory capacity and the impact of probiotic strains on physicochemical and sensory characteristics: Case of pasta filata soft cheeses. Food Res Int 125:108606

Cuffia F, George G, Renzulli P, Reinheimer J, Meinardi C, Burns P (2017) Technological challenges in the production of a probiotic Pasta filata soft cheese. LWT – Food Sci Technol 81:111-117

Felfoul I, Attia H, Bornaz S (2018) Shelf life determination of fresh cheese subjected to different modified atmospheres packaging. J Agric Sci Technol 19(7):847-860

Huang X, Nzekoue FK, Renzi S, Alesi A, Coman MM, Pucciarelli S, ... Silvi S (2022) Influence of modified governing liquid on shelf-life parameters of high-moisture Mozzarella cheese. Food Res Int 159:111627

ISO (1998) Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of mesophilic lactic acid bacteria – Colony-count technique at 30°C. ISO 15214:1998, Geneva, Switzerland

Jana AH, Mandal PK (2011) Manufacturing and quality of Mozzarella cheese: A review. Int J Dairy Sci 6(4):199-226

Jana AH, Tagalpallewar GP (2017) Functional properties of Mozzarella cheese for its end use application. J Food Sci Technol 54(12):3766-3778

Kosikowski FV, Dahlberg AC (1946) A rapid direct-distillation method for determining the volatile fatty acids of cheese. J Dairy Sci 29(12):861-871

Mamo A (2017) Cheddar cheese characterization and its biochemical change during ripening. Int J Adv Sci Res Manag 2(5):53-59

Mc Sweeney, P.L.H., & Fox, P.F. (2004). Metabolism of residual lactose and of lactate and citrate. In P.F. Fox (ed.) Cheese: chemistry, physics and microbiology (pp. 361-371), Springer: Boston

Niamah AK (2017) Physicochemical and microbial characteristics of yogurt with added *Saccharomyces boulardii*. Curr Res Nutr Food Sci 5(3):300-307

Ortakci F, Broadbent, JR, McManus, WR, Mc Mahon DJ (2012) Survival of microencapsulated probiotic *Lactobacillus paracasei* LBC-1e during manufacture of Mozzarella cheese and simulated gastric digestion. J Dairy Sci 95(11):6274-6281

Patel GC, Vyas SH, Upadhyay KG (1986) Evaluation of Mozzarella cheese made from buffalo milk using direct acidification technique. Ind J Dairy Sci 39(4):394-403

Patel HR (2022) Quality improvement of Mozzarella cheese by admixing homogenized milk with unhomogenized milk. MTech Thesis, Kamdhenu University, Gandhinagar, Gujarat

Rajani B, Jana AH, Bihola A, Parmar SC, Shaikh A (2024a) Process standardization and characterization of pizza cheeses prepared employing 'dual acidification' method. J Food Sci Technol (In press)

Rajani B, Jana AH, Bihola A, Shaikh A (2024b) Changes in physico-chemical and functional properties of pizza cheeses made using 'dual acidification' method during refrigerated storage. *Discov Food* 4:157

Rajani BM (2021) Pizza cheese making employing starter culture technique using GDL as an adjunct. MTech Thesis, Anand Agricultural University, Anand, Gujarat

Singh P, Goyal GK (2010) Modified atmosphere packaging and storage on sensory characteristics of ready to bake pizza Nutr Food Sci 40(3):299-304

Staniszewski A, Kordowska-Wiater M (2021) Probiotic and potentially probiotic yeasts – Characteristics and food application. Foods 10(6):1306

Steel RGD, Torrie JH (1980) Analysis of Variance - I: The one-way classification. Principles and Procedure of Statistics – A Biometrical Approach, 2nd edn Mc Graw Hill, Kogakusha Ltd, Japan, pp 137-167

Unique Biotech (2023) *Saccharomyces boulardii* Unique 28. Retrieved from https://www.uniquebiotech.com/probiotic-strains/saccharomyces-boulardii

Indian J Dairy Sci 78(2): 114-120
https://doi.org/10.33785/IJDS.2025.v78i02.002

RESEARCH ARTICLE

Flavoured sterilized milk enriched with alpha linolenic acid: physico-chemical properties and evaluation of storage stability

Pramod B Tambade [1], Monika Sharma (✉)[2] and Ashish K Singh[3]

Received: 01 August 2023 / Accepted: 25 January 2024 / Published online: 23 April 2025

Abstract: Milk is often referred as a complete food, but it lacks certain essential fatty acids like omega-3 fatty acids. Omega-3 fatty acids have been associated with reduced risk of several health diseases. Milk is being widely consumed throughout the masses; it would serve as an ideal vehicle for omega-3 fatty acid fortification. Flaxseed oil is one of the richest vegetarian sources of omega-3 fatty acids. However, due to high susceptibility to oxidation, the use of flaxseed oil is limited in food and dairy products. But this problem can be overcome by encapsulating flaxseed oil where a protective coat is formed on oil droplets. In the present study microencapsulated flaxseed oil was used to fortify milk. The selected microcapsule was used to fortify milk with the aim of providing 25 and 50% RDA of alpha linolenic acid in the milk. Based upon better sensory acceptability, 25% RDA level was selected for further study. Upon addition of flaxseed microcapsules, the sterilized flavoured milk samples were analysed for physico-chemical, sensory and compositional parameters. The samples were also evaluated for storage stability for 28 days. The pH and viscosity of the fortified flavoured milk samples differ significantly ($p < 0.05$) from the control. There was no yeast and mould, coliform count in the samples during storage. Also, the total bacterial count was within the permissible limits for fortified sterilized milk. During storage, the seven, fourteen and twenty-one days of storage depicted non-significant difference ($p > 0.05$) in the flavour score of fortified and control sterilised milk samples. But on 28th day of storage there is significant ($p < 0.05$) difference in sensory score of taste and mouthfeel; however, it was still in the acceptable range. The moisture, fat, protein, ash and total carbohydrates for fortified sterilized milk were 87.30, 3.42, 3.57, 0.85, 4.85%, respectively. Therefore, it can be concluded that the milk can be successfully fortified with alpha linolenic acid fatty acids using the modified starch and soy protein isolate based flaxseed oil microencapsulated powder showed excellent storage stability. The flavoured milk thus developed would provide 0.612 g of ALA in one serving.

Keywords: Fortified milk, Omega-3 fatty acids, Alpha linolenic acid, Storage stability, Sensory acceptability

[1]Techno Commercial, Gujarat Enterprise, Pune, Maharashtra, India

[2] ICAR- National Dairy Research Institute, SRS, Bengaluru, India

[3] ICAR-National Dairy Research Institute, Karnal (Haryana), India

Monika Sharma (✉)

ICAR- National Dairy Research Institute, SRS,

Adugodi, Bengaluru-560030

E-mail: Monika.Sharma@icar.gov.in, sharma.monikaft@gmail.com

Introduction

Milk is very nutritious and perhaps requisite food for human being. Milk is fundamental contributor to improve food security and nutrition throughout the world. Predominantly in developing countries, it may serve as a promising food source in reducing malnutrition. However, milk is devoid of omega-3 (ω-3) fatty acids and alpha linolenic acids (ALA), which are considered to be functional ingredients owing to several physiological health benefits. Thus, ALA and Omega-3 fatty acids serve as vital ingredient in developing the nutraceutical and functional food. The Indian Council of Medical Research (ICMR 2010) recommends 1.6 g/ day of ALA and 250 mg of EPA plus DHA per day. This can be achieved through fortification of food products. The rich sources of omega-3 fatty acids include fish oil, flaxseed oil, algal oil, canola oil etc.

Although fish is the greatest contributor of ω-3 fatty acids, but the Indian diets do not include enough oily fish to meet dietary recommendations of ω-3 fatty acids. Moreover, addition of fish oil preparations (in the form of emulsions or spray dried powder) in food is literally impossible for vegetarians due to their religious beliefs and practices. In such a situation, it is very difficult to meet recommended intakes of ω-3 for vegetarians. It has been reported that ω-6: ω-3 ratio in current Indian urban and Western diets is 38-50:1 and 20:1, respectively (Singh et al. 2011; Simopoulos 2011) which appears to be very high as compared to the recommended ratio, i.e. 5:1 (FAO/WHO 2010).

Flaxseed (*Linum usitatissimum*) oil, also known as linseed oil, is the rich source of ω-3 fatty acids, having 50-60% á-linolenic acid (ω-3, C18:3). Flaxseed oil comprises the essential fatty acid; alpha-linolenic acid (ALA) which the body converts into eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Due to its highly polyunsaturated nature, flaxseed oil is highly susceptible to oxidation and leads to production of off-flavours and toxic peroxides during heating, processing and handling of the products. So, working stabilization of the flaxseed oil for its food applications is a challenging job. Microencapsulation is the most commonly used technique for the stabilization of active compounds. Spray drying is a common method of encapsulation in the food industry (Phisut 2012; Renata et al. 2014). In an effort to stabilize flaxseed oil, the authors have developed flaxseed oil microcapsules (Tambade et al. 2020) using modified starch and soy protein isolate as matrix materials. The developed capsules are stable in nature to be utilized in dairy products.

Lin et al. (2014) fortified dairy products with soy lecithin-stabilized emulsions containing 50 % algal oil, 6 % soy lecithin, and 44 % Milli-Q water. Efforts have been made to develop omega-3 fatty acid fortified baked products, processed cheese, fruit yoghurt, market milk (Goyal et al. 2017; Veena and Nath 2017) but limited work has been done on the utilization of flaxseed oil as a useful functional ingredient in sterilized flavoured milk which is consumed by the larger segment of the population. Secondly, although some of the studies have been carried out for encapsulation of flaxseed oil but concerted efforts are lacking for the development of omega-3 fatty acids fortified dairy products utilizing flaxseed oil, which have good potential as a functional ingredient in dairy products. As milk and milk products are widely consumed by all age group of people and by various standards of livings they play a vital role in nutritional security. So, flavoured milk can serve as an ideal vehicle for fortification of alpha-linolenic acids due to higher palatability among different age groups. Thus, with this backdrop, the present investigation is really required for development of omega-3 fatty acid fortified flavoured sterilized milk. Thus, the present study was planned to study the effect of microencapsulated flaxseed oil in sterilized flavoured milk upon storage.

Materials and methods

Materials

We received cold pressed flaxseed oil as a gift from AAK Kamani Pvt. Ltd., Andheri, Mumbai (Maharashtra, India). Soy protein isolate (SPI) and modified starch (NC-46) used for microencapsulation were procured from Shridurga Sales Corporation, Bangalore, India and Ingredion India Private Ltd, Thane, Maharashtra, India, respectively. In this study, all other chemicals and reagents were of AR grade. Toned milk was obtained from the experimental dairy plant of SRS, ICAR-NDRI. Sugar was procured from local market.

Preparation of flaxseed oil microcapsules

Flaxseed oil microcapsules were prepared using the methodology of Tambade et al. (2020). Firstly, the emulsion was prepared by mixing the calculated amount of 25% flaxseed oil (on Total solids basis), 5% soy protein isolate and 20 % modified starch using hand blender (Phillips, India) for approximately 5 minutes and then prepared solution was homogenised with high shear mixer (IKA T-18, Germany) at 18000 rpm for 5 minutes. The prepared emulsion was preheated in waterbath to 40ÚC for microencapsulation in order to decrease the viscosity for proper atomisation in drying chamber of spray dryer (Technosearch Instrument, Thane, Mumbai, India). For spray drying, inlet and outlet hot air temperatures were maintained at 180±5ÚC and 85±5ÚC, respectively, while, flow rate was maintained from 40-60 mL/min. The microcapsules were stored in aluminium laminates for further study.

Preparation of control and fortified sterilized milks

Three samples were used for the study. The control was prepared using the toned milk (3.0% fat and 8.5% SNF). The second sample, i.e. fortified sterilised milk was prepared by the addition of flaxseed oil microcapsule to meet at least 25% RDA in 240 mL milk. For preparation of the third sample, namely sterilised flavoured fortified milk, milk was standardised to 3.0% fat and 8.5% SNF, sugar was added @8% and then preheated (65°C) for homogenisation. After homogenisation, the IFF banana flavour was added @ 0.05% and calculated amount of microcapsules were added (at 40°C) and mixed properly. Bottles were filled with some headspace and corked. All the three samples were sterilised (In bottle sterilisation at 121°C for 15 min) followed by cooling to room temperature slowly. The sterilised milk samples were then stored (25°C) for storage study and further analysis.

Physico-chemical properties of fortified flavoured sterilized milk

Titratable acidity

Titratable acidity of control and fortified milk was determined as per IS: SP: 18, Part XI (1981).

Free fatty acids (FFA) content

An extraction titration method devised by Deeth et al. (1975) was followed for the determination of FFA content of milk samples.

pH

pH of milk samples was determined by potentiometric method using digital pH meter (Eutech, India). The pH meter was first calibrated using standard buffers of pH 4.0 and 9.2 and standardized using pH buffer of 7.0 at 25.0 ± 0.1°C.

Viscosity

Viscosity of fortified sterilized milk was measured by the capillary viscometer (Ostwald viscometer) following the methodology of Roy and Sen (1994).

Sensory evaluation of fortified flavoured sterilized milk

Sensory evaluation was done by a semi-trained panel of ten judges of different age groups and gender from Dairy Processing section of SRS-NDRI, Bangalore, India. Omega-3 fortified milk was evaluated by the panellists using the nine-point Hedonic scale for colour and appearance, mouthfeel, taste and flavour and overall acceptability in comparison to control milk. Milk was served at 10-15°C temperature for sensory evaluation.

Microbiological evaluation of fortified flavoured sterilized milk

Microbiological analysis of fortified milk was performed on 0, 7, 14, 21 and 28 days of storage. Standard plate count was enumerated using nutrient agar. Plating of serially diluted fortified milk samples were done and plates were incubated at 37°C for 24-48 hours. Coliform count was enumerated using Violet Red Bile agar and plates were incubated at 37°C for 24-48 hours and Yeast and mold count was enumerated using PDA agar and plates were incubated at 30°C for 48-72 hours. Spore count was enumerated using spore count agar and plates were incubated at 30°C for 24-48 hours. Prior to spore count, sterilised milk was incubated at 63°C for 24 hours.

Proximate composition of fortified flavoured sterilized milk

Total solids (TS), fat, ash and nitrogen content of milk samples were determined by the methods as described in IS: SP-18 (1981). Total carbohydrate content was determined by difference method as per IS: SP-18 (1981). Nitrogen content in milk samples was estimated by Kjeldahl method as given in eq (1):

$$Nitrogen\ \% = \frac{14.07 \times (Vs - Vb) \times Normality\ of\ sulphuric\ acid \times 100}{Wt.of\ sample} \quad (1)$$

Where,

V_s = mL 0.1 N H_2SO_4 titrant used for test portion

V_b = mL 0.1 N H_2SO_4 titrant used for blank

Protein content % in milk= Nitrogen content (%) × 6.38

Alpha linolenic acid (ALA) content of fortified flavoured sterilized milk

The alpha linolenic acid content was determined using the method used by Pandule et al. (2021). Firstly, the fat was extracted and then Fatty acid methyl esters (FAME) were prepared. The internal standard- tridecanoic acid (Sigma Aldrich, India) (200 mL, 25 mg mL^{-1}) was added in the extracted fat before preparation of FAME. The FAME were then subjected to gas chromatography-mass spectrometry (GCMS) for quantification of alpha linolenic acid. The FAME were separated using the DB5 MS (30 m × 0.25 mm × 0.25 mm) capillary column with helium as a carrier gas at a ûow rate of 1 mL min^{-1}. The injector temperature was 230 °C, and column temperature was programmed as follows: 50 °C for initial 1 min, subsequent increase to 220 °C at the rate of 3 °C min^{-1} and maintained it for 1 min. The interface temperature for GC-MS (Agilent Technologies, Santa Clara, CA) was 220 °C. Identification of fatty acids was done based on data from mass spectral libraries (NIST 47, NIST 147 and Wiley 175), literature data and by comparison of retention times with Supalco-37 FAME standards (GLC-85 and FAME Mix GLC-90). Results were expressed as g/ 100mL ALA content.

Statistical analysis

The data obtained were analyzed using analysis of variance technique for three replicates with the help of SPSS software and statistical significance was set at $p<0.05$. The least significant difference (LSD) test was used to find out significant differences between sample means. Analysis of variance (ANOVA) was used to determine differences among treatment means using the Post Hoc Test (Dunkan). Sensory attribute of milk samples data during storage were analysed using 2-way analysis of variance (ANOVA), with main effects of treatments and day of storage.

Results and Discussion

Selection of level of addition of flaxseed oil microcapsules for fortified flavoured sterilized milk preparation

Milk was fortified with flaxseed oil microcapsules for providing at least 25 and 50% recommended dietary allowance (RDA) of alpha linolenic acid (ALA) in one serving (240mL). The sensory acceptability of fortified samples is represented in Table 1. It is evident from the table data, that there was no significant ($p>0.05$) difference among the scores for colour, mouthfeel of control milk and F25 milk sample, however, the mouthfeel of F50 milk was lower than control milk. Further, the taste and flavour and overall acceptability decreased significantly ($p<0.05$) upon addition of microcapsule. Based upon higher sensory acceptability, 25% RDA level was selected. In order to further increase the acceptability by a wider group of consumers, flavoured fortified milk was also developed and compared for various physico-chemical parameters, sensory acceptability, rancidity and microbiological spoilage during storage.

Compositional analysis of alpha linolenic acid fortified flavoured sterilized milk

The proximate composition of fortified and control sterilized milk were analysed and the results obtained are presented in Table 2.

From the table, it can be interpreted that the control sample and fortified sample were significantly different ($p<0.05$) from each other. The moisture, fat, protein, ash and total carbohydrates for control milk were 88.52, 3.10, 3.47, 0.81, 4.10% respectively. While the fat, protein and total carbohydrates difference in control and fortified milk sample were due to addition flaxseed oil microcapsules made from soy protein isolates and modified starch. The constituents were similar in both milks except for higher carbohydrate content in the flavoured fortified milk (Table 2) owing to the addition of sugar. Further, it is vital to note that 3 g of microcapsules added to 240 mL of milk were sufficient to provide 0.612 g of ALA (Table 2), which accounts to 38.25% of RDA as per ICMR (Indian Council of Medical Research), 2010 guidelines and 27.81 % as per ISSFAL (International Society for the Study of Fatty Acids and Lipids), 2004 guidelines.

Sensory acceptability of fortified flavoured sterilized milk during storage

The changes in the score of colour and appearance are shown Table 3. It is evident from the data that on 0th day, score of plain sterilised milk was slightly higher than the fortified sterilised and fortified flavoured sterilised milk. The sensory scores for colour and appearance were statistically same for first and seventh day of storage and thereafter the score were found to decrease over the period of storage. The colour and appearance score was less for the fortified flavoured sterilised milk because of maillard browning taking place during the heat treatment as addition of sugar was done in to milk. While control and fortified sterilized milk has almost same colour and appearance score over the storage period. Also, the score for mouthfeel of fortified and control milk samples decreased with storage period. It can be seen that the mouthfeel scores followed following trend: fortified flavoured milk > Plain sterilised > fortified sterilised. The mouthfeel score for fortified flavoured sterilised milk was more may be because of smoothness in texture of fortified flavoured milk, while followed by plain sterilised and lastly fortified sterilised, because of addition of microcapsules.

Effect of storage on sensory scores of taste and flavour and overall acceptability of fortified and control sterilized milk are given in Table 3. The taste and flavour scores were maximum on 0 day for all the three samples. Acceptability of fortified milk on zero day was comparable to the control milk while sterilised flavoured milk had slightly higher taste and flavour scores than the fortified milk. Upon storage for seven, fourteen and twenty-one days there were non-significant ($p>0.05$) differences in the taste and flavour score of fortified and control sterilised milk samples. But on 28th day of storage, there were significant ($p<0.05$) differences in the sensory scores for taste and flavour of fortified and control samples.

The fortified flavoured sterilised milk was liked the most by the sensory panellist as compared to control and fortified sterilised milk samples. The overall acceptability scores on the 0th day for plain sterilised and fortified sterilised milk were 8.05 and 7.88

Table 1 Effect of level of microcapsules on sensory acceptability of fortified flavoured sterilized milk

Sensory Parameters	control	F25	F50
Colour and appearance	8.78±0.25^{a}	8.72±0.56^{a}	8.81±0.12^{a}
Mouthfeel	8.61±0.65^{a}	8.58±0.78^{a}	8.39±0.77^{b}
Taste and Flavour	8.72±0.23^{a}	8.47±0.46^{ab}	7.39±0.82^{b}
Overall Acceptability	8.67±50^{a}	7.78±0.72^{b}	7.28±0.24^{c}

F25- sample having microcapsules providing 25% RDA of ALA, F50- sample having microcapsules providing 50% RDA of ALA, Results are expressed as Mean±SD, n=10; Means with different small letters superscript (a,b,c) within row differ significantly ($p<0.05$) among the samples

Table 2 Proximate composition of fortified flavoured sterilized milk

Constituents (%)	Control	Sterilized Milk	
		Plain fortified sterilized milk	Flavoured fortified sterilized milk
Moisture content	88.52±0.13^{a}	87.30±0.21^{b}	79.56±0.47^{c}
Fat	3.10±0.03^{b}	3.42±0.02^{a}	3.37±0.02^{a}
Protein	3.47±0.05^{b}	3.57±0.03^{a}	3.57±0.03^{ab}
Ash	0.81±0.13^{c}	0.85±0.21^{b}	0.99±0.47^{a}
Total Carbohydrates	4.10±0.11^{c}	4.85±0.19^{b}	12.51±0.44^{a}
ALA, g/100mL	---	0.255	0.255

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row differ significantly ($p<0.05$) among the samples

respectively, while on 28th day, it was 7.11 and 7.94. Whereas score for the fortified flavoured sterilised was 8.61 on first day while, at end of storage it was 7.55 (Table 3). The higher overall acceptability may be due to the addition of sugar and artificial flavour in fortified flavoured sterilised milk.

Viscosity of fortified flavoured sterilized milk during storage

Viscosity is the most important physical change taking place during the storage of market milk. The extent depends on temperature and time of storage and history of heat treatment (Usarek et al. 1997). It may be seen from the results (Table 4) that the viscosity of the fortified milk was significantly ($p<0.05$) higher than the control sample throughout the storage period. The viscosity of both control and fortified milk increased significantly ($p<0.05$) with increasing storage period. Cano-ruiz and Richter (1998) reported that the apparent viscosity of the samples increased as milk solids non-fat increased. The highest viscosity was found in fortified flavoured sterilised milk (5.290 mPas), followed by fortified sterilised milk (2.833 mPa-s) and sterilised milk (2.012 mPa-s) while, at the end of storage it was 6.009, 3.796 and 2.509 mPa-s in fortified flavoured, fortified, plain sterilised milk, respectively. This observation indicated that the interaction of added sugar, milk proteins and milk fat caused a significant increase in the viscosity of the fortified milk. Similar findings were also reported by Veena and Nath (2017) for increase in the viscosity for fortified milk. A higher volume fraction of milk solids would result in greater viscosity. Thus, higher fat and total solids content in fortified milk might have contributed for a higher viscosity. Further, Rauh et al. (2014) attributed the rise in viscosity to proteolysis, resulting in gel formation during the storage of UHT milk due to protein interactions and imbibition of water leading network of bonds.

Acidity and pH of fortified flavoured sterilized milk during storage

The chemical properties viz. acidity and pH of control and fortified milk samples during storage are shown in Table 4. Lactic acid is the principal acid produced due to which titratable acidity of milk rises. Increase in free fatty acids is also responsible for increasing the total titratable acidity of milk (Swartzel 1983). From the results, it can be seen that titratable acidity increased significantly ($p< 0.05$) with storage in both control and fortified sample. Titratable acidity of control and fortified sterilised and flavoured fortified sterilised milk increased from 0.159 to 0.339% LA, 0.168 to 0.369% LA and 0.168 to 0.375% LA, respectively, at

Table 3 Sensory attributes of control and fortified flavoured sterilized milk samples during storage (at 25°C)

Colour and appearance	Days	Sample		
		PS	FS	FFS
Mouth feel	0	8.27 ± 0.26^{aA}	8.16 ± 0.25^{aA}	8.16 ± 0.25^{aA}
	07	8.05 ± 0.63^{aA}	8.05 ± 0.30^{aA}	8.12 ± 0.20^{aA}
	14	7.66 ± 0.75^{aAB}	7.55 ± 0.68^{aAB}	8.05 ± 0.05^{aAB}
	21	8.00 ± 0.43^{aB}	7.88 ± 0.22^{aB}	8.11 ± 0.22^{aB}
	28	7.20 ± 0.26^{aC}	7.22 ± 0.26^{aC}	7.22 ± 0.36^{aC}
Taste and Flavour	0	7.94 ± 0.30^{bA}	7.88 ± 0.33^{bA}	8.23 ± 0.27^{aA}
	07	7.94 ± 0.39^{bA}	7.94 ± 0.30^{bA}	8.33 ± 0.25^{aA}
	14	7.55 ± 0.58^{bAB}	7.27 ± 0.66^{bAB}	8.16 ± 0.50^{aAB}
	21	7.75 ± 0.35^{bB}	7.83 ± 0.25^{bB}	7.88 ± 0.33^{aB}
	28	7.33 ± 0.35^{bC}	7.33 ± 0.35^{bC}	7.44 ± 0.46^{aC}
Overall acceptability	0	7.94 ± 0.30^{cA}	7.77 ± 0.26^{bA}	8.61 ± 0.48^{aA}
	07	7.56 ± 0.62^{cB}	7.33 ± 0.66^{bB}	8.55 ± 0.30^{aA}
	14	7.44 ± 0.39^{cAB}	6.88 ± 0.65^{bAB}	8.05 ± 0.52^{aAB}
	21	6.34 ± 0.33^{cAB}	7.22 ± 0.61^{bAB}	7.77 ± 0.66^{aAB}
	28	7.31 ± 0.45^{cC}	7.16 ± 0.35^{bC}	7.72 ± 0.44^{aC}
	0	8.05 ± 0.30^{bA}	7.88 ± 0.33^{bA}	8.61 ± 0.41^{aA}
	07	7.55 ± 0.60^{bA}	7.84 ± 0.32^{bA}	8.61 ± 0.22^{aA}
	14	7.44 ± 0.46^{bB}	7.16 ± 0.55^{bB}	8.11 ± 0.48^{aB}
	21	7.27 ± 0.06^{bB}	7.38 ± 0.54^{bB}	7.83 ± 0.61^{aB}
	28	7.11 ± 0.33^{bC}	7.94 ± 0.39^{bC}	7.55 ± 0.39^{aC}

PS- Plain sterilized milk (Control), FS- fortified sterilized milk, FFS- fortified flavoured sterilized milk, Results are expressed as Mean±SD, n=10; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

28 days of storage. Similar findings were reported by Goyal et al. (2017) for fortified market milk. According to Cais-Sokoliñska et al. (2002), acidity changes in sterilised milk could be attributed to the course of enzymatic (mainly lipolytic) reactions, which result in the formation of free fatty acids. A similar phenomenon, in which pH decreased and potential acidity increased with the time of UHT milk storage, was described by Biliñska et al. (1998). A certain effect on the acidity of sterilized milk (mainly milk sterilized by the long-time method) can be exerted by small amounts of formic, acetic and other acids and maillard-type reactions (Fink and Kessler 1986). As reported in the literature, acidity of sterilised and UHT milk reflects product freshness and, to a certain extent, the quality of raw milk used for processing and the intensity of heat treatment used during the technological process (Kruk et al. 1995).

It can be inferred from results (Table 4) that the pH of the control and fortified milk decreased significantly ($p<0.05$) with storage period, which corresponds to the increasing acidity. The results are in agreement with Veena and Nath (2017) for milk fortified with omega-3 fatty acids, phytosterols and soluble dietary fibre. Between zero and the 28th day of storage, significant differences ($p<0.05$) in pH value were noticed in both control and fortified milk samples. Fortified milk had the lowest pH value throughout storage period and did not differ significantly ($p>0.05$) from the control sample. The initial pH of the control and fortified sterilised and flavoured fortified sterilised milk was 6.68, 6.67 and 6.65, respectively and at the end of storage (28th day) it was 6.42, 6.36 and 6.33, respectively. Venkatachalm and McMahon (1991) verified a drop in pH during storage of UHT milk and associated it with browning reactions. Andrews et al. (1977) confirmed similar effects and concluded that the level and extent of pH decrease was related to age-gelation. When milk is heated at a temperature above 100^{o}C and subsequently stored, lactose is degraded to acids.

Hydrolytic rancidity of fortified flavoured sterilized milk during storage

The hydrolytic rancidity of control and fortified milk during storage was evaluated by determining FFA content and the results are presented in Table 4. The level of un-esterified fatty acids in milk provides a measure of the extent of lipolysis in milk. The free fatty acids (FFA) content in milk is dependent on the changes in the lipolytic activity of sterilized milk. From the results, it can be seen that FFA content increased significantly ($p<0.05$) with storage in both control and fortified milk samples. Between control and fortified milk samples, significant ($p<0.05$) difference in FFA content was observed. The increase in the FFA content was 0.170 ìEq/ml, 0.315 ìEq/ml and 0.166 ìEq/ml for control, fortified sterilised and fortified flavoured sterilised milk, respectively after 28 days of storage. After 28th day of storage at 25^{o}C, the FFA content in control, fortified milk and fortified flavoured sterilised milk samples was 0.417 ìEq/ml, 0.644 ìEq/ml and 0.517 ìEq/ml, respectively. The results are in agreement with the findings of Deeth et al. (1975) for milk with low levels of lipolysis. They reported FFA content of d" 1.0 ìEq/ml for low lipolysis milk.

Table 4 Effect of storage (4-7^{o}C) on physico-chemical properties of fortified flavoured sterilized milk

Parameter	DAY	PS	FS	FFS
Acidity (% Lactic acid)	0	0.159 ± 0.005^{cE}	0.168 ± 0.005^{bE}	0.168 ± 0.005^{aE}
	7	0.180 ± 0.009^{cD}	0.192 ± 0.005^{bD}	0.204 ± 0.014^{aD}
	14	0.219 ± 0.005^{cC}	0.225 ± 0.009^{bC}	0.234 ± 0.009^{aC}
	21	0.285 ± 0.005^{cB}	0.303 ± 0.005^{bB}	0.318 ± 0.005^{aB}
	28	0.339 ± 0.005^{cA}	0.369 ± 0.016^{bA}	0.375 ± 0.010^{aA}
pH	0	6.68 ± 0.01^{aA}	6.67 ± 0.01^{bA}	6.65 ± 0.00^{cA}
	7	6.64 ± 0.00^{aB}	6.64 ± 0.00^{bB}	6.63 ± 0.02^{cB}
	14	6.57 ± 0.01^{aC}	6.55 ± 0.01^{bC}	6.51 ± 0.01^{cC}
	21	6.49 ± 0.01^{aD}	6.45 ± 0.01^{bD}	6.41 ± 0.01^{cD}
	28	6.42 ± 0.01^{aE}	6.36 ± 0.01^{bE}	6.33 ± 0.01^{cE}
Viscosity (m Pas)	0	2.012 ± 0.033^{cD}	2.833 ± 0.042^{bD}	5.290 ± 0.247^{aD}
	7	2.062 ± 0.01^{cC}	2.883 ± 0.028^{bC}	5.793 ± 0.028^{aC}
	14	2.093 ± 0.042^{cBC}	2.978 ± 0.047^{bBC}	5.827 ± 0.074^{aBC}
	21	2.259 ± 0.024^{cB}	3.099 ± 0.053^{bB}	5.938 ± 0.039^{aB}
	28	2.509 ± 0.250^{cA}	3.796 ± 0.070^{bA}	6.009 ± 0.070^{aA}
FFA (µEq/ml)	0	0.247 ± 0.000^{cD}	0.329 ± 0.005^{bE}	0.351 ± 0.004^{aE}
	7	0.272 ± 0.001^{cD}	0.464 ± 0.012^{bD}	0.356 ± 0.007^{aD}
	14	0.329 ± 0.004^{cC}	0.498 ± 0.001^{bC}	0.433 ± 0.004^{aC}
	21	0.360 ± 0.002^{cB}	0.496 ± 0.005^{bB}	0.430 ± 0.000^{aB}
	28	0.417 ± 0.005^{cA}	0.644 ± 0.003^{bA}	0.517 ± 0.003^{aA}

PS- Plain sterilized milk (Control), FS- fortified sterilized milk, FFS- fortified flavoured sterilized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

Microbiological quality of fortified flavoured sterilized milk during storage

The application of heat at high temperatures for a sufficient time renders milk or milk products commercially sterile, thus resulting in products that are safe and microbiological stable at room temperature. The control and fortified sterilised milks were analysed for standard plate count, yeast and mold, coliform count and spore count during the 28 days' storage period and were not detected throughout the storage period. Thus, it could be interpreted that the sterilisation treatment to milk was carried out successfully.

Conclusions

The present study resulted in the development of a sensorially acceptable alpha linolenic acid fortified sterilized milk. It can be inferred that the microencapsulated flaxseed oil powder can be suitably used to fortify milk to provide at least 25% recommended dietary allowance of alpha-linolenic acid in one serving of fortified milk with acceptable physico-chemical characteristics. The developed sterilized milk had acceptable sensory acceptability and was microbiologically safe throughout the storage period of four weeks at 25°C. Further, encapsulated flaxseed oil powder can also be used for the fortification of several other products such as bakery products, confectionery, fruit juices and other dairy and food products.

Acknowledgments

The authors gratefully acknowledge Director, ICAR-National Dairy Research Institute, Karnal, India and Head, SRS, ICAR-NDRI, Bengaluru for financial assistance and AAK Kamani Oil Industry, Mumbai, India for gifting flaxseed oil to conduct the present study.

References

Andrews AT, Brooker BE and Hobbs G (1977) Properties of aseptically packed UHT milk. Electron microscopic examination of changes occurring during storage. J Dairy Res 44: 283-285

Biliñska S, Baranowski A, Pawlik S, Zuczkowa J and Iwaszek M (1998) Research on quality and durability of milk sterilised by means of the UHT method. Rocz Instytutu Mleczarstwa, 72(1): 55–71.

Cais-Sokoliñska D, Danków R and Pikul J (2002) Influence of temperatureconditions during storage on physico-chemical and sensory changes of the UHT milk. Ch³odnictwo 37(10): 40–43

Cano-Ruiz ME and Richter RL (1998) Changes in physicochemical properties of retort-sterilized dairy beverages during storage. J Dairy Sci 81: 2116-2123

Deeth HC, Fitzgerald CH and Wood AF (1975) A convenient method for determining the extent of lipolysis in milk. Aust J Dairy Technol 30: 109–111

FAO/WHO (2010) Fats and Fatty Acids in Human Nutrition Rome: FAO Food and nutrition paper # 697 91 Report of an expert consultation Geneva, November 10–14, 2008.

Fink R and Kessler HG (1986) Hydroxy methyl furfural values in heat treated and stored milk. Milchwis sens chaft 41: 638–641

Goyal A, Sharma V, Sihag MK, Singh AK, Arora S and Sabikhi L (2017) Oxidative stability of alpha-linolenic acid (·§-3) in flaxseed oil microcapsules fortified market milk. Int J Dairy Technol 70(2): 188-196

ICMR (2010). Nutrient requirements and recommended dietary allowances for Indians. A report of the expert group of the Indian Council of Medical Research 2009. Available from: http://icmr.nic.in/final/RDA-2010.pdf Last accessed on 20/01/2014

IS:SP: 18 (Part XI) (1981) Handbook of Food Analysis. Dairy Products. Bureau of Indian Standards, New Delhi.

ISSFAL (2004) Recommendations for intake of polyunsaturated fatty acids in healthy adults. (International Society for the Study of Fatty acids and Lipids (ISSFAL) News 11: 12-18

Kruk A, Czerniewicz M and Haponiuk E (1995) Effects of high-pressure homogenization on the physicochemical properties of milk with various fat concentrations. Pol J Food Nutr Sci 15/56: 91-94

Lin X, Wang Q, Li, W, Wright AJ (2014) Emulsification of algal oil with soy lecithin improved DHA bio accessibility but did not change overall in vitro digestibility. Food & Function 5 (11): 2913-2921

Pandule VS, Sharma M, Devaraja, HC and B Surendra Nath (2021) Omega-3 fatty acid fortified butter: Preparation and characterisation of textural, sensory, thermal and physic-chemical properties. Int J Dairy Technol 74(1): 181-191

Phisut N (2012) Spray drying technique of fruit juice powder: some factors influencing the properties of product. Int Food Res J 19: 1297-1306

Renata V, Nunes GL, Boaventura BCB, Pinto SS, Verruck S, Murakami FS, Prudêncio ES and Amboni RDDMC (2014) Microencapsulation of freeze concentrated Ilex paraguariensis extract by spray drying. J of Food Eng 151: 60-68

Roy D and Sen P (1994) Fluorescence anisotropy decay and solvation dynamics in a nanocavity: coumarin 153 in methyl â-cyclodextrins. J Phys Chem A 109(43): 9716-9722

Rauh VM, Sundgren A, Bakman M, Ipsen R, Paulsson M, Larsen LB, Hammershøj M (2014) Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion. Int Dairy J 38: 199-207

Simopoulos AP (2011) Evolutionary aspects of diet: the omega-6/omega-3 ratio and the brain. Mol Neurobiol 44(2): 203-215

Singh RB, Moshiri M, De Meester F, Juneja L, Muthusamy V and Manoharan S (2011) The evolution of low ù-6/ ù-3 ratio dietary pattern and risk of cardiovascular diseases and diabetes. J Altern Med Res 3: 45-70

Swartzel KR (1983) The role of heat exchanger fouling in the formation of sediment in aseptically processed and packed milk. J Food Process 7: 247-251

Tambade PB, Sharma, M, Singh AK, Surendra Nath B (2020) Flaxseed oil microcapsules prepared using soy protein isolate and modified starch: process optimization, characterization and in vitro release behaviour. Agric Res 9: 652–662.

Usarek A, Wêgrzynowski T and Œwitka J (1997) Qualitative changes of sterilized UHT milk during storage. Przegl Mlecz (4): 104–109

Venkatachalm N, MacMahon DJ (1991) Effect of lactose concentration on age-gelation of UHT sterilized skim milk concentrate. J Dairy Sci 74: 101107

Veena N and Surendra Nath B (2017) Effect of fortification of milk with omega-3 fatty acids, phytosterols and soluble fibre on the sensory, physicochemical and microbiological properties of milk. J Sci Food Agric 97(12): 4160-4168

Indian J Dairy Sci 78(2): 121-125
https://doi.org/10.33785/IJDS.2025.v78i02.003

RESEARCH ARTICLE

Preparation and quality assessment of Mozzarella cheese from Buffalo milk with addition of specific type of LAB

Md. Ashik Uz-Zaman[a] (✉), **Junayed Ahmed**[a], **Md. Abu Hanif Ruman**[a], **Shimul Mojumder**[a], **Anzuman Ara**[a], **Sajib Paul**[b], **Md. Irtija Ahsan**[c]

Received: 17 September 2024 / Accepted: 13 January 2025 / Published online: 23 April 2025

Abstract: This study investigates the chemical composition, microbiological attributes, and sensory properties of Mozzarella cheese produced from buffalo milk collected from selected farms in Haripur, Jaintapur Upazila at Sylhet district. The cheese was prepared using varying concentrations of lactic acid bacteria (LAB) starter cultures (0.0%, 0.5%, 1.0%, and 2.0%), followed by enzymatic coagulation with rennet. The physico-chemical analysis revealed that the addition of LAB significantly ($P<0.05$) increased the dry matter (DM%) and ether extract (EE%) content, with the highest values observed in the T3 treatment (2.0% LAB). Conversely, nitrogen-free extract (NFE%) decreased significantly with increasing LAB concentration. The microbiological analysis demonstrated a significant increase ($P < 0.05$) in LAB count across treatments, while the standard plate count (SPC) showed an upward trend, though not statistically significant. Coliform bacteria were absent in all samples. Sensory evaluation indicated substantial improvements ($P < 0.05$) in color, taste, texture, flavor, and appearance with increasing LAB levels, particularly in T2 (1.0% LAB) and T3 (2.0% LAB). These findings suggest that the incorporation of LAB enhances both the quality and sensory attributes of Mozzarella cheese, making it a promising approach for cheese production.

Key words: Mozzarella cheese, Buffalo milk, quality and Lactic acid bacteria

[a] Department of Dairy Science, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet-3100, Bangladesh
[b] Department of Animal Nutrition, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet-3100, Bangladesh
[c] Department of Epidemiology and Public Health, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet-3100, Bangladesh

(✉)Md. Ashik Uz-Zaman
Department of Dairy Science, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet-3100, Bangladesh.
E-mail: ashik.ds@sau.ac.bd

Introduction

Buffalo is known as the black gold of South Asia. It plays a very important role in the South Asia along with cattle, which constitutes 73.77% of world buffalo population. The South Asian countries share 93.19% of world buffalo milk production where India and Pakistan contributes 67.99 and 23.96%, respectively (Hamid et al. 2016).In Bangladesh, recent year's dairying has been transforming from customary subsistence to market oriented and enterprise driven approach in the dairy production system (Uddin et al. 2021) The rapid growth of urbanization, poverty reduction, increase in middle class and their increased income have changed their food habit. These recent developments have major impacts on demand for animal derived products like as milk, meat, cheese, butter, ghee, ice-cream, yoghurt and other traditional sweetmeats which are merely dependent on milk. The major market players in the country are Milk vita, Pran Dairy Ltd, BRAC Dairy and Food (Arong) and Akij Dairy Ltd (Farm fresh) corresponding to only 5% share to the total milk production in the country. In Bangladesh, the contribution of buffalo in total milk production is more or less stagnant due to absence of any milk improvement program. Buffalo milk has much total solids than cow milk that is useful for making cheese, butter and other dairy products.

Cheese is the curd or substance formed by the coagulation of milk of certain mammals by rennet or similar enzymes in the presence of lactic acid produced by added or adventitious microorganisms (Mirsalami SM, Alihosseini A, 2023). The moisture has been removed by cutting, warming and pressing, which has been shaped in mould and then ripened (also unripened) by holding for sometime at suitable temperatures and humidity (Huang X et al. 2022). Cheese has high protein content and it is commonly known as milk meat. Mozzarella cheese is a soft, unripened cheese variety of the Pasta-filata family which had its origin in the Battipaglia region of Italy (Citro, 1981). Conventionally, mozzarella cheese was made from buffalo milk. The cheese is soft, white with a glossy surface is valued for its stretch property (Deshwal et al. 2023). Day by day it is becoming popular in Bangladesh for preparing many delicious food items like pizza, sandwitch, salad and other items. Cheese would be one of the economically valuable dairy products in Bangladesh.

If the buffalo farmers of haor areas get proper scientific support for the production of milk and cheese, then they will contribute in national economy through cheese marketing. Considering those facts, the objectives of this study were to investigate the effects of different concentration of lactic acid bacteria (LAB) starter cultures on the chemical composition, microbiological characteristics and sensory attributes of Mozzarella cheese produced from buffalo milk.

Materials and Methods

Sample collection

Milk samples were collected from selected farms of Haripur, Jaintapur Upazila for both manufacturing and quality analysis of Mozzarella cheese. For sample collection once in a week buffalo milk was collected in a large plastic bottle and kept it in a cool box for transportation. Samples were transported via CNG vehicle from Haripur to Sylhet Agricultural University.

Preparation of Mozzarella cheese

The cheese was produced by enzymatic coagulation of milk by following steps (Fig. 1)

Physico-chemical examination

Chemical composition of milk samples were analyzed by milk analyzer (Lactoscan, Bulgaria). The organoleptic tests of manufactured cheese sample were carried out by the panel of judges using score card. And chemical compositions of cheese samples were analyzed according to AOAC (2003).

Microbial examination

The experimental procedure was followed for the determination of the number of total viable bacteria in a sample and the detection, LAB and enumeration of coliform bacteria as per recommendation of APHA (1998).

Statistical analysis

The differences between four groups (six replications in each group) among physical, chemical and microbial parameters were analyzed by ANOVA using SPSS version 28 software from IBM.

Results and Discussion

The chemical composition of mozzarella cheese, such as dry matter (DM%), ash (Ash%), crude protein (CP%), crude fiber (CF%), ether extract (EE%), and nitrogen-free extract (NFE%), is depicted in Table 1. The chemical analysis of the cheese revealed the mean DM% (50.94 ± 0.33) in the control group (T_0). After adding LAB, the DM% gradually increased in all treatment groups (53.01 ± 0.88) in T_1, (54.35 ± 0.72) in T_2, and the highest was found in T_3 (56.23 ± 0.44). However, the result showed a significant ($P < 0.05$) variation between the control group (T_0) and all treatment groups. This suggests that adding LAB to cheese enhances its solids content.

Further, the ash content was demonstrated to be (3.63 ± 0.13) in the control group (T_0), T_1 (3.67 ± 0.33), and T_2 (3.55 ± 0.16), respectively, and was relatively consistent across treatments except T_3 (3.02 ± 0.03). T_3 revealed a significant decrease ($P < 0.05$) in the ash content compared with the control groups (T_0), T_1, and T_2.Moreover, the crude protein (CP%) content was determined to be highest in T_2 (17.36 ± 0.27) and T_0 (17.25 ± 0.46), with significant differences ($P < 0.05$)observed in T_1 (15.89 ±

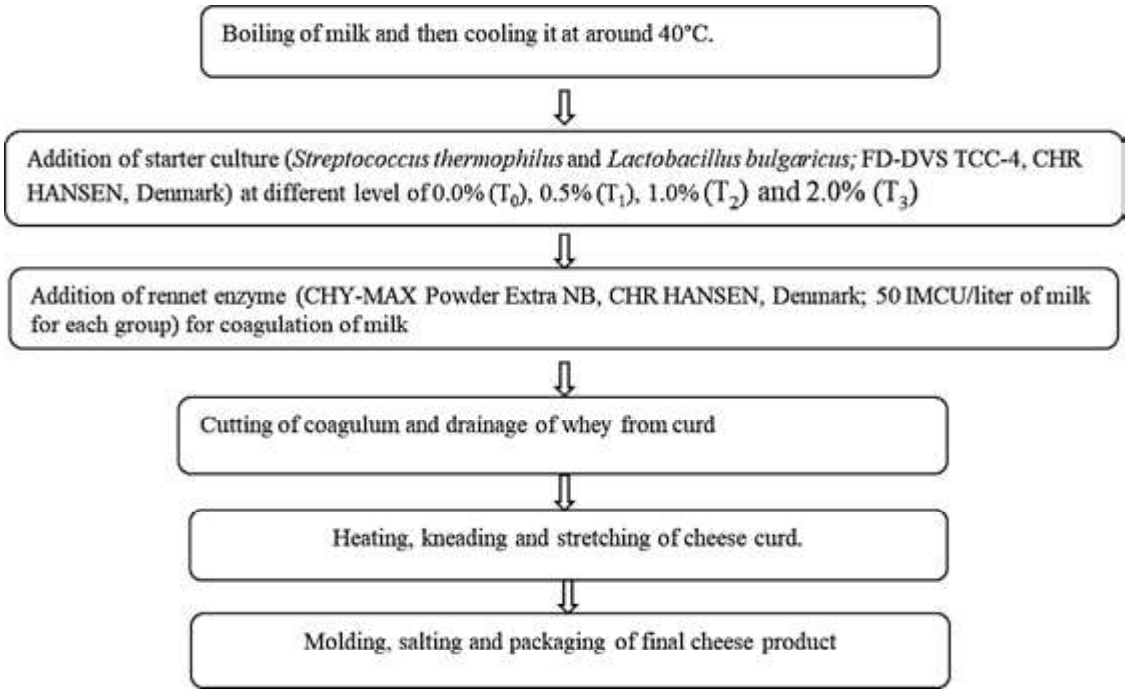


Fig. 1

0.40) and T_3 (16.38 ± 0.10). The crude fiber (CF%) content exposed slight variations across treatments, with the maximum value in T_2 (0.032 ± 0.004) and the minimum in T_3 (0.022 ± 0.004). However, these changes were not statistically significant (P>0.05).In addition, the chemical analysis of cheese revealed a notable increase in ether extract percentage after the addition of lactic acid bacteria (LAB) from (6.54 ± 0.41) in the control (T_0) to (17.50 ± 0.14) in T_1, (17.75 ± 0.06) in T_2, and (21.26± 0.29) in T_3 respectively, which indicated substantial changes (P<0.05)among treatments. Finally, the nitrogen-free extract (NFE) was reduced significantly following the inclusion of LAB from (72.78 ± 0.35) in the control $T_0$to (62.82 ± 0.24) in T_1, (61.26 ±0.34) in T_2 and (59.32 ± 0.54) in T_3consecutively. Even so, there were significant differences (P < 0.05) observed between the treatments.

Table 2, provides insightful data on the microbiological properties of mozzarella cheese as well as changes after the addition of LAB. First of all, throughout all treatments (T_0, T_1, T_2, and T_3), the coliform count was continuously found to be nil.In terms of the standard plate count (SPC), which measures the total number of viable bacteria present in cheese, there was an apparent increase from the control to the maximum treatment level. The SPC in the control (T_0) was found to be (6.28 ± 14.82), while it increased to (7.24± 17.49) in T_1, (7.82 ± 24.35) in T_2, and reached (8.90 ± 14.92) in T_3. Although this difference was not statistically significant (P e”0.05). In the end, the most significant alterations were noticed in the lactic acid bacteria (LAB) count, which increased significantly with the addition of more lactic acid bacteria. The LAB count was found at (2.18 ± 8.07) in T_0, which was raised to (2.34 ± 11.37) in T_1, (5.58 ± 25.67) in T_2, and elevated to (8.20 ± 16.02) in T_3. This increase was statistically significant (P<0.05)

By incorporating lactic acid bacteria (LAB), the sensory quality of the mozzarella cheese significantly improved, as illustrated in Table 3.

At first, the color scores were observed (5.20 + 0.84) in T_0 and gradually increased in all treatments, with the maximum observed in T_2 (6.60 + 0.55). This study revealed a significant variation (p<0.05) in color among different treatments. Repeatedly, a similar result was found in case of taste (5.00 ± 0.71) in T_0, and the highest improvement was seen in T_3 (6.60 ± 0.55) that was significantly (p<0.05) different between treatments while compared with T_0.Notably, texture and flavor had the most significant (p<0.05) improvements, with texture scoring (7.60 ± 0.55) and flavor scoring (7.40 ± 0.55) in T_3, compared to the control (T_0) with scores of (4.60 ± 0.55) and (4.80 ± 0.84), respectively. Finally, the appearance score was noted (5.40 ± 0.55) in T_0 and progressively elevated (6.00 ± 0.71) in T_1, (7.00 ± 0.71) in T_2, and T_3 had the highest score (7.40 ± 0.55) and exhibited substantial (p<0.05) variation among treatments.

The present study emphasized the substantial effects on the chemical composition, microbiological characteristics, and sensory qualities of mozzarella cheese by incorporating lactic acid bacteria (LAB). The chemical composition of mozzarella cheese, as illustrated in Table 1, revealed the significant (P < 0.05) differences among different treatments with the incorporation of LAB. Initially, the DM% increased significantly from (50.94 ± 0.33) in T_0 to (56.23 ± 0.44) in T_3. This increase indicates that LAB enhances the cheese solids content, maybe as a result of better fermentation and retention of moisture accordance with the findings of (McSweeney et al. 2013)and Parvez et al. (2006).This result is consistent with earlier studies by Settanni and Moschetti, (2010), who noticed that adding LAB to cheese manufacturing resulted in substantial increases in DM%.A similar trend was observed in the case of ether extract, which significantly increased from 6.54% in T_0 to 21.26% in T_3, demonstrating the substantial improvement of fat% in cheese. However, the NFE% decreased significantly across the treatments, from 72.78% in T_0 to 59.32% in T_3, which showed a negative relationship between fat content and the NFE% of cheese. The existing study correlated with the findings Kondyli et al. (2022), who reported that LAB can impact lipid metabolism in cheese, resulting in increased ether extract values.In line with studies by Beresford et al. (2001), that show LAB fermentation results in decreased NFE concentrations in dairy products, the decrease in NFE% suggests that LAB uses more nitrogen-free substances.

Table 1. Chemical composition of mozzarella cheese

Parameters	T_0	T_1	T_2	T_3	P-value
DM%	50.94 ± 0.33^{d}	53.01 ± 0.88^{c}	54.35 ± 0.72^{b}	56.23 ± 0.44^{a}	< 0.05
ASH%	3.63 ± 0.13^{a}	3.67 ± 0.33^{a}	3.55 ± 0.16^{a}	3.02 ± 0.03^{b}	< 0.05
CP%	17.25 ± 0.46^{a}	15.89 ± 0.40^{c}	17.36 ± 0.27^{a}	16.38 ± 0.10^{b}	< 0.05
CF%	0.026 ± 0.011^{ab}	0.028 ± 0.004^{ab}	0.032 ± 0.004^{a}	0.022 ± 0.004^{b}	0.183
EE%	6.54 ± 0.41^{c}	17.50 ± 0.14^{b}	17.75 ± 0.06^{b}	21.26 ± 0.29^{a}	< 0.05
NFE%	72.78 ± 0.35^{a}	62.82 ± 0.24^{b}	61.26 ± 0.34^{c}	59.32 ± 0.54^{d}	< 0.05

Parameters' values were shown as Mean ± Standard Deviation

[a,b,c.d]Means in the same row with different superscript letters differ significantly (P < 0.05), DM= Dry Matter, CP= Crude Protein, CF= Crude Fiber, EE= Ether Extract, NEF= Nitrogen Free Extract, T_0= contain no lactic acid bacteria, T_1= contain 0.5% lactic acid bacteria, T_2= contain 1% lactic acid bacteria, T_3= contain 2% lactic acid bacteria.

Table 2. Microbiological quality of mozzarella cheese

Parameters (cfu/ml)	T_0 (Mean±SD×10^8)	T_1 (Mean ±SD×10^8)	T_2 (Mean ±SD×10^8)	T_3 (Mean ±SD×10^8)	*P*-value
Coliform	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-
SPC	6.28 ± 14.82^{b}	7.24 ± 17.49^{ab}	7.82 ± 24.35^{ab}	8.90 ± 14.92^{a}	0.189
LAB	2.18 ± 8.07^{c}	2.34 ± 11.37^{c}	5.58 ± 25.67^{b}	8.22 ± 16.02^{a}	< 0.05

Parameters' values were shown as Mean ± Standard Deviation

[a,b,c,d]Means in the same row with different superscript letters differ significantly ($P < 0.05$), T0= no lactic acid bacteria, T_1= contain 0.5% lactic acid bacteria, T_2= contain 1% lactic acid bacteria, T_3= contain 2% lactic acid bacteria, cfu= Colony Forming Unit, SPC= Standard Plate Count, LAB= Lactic Acid Bacteria.

Table 3. Sensory quality of mozzarella cheese

Parameters	T_0	T_1	T_2	T_3	*P*-value
Color	5.20 ± 0.84^{b}	6.40 ± 0.55^{a}	6.60 ± 0.55^{a}	6.20 ± 0.45^{a}	0.011
Taste	5.00 ± 0.71^{c}	5.80 ± 0.84^{bc}	6.40 ± 0.55^{ab}	6.60 ± 0.55^{ab}	0.007
Texture	4.60 ± 0.55^{c}	5.80 ± 0.84^{b}	6.20 ± 0.84^{b}	7.60 ± 0.55^{a}	< 0.05
Flavor	4.80 ± 0.84^{c}	5.20 ± 0.84^{bc}	6.20 ± 0.84^{b}	7.40 ± 0.55^{a}	< 0.05
Appearance	5.40 ± 0.55^{b}	6.00 ± 0.71^{b}	7.00 ± 0.71^{a}	7.40 ± 0.55^{a}	< 0.05

Parameters' values were shown as Mean ± Standard Deviation

[a,b,c]Means in the same row with different superscript letters differ significantly ($P < 0.05$),T_0= no lactic acid bacteria, T_1= contain 0.5% lactic acid bacteria, T_2= contain 1% lactic acid bacteria, T_3= contain 2% lactic acid bacteria

Interestingly, the ash content was found to range from (3.63 ± 013 to 3.02 ± 0.03) relatively consistent in all treatments, following a significant decrease in (3.02 ± 0.03) in T_3. This finding is aligned with the result of (Bintsis et al. 2002) who observed variations in ash content with different microbial cultures of cheese. In contrast with the result of Bhat et al. (2022), who found the ash content of buffalo milk mozzarella cheese ranged from 5.30 and 7.80%, which are higher than the current study. The substantial decrease in T_3 indicates that increased levels of LAB could potentially impact the mineral composition of mozzarella cheese. Moreover, the CP content exhibited a significant decrease. Significant differences ($P < 0.05$) were observed in T_1 (15.89 ± 0.40%) and T_3 (16.38 ± 0.10%) due to the proteolytic function of LAB. According to Martinez-Martínez & Velez-Ruiz, (2019), who noted the CP% of mozzarella cheese was 13.2–25.2%, higher than a recent study. Finally, a non-significant variation in CF% was observed across the treatments, which was in harmony with the result of Awad et al. (2005), reported slight variation in crude fiber with LAB inclusion cheese.

Table 2 presents the microbiological characteristics of mozzarella cheese and the effects of introducing lactic acid bacteria (LAB). The coliform count remained zero in all treatments (T_0, T_1, T_2, and T_3), exhibiting appropriate hygiene practices and efficient microbial control during cheese manufacture. This is in line with the results of several studies on dairy hygiene standards Giraffa et al. (2010); Quigley et al. (2013); Mirsalami et al. (2024) and Rehman et al. (2017), who observed no *E. coli* in cheese.The current study revealed that the standard plate count (SPC) was increased from T_0 (6.28 ± 14.82) to T_3 (8.90 ± 14.92), although the increase was not statistically significant (P e" 0.05). This trend suggests that the addition of LAB may have increased microbial quantity.In this study, the most significant ($P < 0.05$) change was observed in the LAB count, which increased significantly from 2.18 ± 8.07 in T_0 to 8.22 ± 16.02 in T_3. This results in coherence with the findings of Rehman et al. (2017) and Fontana et al. (2013), who claimed substantial increases in LAB counts in dairy products fortified with probiotics.

The sensory evaluation of mozzarella cheese is depicted in Table 3. This study revealed that the sensory qualities, including color, taste, texture, flavor, and appearance of the mozzarella cheese, were significantly ($P < 0.05$) increased by the inclusion of LAB. All sensory parameters showed enhanced scores with an increasing concentration of LAB. Notably, the most significant ($P < 0.05$) increases were found in texture and flavor, scoring 7.60 ± 0.55 and 7.40 ± 0.55 in T_3 compared to 4.60 ± 0.55 and 4.80 ± 0.84 in T_0, respectively. The study conveyed by (Mijan et al. 1970), who determined the color score, were 8.2 ± 0.2 and 8.2 ± 0.1, manufactured from buffalo and cow milk, which is somewhat higher than recent studies. A study was carried out by (Bhattarai et al. 2013), who exhibited the sensory scores of mozzarella cheese made from buffalo milk for flavor (7.00), appearance (7.00), taste (7.00), and texture (7.00), which was in accordance with the current study. Cheese quality can be measured mostly by its texture. Additionally, the texture and overall acceptance of a cheese are

more important than its taste. Consumers should prioritize these components Aday & Yuceer, (2014). According to Cosentino et al. (2016), there is a considerable difference in the odour and flavour of mozzarella cheeses depending on the variety of milk utilized during the manufacturing process. The colour scores of mozzarella cheeses made from water buffalo milk were shown to be greater than those made from cow's milk by Fasale et al. (2017). Finally, the existing study emphasizes the importance of LAB strains on the quality of Mozzarella cheese, which not only impacts microbiological and nutritional qualities but also maintains or improves the sensory quality of the Mozzarella cheese.

Conclusion

This study highlights that the incorporation of varying levels of lactic acid bacteria (LAB) starter cultures significantly enhances the chemical composition, microbiological safety, and sensory qualities of Mozzarella cheese made from buffalo milk. The addition of LAB improved the dry matter and ether extract content while reducing nitrogen-free extract, leading to a more concentrated cheese. Microbiological assessments confirmed a notable increase in LAB counts without the detection of coliform bacteria, ensuring product safety. Sensory analysis showed that Mozzarella cheese with 1.0% and 2.0% LAB concentrations achieved the highest scores in parameters such as color, taste, texture, flavor, and appearance, reflecting its superior quality. These findings underscore the potential of LAB incorporation as an effective approach to enhance the overall quality and marketability of Mozzarella cheese.

Acknowledgments

The author acknowledges financial support from the Sylhet Agricultural University Research System (Project ID: SAURES-UGC-2023-2024-Vet 07) and their guidance throughout the research work as well as the whole personnel of the Dairy Science Department of SAU.

References

Aday, Serpil and Yuceer, Yonca Karagul (2014) Physicochemical and Sensory Properties of Mihalic Cheese. Int J Food Prop 17:2207–2227. doi:10.1080/10942912.2013.790904

AOAC (2005). Dairy Products, in Official Methods of Analysis, 18th edition, chapter 33, W Horwitz editor, pp. 1-4, 72-73. AOAC International, Gaithersburg, USA.

APHA (1998). American Public Health Association.

Awad, S, Hassan, AN, Science, K Muthukumarappan (2005) Application of Exopolysaccharide-Producing Cultures in Reduced-Fat Cheddar Cheese: Texture and Melting Properties. Journal of Dairy Sci 2005. Elsevier.

Beresford, Tom P, Fitzsimons, Nora A, Brennan, Noelle L and Cogan, Tim M (2001) Recent Advances in Cheese Microbiology. Int Dairy J 11:259–274. doi:10.1016/S0958-6946(01)00056-5

Bhat, Abdul Rauf, Shah, Atta Hussain, Ayoob, Mansoor, Ayoob, Muhammad Faisal, Saleem, Farrukh, Ali, Muhammad Mohsin and Fayaz, Muhammad (2022) Chemical, Rheological, and Organoleptic Analysis of Cow and Buffalo Milk Mozzarella Cheese. Ankara Univ Vet Fak Derg 69:51–60. doi:10.33988/auvfd.813215

Bhattarai, Rewati Raman and Acharya, Pushpa Prasad (2013) Preparation and Quality Evaluation of Mozzarella Cheese from Different Milk Sources. J Food Sci Technol Nepal 6:94–101. doi:10.3126/JFSTN.V6I0.8268

Bintsis, T, Dairy, P Papademas (2002) Microbiological Quality of White-brined Cheeses: A Review. International Journal of Dairy Technology. Wiley Online Libr 55:113–120. doi:10.1046/j.1471-0307.2002.00054.x

Citro, V (1981). Atypical local product obtained from buffalo milk. Scienza-e- Tecnica-Lattiero-Casearia, 32: 263-273.

Cosentino, C, Faraone, D, Paolino, R, Freschi, P and Musto, M (2016) Short Communication: Sensory Profile and Acceptability of a Cow Milk Cheese Manufactured by Adding Jenny Milk. J Dairy Sci 99:228–233. doi:10.3168/JDS.2015-10107

Deshwal GK, Gómez-Mascaraque LG, Fenelon M, Huppertz T (2023) A Review on the Effect of Calcium Sequestering Salts on Casein Micelles: From Model Milk Protein Systems to Processed Cheese. Molecules 28:2085.

Fasale, Abhijeet B, Patil, Vaibhav S and Bornare, DT (2017) Process Optimization for Mozzarella Cheese from Cow and Buffalo Milk. Int J Food Ferment Technol 7:165. doi:10.5958/2277-9396.2017.00018.6

Fontana, Luis, Bermudez-Brito, Miriam, Plaza-Diaz, Julio, Muñoz-Quezada, Sergio and Gil, Angel (2013) Sources, Isolation, Characterisation and Evaluation of Probiotics. Br J Nutr 109:S35–S50. doi:10.1017/S0007114512004011

Giraffa, G, Chanishvili, N, Microbiology, Y Widyastuti (2010) Importance of Lactobacilli in Food and Feed Biotechnology. Elsevier.

Hamid, M A, Ahmed, S, Rahman, M A and Hossain, K M (2016) Status of Buffalo Production in Bangladesh Compared to SAARC Countries. Asian J Anim Sci 10:313–329. doi:10.3923/AJAS.2016.313.329

Huang X, Nzekoue FK, Renzi S, Alesi A, Coman MM , Pucciarelli S, Sagratini G, Silvi S (2022) Influence of modified governing liquid on shelf-life parameters of high-moisture mozzarella cheese. Food Res Int 159:111627. https://doi.org/10.1016/j.foodres.2022.111627

Kondyli, Efthymia, Pappa, Eleni C, Arapoglou, Dimitris, Metafa, Maria, Eliopoulos, Christos and Israilides, Cleanthes (2022) Effect of Fortification with Mushroom Polysaccharide Beta -Glucan on the Quality of Ovine Soft Spreadable Cheese. Foods 11:1–13. doi:10.3390/foods11030417

Martinez-Martínez, Myrna and Velez-Ruiz, Jorge F (2019) Development and Physicochemical Characterization of a Functional Mozzarella Cheese Added with Agavin. J Food Sci Nutr Res 02:87–107. doi:10.26502/jfsnr.2642-11000012

McSweeney, PLH, Lait, MJ Sousa - Le and 2000, Undefined (2013) Biochemical Pathways for the Production of Flavour Compounds in Cheeses during Ripening: A Review. lait.dairy-journal.orgPLH McSweeney, MJ SousaLe Lait, 2000•lait.dairy-journal.org.

Mijan, MA, Haque, MA, Habib, MA and Wadud, MA (1970) Evaluation of Quality of Mozzarella Cheese. Bangladesh Vet 27:36–42. doi:10.3329/bvet.v27i1.5913

Mirsalami SM, Alihosseini A (2023) The effect of *Lactobacillus plantarum* LP-115 strain on improving the savor and aroma of milk containing grape sap through fermentation. Food and Humanity 1: 404–414. https://doi.org/10.1016/j. foohum.2023.06.013

Mirsalami SM, Mirsalami M, Alihosseini A, Ghodousian A (2024) The distribution of rennet activity between the cheese aging process and whey is not influenced by the association of enzymes with caseins. Heliyon 10: e32263. https://doi.org/10.1016/j.heliyon.2024.e32263

Parvez, S, Malik, K A, Ah Kang, S and Kim, H Y (2006) Probiotics and Their Fermented Food Products Are Beneficial for Health. J Appl Microbiol 100:1171–1185. doi:10.1111/j.1365-2672.2006.02963.x

Quigley, Lisa, O'sullivan, Orla, Stanton, Catherine, Beresford, Tom P, Ross, R Paul, Fitzgerald, Gerald F and Cotter, Paul D (2013) The Complex Microbiota of Raw Milk. Acad Quigley, O O'Sullivan, C

Indian J Dairy Sci 78(2): 126-133
https://doi.org/10.33785/IJDS.2025.v78i02.004

RESEARCH ARTICLE

Replacement of sugar with common salt and black pepper in low-fat frozen yoghurt

Mitrajsinh R Gohil[1], Chetan N Dharaiya[2](✉), Jarita M Mallik[3], Ajay J Gokhale[4]

Received: 28 November 2023 / Accepted: 12 January 2024 / Published online: 23 April 2025

Abstract: Common salt, black pepper and maltodextrin were used to replace milk fat and sugar in low-fat frozen yoghurt. Response Surface Methodology (RSM) was used to optimize the level of maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture. The responses in RSM were sensory attributes of frozen yoghurt such as flavour, body & texture, melting characteristics, colour & appearance and total score as well as physical properties such as melting rate and overrun. Based on the output, RSM suggested the rate of addition of maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture to be 6.12, 0.65 and 0.45 per cent respectively. The experimental frozen yoghurt was prepared as per the suggestion of RSM and compared with control low-fat frozen yoghurt which was prepared using vanilla flavour. The experimental frozen yoghurt was statistically similar with control sample for compositional parameters, physical properties, sensory attributes and microbiological quality.

Keywords: common salt, black pepper, frozen yoghurt, response surface methodology, sensory, overrun

Introduction

Frozen yoghurt is a frozen and fermented dairy dessert which possesses the characteristics of ice-cream and yoghurt both. Hence, it displays sensory characteristics of ice-cream and nutritive benefits of yoghurt. Frozen yoghurt is prepared by three different methods. Traditonally, ice-cream mix was fermented with yoghurt starter culture *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*. The set yoghurt was stirred and frozen. In another method, ice-cream and yoghurt were prepared separately followed by mixing in specific proportion. The mixture was frozen in ice-cream freezer. In third method, ice-cream mix is partially fermented with yoghurt culture followed by freezing (Granato et al. 2018).

From time immemorial, spices have played a vital role in world trade due to their varied properties and applications. We primarily depend on spices for flavour, colour, preservation and inherent medicinal qualities. One of the major spices, black pepper (*Piper nigrum*), king of spices, contains an array of phenolic components which are a mixture of the glycosides of phenolic acids and flavonol glycosides. Black pepper has found therapeutic applications in curing atherosclerosis, gangrene, earache, abdominal tumors, constipation, tooth decay, liver disorders, joint pain, lung diseases, insect bites etc. (Sharif et al. 2018).

Response Surface Methodology (RSM) has been widely used in recent years for the development of new products as well as improvement in existing products. RSM delineates the effect of the independent variables on responses of importance and is regarded as an effective method to optimize the new product formulations. It is a robust tool for data analysis that focuses on an adequate approximation relationship between input and output variables and determines the best operating circumstances for a system (Dean et al. 2017).

Around 5 per cent people worldwide and 11 per cent in India are suffering from diabetes and increasing at the rate of 4 per cent annually (WHO, 2023). Sugar has been replaced by artificial intense sweeteners in variety of products but with the development of science, artificial sweeteners are now being linked with several diseases from mild headache to cancer (More et al. 2021). Therefore, replacement of sugar with natural ingredients is need of the hour. In addition, with a changing lifestyle and increasing awareness towards health and nutrition, consumers are moving towards low-fat diet to reduce the risk of obesity, coronary heart disease, atherosclerosis and hypertension (Dharaiya et al. 2021). High fat diet is also linked with psychiatric disorders (Jeong et al. 2019). Fat, being a costliest constituent in

[4]Dairy Technology Department, SMC College of Dairy Science, Kamdhenu University, Anand-388110, Gujarat, India
Email: ajay.gokhale@kamdhenuuni.edu.in

Chetan N Dharaiya(✉)
Dairy Technology Department, SMC College of Dairy Science, Kamdhenu University, Anand-388110, Gujarat, India
Email: chetandharaiya@gmail.com
Mobile #: +91 75730 13697

milk, increases the cost of final product and make the product unaffordable by low-income group people. However, reduction in fat content of frozen yoghurt influences sensory and rheological characteristics of the product. Incorporation of salt and black pepper will make up for the deterioration taken place in the quality of frozen yoghurt by reduction of fat along with improvement in the nutritional quality of the final product. Hence, in current investigation, sugar has been replaced by salt and black pepper in low-fat frozen yoghurt.

Materials and Methods

Fresh, raw mixed (cow and buffalo) milk was procured from Livestock Research Station (LRS) of the University and calculated quantity of whole milk was subjected to cream separation to obtain skimmed milk. Skimmed milk powder (Sagar brand, marketed by Gujarat Cooperative Milk Marketing Federation Ltd., Anand, India), Cane sugar (Madhur brand, Karnataka), black pepper (Keya Foods International Pvt. Ltd., Kerala) and common salt (Tata Chemicals Ltd., Mumbai) was purchased from local market. Maltodextrin was supplied by Cargill India Pvt. Ltd., New Delhi. Stabilizers such as pectin, sodium alginate, guar gum and carrageenan were obtained from HiMedia, Mumbai. Emulsifier Glyceromonostearate (GMS) of Loba Chemicals, Mumbai was used. Starter cultures for yoghurt making such as *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were obtained from Dairy Microbiology Division of the institute. Vanilla flavour for control sample was obtained from International Flavors and Fragrances (IFF), Mumbai.

Preparation of stabilizer-emulsifier blend: A blend of stabilizers (such as sodium alginate, guar gum, carrageenan and pectin) as well as emulsifier (such as glyceromonostearate) was used in preparation of frozen yoghurt as per the suggestion of Response Surface Methodology (RSM). The blend contained sodium alginate, guar gum, carrageenan, pectin and GMS in the ration of 2:2:1:2:2 on the basis of preliminary trials. A combination of different stabilizers has synergistic effect on the quality of frozen yoghurt and can reduce their use (Kugashiya et al. 2023). Hence, a combination of stabilizers and emulsifiers has been used in current investigation.

Preparation of salt and black pepper blend: Salt and black pepper were mixed in the ratio of 1:1 based on preliminary trials and used in preparation of frozen yoghurt.

Preparation of frozen yoghurt: Frozen yoghurt has been prepared using the method suggested by Kugashiya et al (2023) with minor modifications. The detailed method is illustrated hereunder:

Calculated quantity of whole milk and skimmed milk were mixed at 45°C followed by heating to 55°C. All the dry ingredients such as skimmed milk powder, maltodextrin, sugar, salt and black pepper blend as well as stabilizer-emulsifier mixture were blended together before addition to whole milk and skimmed milk blend. The mixture was then homogenized at 65°C. The homogenization pressures used were 2000 and 500 psi in first and second stage respectively. It was then heat treated at 85°C for 30 min followed by cooling to 42±2°C and inoculation of starter cultures *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* at the rate of 2 per cent (w/w) of the quantity of yoghurt mix and incubated till the acidity reached to 0.6 per cent LA. The yoghurt mix was then cooled to 4°C and stirred. The stirred yoghurt mix was aged at 4°C for 6 h followed by freezing, packaging and hardening at -25°C for 24 h. The frozen yoghurt was stored at -18°C after hardening.

Analysis of milk and experimental frozen yoghurt: Whole milk and skimmed milk were analysed for fat, total solids and acidity as per the method described by FSSAI (2015). The prepared frozen yoghurt was analysed for fat, protein, ash, total solids and pH as per the method described by FSSAI (2015). Carbohydrates are calculated by difference. Viscosity of yoghurt mix, using Brookfield viscometer, as well as melting rate of frozen yoghurt was analysed by the method suggested by Shahein et al. (2022). Overrun of frozen yoghurt was calculated as per the formulae used by Ilansuriyan and Shanmugam (2018). Aerobic plate count, coliform count and yeast and Mold count were analysed using the method given by Shahein et al. (2022).

Sensory evaluation of experimental frozen yoghurt: The frozen yoghurt samples were stored at -13±2°C for 24 h before serving to the semi-trained judges (n=12). The judges were from the faculty of the institute who have basic idea about the product. Sensory analysis of the product was performed in isolated sensory booths illuminated with incandescent light maintained at 22±2°C. The well-labelled samples were presented in polystyrene cups in completely randomized order. The frozen yoghurt samples were evaluated using 100-point score card (Hussein et al. 2023).

Statistical analysis: A Central Composite Rotatable Design (CCRD) of the Response Surface Methodology (RSM) technique was adopted for the optimization of maltodextrin, salt and black pepper blend as well as stabilizer-emulsifier blend. The minimum and maximum levels of maltodextrin, salt and black pepper blend as well as stabilizer-emulsifier blend were selected as 4 and 8 per cent, 0.5 and 1.0 per cent as well as 0.25 and 0.75 per cent respectively, on the basis of preliminary trials. The CCRD of three factors contained 20 combinations, including lower and upper limits, along with their responses for sensory parameters as well as melting rate and overrun are displayed in Table 1. The data generated for different responses were analysed using Design Expert® software (13.0.2 version) (Stat-Ease, Inc., 2021 E. Hennepin Avenue, Minnepolis, USA). A general polynomial equation given below was fitted for each response.

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_{12} + a_{22}x_{22} + a_{33}x_{32} + a_{12}x_1x_2 + a_{23}x_2x_3 + a_{13}x_1x_3 + \text{Error term}$$

where Y represents the predicted response; a_0 the constant coefficient; a_{11}, a_{22} and a_{33} denote quadratic coefficients; a_{12}, a_{23} and a_{13} denote interaction coefficients; x_1, x_2 and x_3 denote rate of addition

of maltodextrin, salt and black pepper blend as well as stabilizer-emulsifier blend respectively.

Adequacy of the model was evaluated using coefficient of determination (R^2) and statistical significance was examined by F value. The effect of independent variables and individual responses was described at $P<0.05$. t-test for two samples assuming equal variance was applied using Microsoft Excel for comparison of predicted values with the actual values of the responses. The variation between control sample prepared using vanilla flavour and low-fat frozen yoghurt added with salt and black pepper was analysed using independent t-test.

Results and Discussion

The optimization of rate of addition of maltodextrin, salt and black pepper blend as well as stabilizer-emulsifier blend were carried out on the basis of sensory attributes such as flavour, body & texture, melting characteristics, colour & appearance and total score as well as melting rate and overrun. The successive regression analysis of the responses produced the quadratic models for each response. The variation in experimental data of fitted quadratic model was given by coefficient of determination (R^2) which ranged from 82 to 91 per cent (Table 2). The model F-value of the fitted quadratic model for all responses was found to be significant. The sufficient accuracy for predicting all response variables of the frozen yoghurt prepared from any combinations of variables within the range was evaluated by non-significant lack of fit. These indicate that the obtained quadratic model fitted the data strongly. The signal to noise ratio called *Adequate Precision Value (APV)* for a well fitted model should be above four. This measure also fulfilled for the obtained mode with APVs ranging from 8.16 to 13.78. All these results firmly recommended that the model could be used to develop low-fat frozen yoghurt added with salt and black pepper. Regression equation for predicting sensory score, melting rate and overrun of the experimental frozen yoghurt is depicted in Table 3.

Effect of variables on flavour: Flavour is the most important sensory characteristics for majority of dairy products which includes taste, odour and mouthfeel. The flavour score for the frozen yoghurt ranged from 35.14 to 40.57. The minimum flavour score was obtained when maltodextrin, salt & black pepper blend and stabilizer-emulsifier blend were added at the rate of 6.00, 1.17 and 0.50 per cent respectively. Similarly, the maximum flavour score was obtained when maltodextrin, salt & black pepper blend and stabilizer-emulsifier blend were added at the rate of 4.00, 0.50 and 0.75 per cent respectively (Table 1). Salt and black pepper blend significantly ($P<0.05$) improved flavour of the frozen yoghurt at linear level which could be attributed to

Table 1: Experimental design matrix showing factors and their responses for the development of low-fat frozen yoghurt added with salt and pepper

Std Run	A: Malto-dextrin (% w/w)	B: S+P@ (% w/w)	C: S+E** (% w/w)	Response 1: Flavour	Response 2: Body & Texture	Response 3: Melting character-ristics	Response 4: C&A#	Response 5: Total score*	Response 6: Melting rate, %	Response 7: Overrun, %
1	8.00	0.50	0.75	39.14	25.71	3.18	4.28	87.31	43.75	85.42
2	6.00	0.33	0.50	36.42	25.42	3.72	4.07	84.63	48.19	88.92
3	8.00	1.00	0.75	35.57	25.50	3.32	4.08	83.47	47.26	84.06
4	4.00	1.00	0.75	36.71	24.71	3.71	4.02	84.15	49.11	88.24
5	6.00	0.75	0.50	39.85	26.42	4.07	4.32	89.66	50.98	91.05
6	6.00	0.75	0.50	39.14	26.34	4.05	4.27	88.80	50.44	90.36
7	4.00	0.50	0.75	40.57	26.01	3.64	4.28	89.50	43.92	88.29
8	6.00	0.75	0.50	39.52	26.38	4.25	4.29	89.44	51.12	89.94
9	6.00	0.75	0.08	40.14	23.42	3.72	4.07	86.35	59.23	80.19
10	6.00	1.17	0.50	35.14	25.42	3.52	3.97	83.05	54.29	92.76
11	9.36	0.75	0.50	35.28	24.85	3.71	4.07	82.91	45.68	94.05
12	8.00	1.00	0.25	36.42	24.42	3.28	4.07	83.19	55.81	87.26
13	2.63	0.75	0.50	35.37	24.12	3.43	4.02	81.94	53.96	84.32
14	6.00	0.75	0.92	38.51	23.76	3.75	4.25	85.27	43.82	83.87
15	6.00	0.75	0.50	39.23	26.35	3.98	4.26	88.82	49.88	90.55
16	6.00	0.75	0.50	40.18	26.25	3.95	4.36	89.74	50.24	90.28
17	4.00	0.50	0.25	40.05	24.50	3.74	4.12	87.41	54.81	82.60
18	6.00	0.75	0.50	39.63	26.26	4.02	4.17	89.08	50.71	91.15
19	8.00	0.50	0.25	40.25	25.62	3.62	4.25	88.74	56.74	86.15
20	4.00	1.00	0.25	35.87	24.12	3.81	4.02	82.82	57.33	84.25

*Score for bacteria (15) was added in the Total score; @ Salt and pepper blend; **Stabilizer-emulsifier blend; #Colour and appearance

development of pleasant flavour due to salt and black pepper while at quadratic level, maltodextrin as well as salt and black pepper significantly (P<0.05) deteriorated the flavour. Maltodextrin produced typical bland flavour when added at higher level. Slow release of the flavour was also reported by the judges when maltodextrin was added at higher level. Addition of salt and black pepper blend in higher amount resulted in intense flavour which was not appreciated by the judges. Incorporation of maltodextrin in low-fat ice-cream improved flavour up to 5 per cent level followed by deterioration at higher level (Sonwane and Hembade, 2014). Solanki et al (2023) reported non-significant influence of rate of addition of stabilizers on flavor when added between 0.15 to 0.25 per cent. Incorporation of tulsi powder (Trivedi et al. 2014) in ice-cream improved flavour at initial level but deteriorated at higher level.

Effect of variables on body and texture: Body and texture is an important sensory characteristic for frozen products. The body and texture score varied between 23.42 and 26.42. The frozen yoghurt samples added with 6.00 per cent maltodextrin, 0.75 per cent salt & black pepper blend and 0.08 per cent stabilizer-emulsifier blend obtained minimum score while the one containing 6.00 per cent maltodextrin, 0.75 per cent salt & black pepper blend and 0.50 per cent stabilizer-emulsifier blend scored maximum (Table 1). Maltodextrin and stabilizer-emulsifier blend had significant (P<0.05) positive impact on body and texture of the product at linear level which could be due to improvement in the firmness of the body and smooth texture but they had significant (P<0.05) negative impact at quadratic level due to development of heavy and soggy body which could be due to higher viscosity of the yoghurt mix. The interaction of maltodextrin and stabilizer-emulsifier blend also had significant (P<0.05) positive impact. Incorporation of maltodextrin in low-fat ice-cream improved body & texture when added up to 5 per cent level. Addition of maltodextrin at higher level resulted in heavy and soggy body (Sonwane and Hembade, 2014). Body and texture score

Table 2: Regression coefficients and ANOVA fitted quadratic model for the responses of low-fat frozen yoghurt added with salt and pepper

Partial coefficients	Flavour	Body & texture	Melting characteristics	Colour & appearance	Total score	Melting rate, %	Overrun, %
Intercept	39.54	25.84	3.99	4.28	88.67	51.12	89.76
A-Maltodextrin	-0.17	0.25*	0.20*	0.03	1.48*	-0.34	0.23
B-S+P@	1.26*	-0.17	-0.02	0.07	0.41*	0.25	0.16
C-S+E#	-0.27	0.34*	0.28*	0.06	0.44*	-1.14*	1.49*
AB	0.03	0.18	-0.04	-0.02	-0.51	-0.12	0.19
AC	-0.48	0.29*	0.24*	-0.01	0.08	-0.95*	0.41
BC	0.02	-0.09	0.06	0.02	0.61	0.21	0.36
A^2	-1.23*	-0.30*	-0.16*	-0.04	-1.74*	-0.96*	1.25*
B^2	-0.96*	-0.09	-0.14*	-0.19*	-1.29*	0.56*	0.26
C^2	0.18	-0.44*	-1.02*	-0.09	-1.20*	-2.34*	-2.13*
Model fit statistics							
Lack of fit	0.002	0.034	0.038	0.027	0.001	<0.0001	<0.0001
Model F value	8.16	9.52	9.46	8.36	11.48	13.78	9.58
R^2	0.82	0.84	0.87	0.87	0.85	0.89	0.91
APV	8.24	9.32	9.25	8.14	11.23	12.89	9.31

@ Salt and pepper blend; #Stabilizer-emulsifier blend; *significant effect at 5% level

Table 3: Regression equation for predicting sensory score, melting rate and overrun of low-fat frozen yoghurt added with salt and pepper

Property	Equation
Flavour	$39.54 - 0.17A + 1.26B - 0.27C + 0.03AB - 0.48AC + 0.02AB - 1.23A^2 - 0.96B^2 + 0.18C^2$
Body & texture	$25.84 + 0.25A - 0.17B + 0.34C + 0.18AB + 0.29AC - 0.09BC - 0.3A^2 - 0.09B^2 - 0.44C^2$
Melting characteristics	$3.99 + 0.2A - 0.02B + 0.28C - 0.04AB + 0.24AC + 0.06BC - 0.16A^2 - 0.14B^2 - 1.02C^2$
Colour & appearance	$4.28 + 0.03A + 0.07B + 0.06C - 0.02AB - 0.01AC + 0.02BC - 0.04A^2 - 0.19B^2 - 0.09C^2$
Total score	$88.67 + 1.48A + 0.41B + 0.14C - 0.51AB + 0.08AC + 0.61BC - 1.74A^2 - 1.29B^2 - 1.20C^2$
Melting rate	$51.12 - 0.34A + 0.25B - 1.14C - 0.12AB - 0.95AC + 0.21BC - 0.96A^2 + 0.56B^2 - 2.34C^2$
Overrun	$89.76 + 0.23A + 0.16B + 1.49C + 0.19AB + 0.41AC + 0.36BC + 1.25A^2 + 0.26B^2 - 2.13C^2$

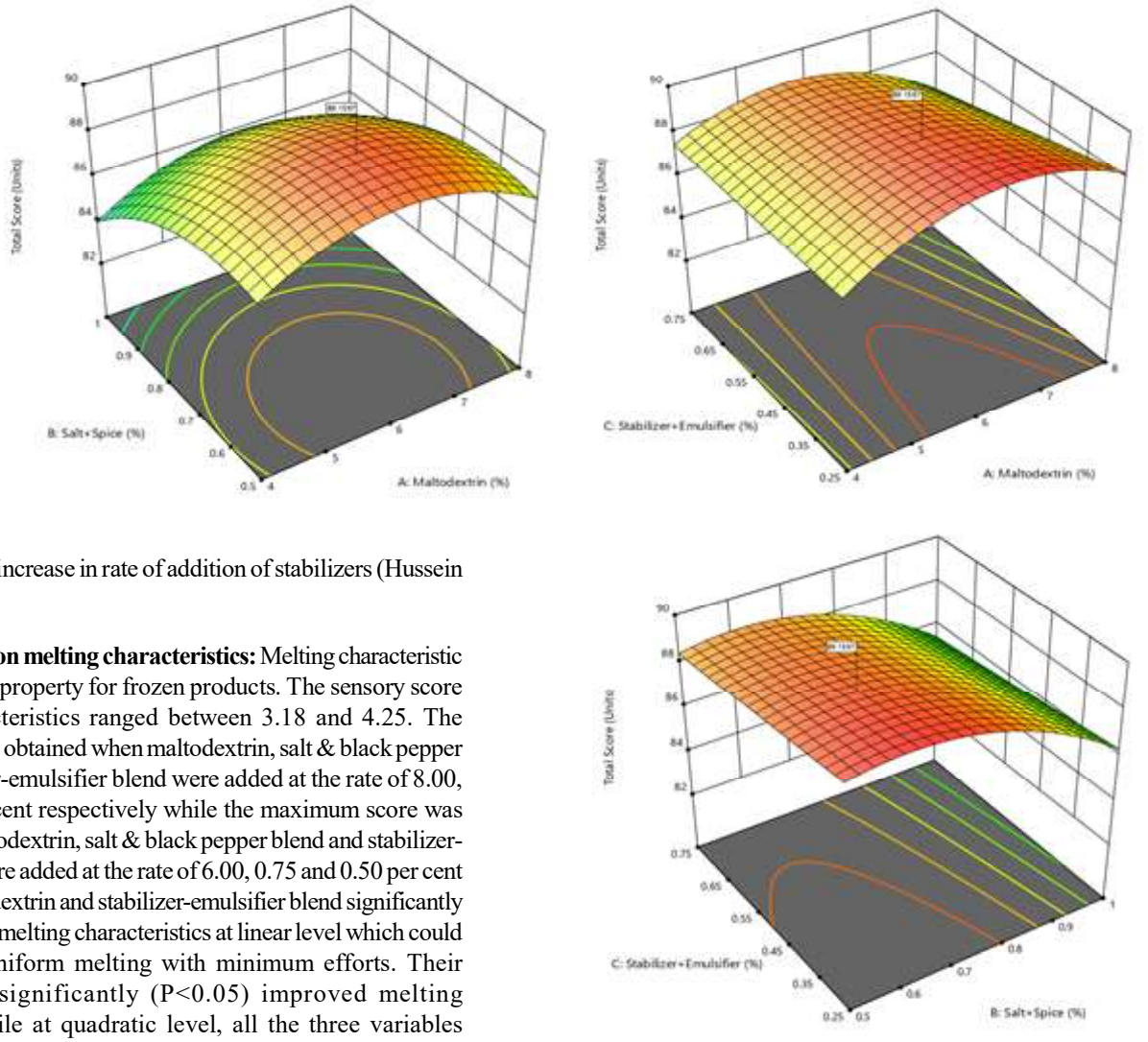


Fig. 1 Effect of different variables on total score of low-fat frozen yoghurt added with salt and pepper

also increased with increase in rate of addition of stabilizers (Hussein et al. 2023).

Effect of variables on melting characteristics: Melting characteristic is a unique sensory property for frozen products. The sensory score for melting characteristics ranged between 3.18 and 4.25. The minimum score was obtained when maltodextrin, salt & black pepper blend and stabilizer-emulsifier blend were added at the rate of 8.00, 0.50 and 0.75 per cent respectively while the maximum score was obtained when maltodextrin, salt & black pepper blend and stabilizer-emulsifier blend were added at the rate of 6.00, 0.75 and 0.50 per cent respectively. Maltodextrin and stabilizer-emulsifier blend significantly ($P<0.05$) improved melting characteristics at linear level which could be attributed to uniform melting with minimum efforts. Their interaction also significantly ($P<0.05$) improved melting characteristics while at quadratic level, all the three variables significantly ($P<0.05$) deteriorated melting characteristics. The product was criticized for very slow and uneven melting when maltodextrin and stabilizer-emulsifier blend were added at higher level while it melted very rapidly when salt & black pepper blend was added at higher level as addition of salt would result in freezing point depression yielding rapid melting. Addition of maltodextrin resulted in improvement in melting characteristics up to 3 per cent level followed by deterioration (Azari-anpar et al. 2017). Blassy et al. (2019) and Trivedi et al. (2014) also observed similar results in ginger ice-cream and basil ice-cream respectively.

Effect of variables on colour and appearance: Colour and appearance is first sensory attribute which is observed during sensory evaluation of a product. The colour and appearance score of the product ranged between 3.97 and 4.36. The minimum score was obtained by a product which contained 6.00 per cent maltodextrin, 1.17 per cent salt and black pepper blend and 0.50 per cent stabilizer-emulsifier mixture while the maximum score was obtained by the frozen yoghurt which contained 6.00 per cent maltodextrin, 0.75 per cent salt and black pepper blend and 0.50 per cent stabilizer-emulsifier mixture. Salt and black pepper blend had significant ($P<0.05$) negative effect at quadratic level which is majorly due to development of slight dark appearance because of the presence of black pepper. Maltodextrin and stabilizer-emulsifier blend failed to exert any impact on colour and appearance of low-fat frozen yoghurt added with salt and black pepper. Incorporation of black pepper in cottage cheese up to 1 per cent level improved colour and appearance followed by deterioration in it (Himabindu and Arunkumar, 2017). Addition of basil powder up to 3 per cent level improved colour and appearance of ice-cream followed by deterioration (Trivedi et al. 2014).

Effect of variables on total score: Total score is summation of the sensory score of all sensory attributes. Total score of the frozen yoghurt ranged from 81.94 to 89.74. The frozen yoghurt which obtained minimum score contained 2.63 per cent maltodextrin, 0.75 per cent salt and black pepper blend and 0.50 per cent stabilizer-emulsifier mixture while maximum score was obtained by a product

which contained 6.00 per cent maltodextrin, 0.75 per cent salt and black pepper blend and 0.50 per cent stabilizer-emulsifier mixture. All the three variables significantly (P<0.05) increased total score at linear level while they significantly (P<0.05) reduced it at quadratic level. Addition of maltodextrin up to 5 per cent level increased total sensory score of low-fat ice-creams followed by reduction at higher level (Sonwane and Hembade, 2014). Several other researchers observed similar trend in ice-cream (Trivedi et al. 2014; Ateteallah et al. 2019; Butt et al. 2023).

Effect of variables on melting rate: Melting rate is an important characteristic for frozen products. The melting should not be too rapid and too slow. The melting rate of the experimental frozen yoghurt varied between 43.75 and 59.23 per cent. The product containing 8.00 per cent maltodextrin, 0.50 per cent salt and black pepper blend and 0.75 per cent stabilizer-emulsifier mixture showed minimum melting while the one containing 6.00 per cent maltodextrin, 0.75 per cent salt and black pepper blend and 0.08 per cent stabilizer-emulsifier mixture showed maximum melting. Stabilizer-emulsifier mixture significantly (P<0.05) reduced melting of the product at linear as well as quadratic level which could be attributed to water binding by stabilizer and increased overrun by emulsifier. At quadratic level, maltodextrin significantly (P<0.05) decreased melting that could be ascribed to increased viscosity and overrun with the addition of maltodextrin while salt and black pepper blend significantly (P<0.05) increased it by increasing freezing point depression. The interaction of maltodextrin and stabilizer-emulsifier mixture significantly (P<0.05) reduced melting rate by increasing water binding, viscosity and overrun. Increasing the rate of addition of stabilizer-emulsifier mixture from 0.144 to 0.198 per cent reduced melting rate of frozen yoghurt (Tawfek, 2021). Increasing κ-carrageenan from 0.05 to 0.15 per cent and corn starch from 1 to 3 per cent reduced melting rate of lactose-free frozen yoghurt (Skryplonek et al. 2019).

Effect of variables on overrun: Overrun is related to the amount of air incorporated during freezing. Frozen product should have optimum overrun. Higher overrun leads to fluffy body while lower overrun results in heavy and soggy body. The overrun of experimental frozen yoghurt varied from 80.19 to 94.05 per cent. The frozen yoghurt containing 6.00 per cent maltodextrin, 0.75 per cent salt and black pepper blend and 0.08 per cent stabilizer-emulsifier mixture had minimum overrun while the one containing 9.36 per cent maltodextrin, 0.75 per cent salt & black pepper blend and 0.50 per cent stabilizer-emulsifier mixture displayed maximum overrun. Stabilizer-emulsifier significantly (P<0.05) increased overrun at linear level by increasing water binding and thus viscosity. At quadratic level, maltodextrin significantly (P<0.05) increased overrun while stabilizer-emulsifier mixture significantly (P<0.05) reduced overrun by excessively increasing viscosity resulting in poor air incorporation (Syed and Shah, 2016). Swelam et al. (2021) reported increase in overrun up to the level of 0.25 per cent. Though the combination of stabilizers and emulsifiers was different as well as the process of preparation of frozen yoghurt was also different. Increase in carrageenan content from 0.05 to 0.15 per cent reduced overrun by almost 5.0 per cent while increasing corn starch from 1 to 3 per cent reduced overrun rate by 6 per cent (Skryplonek et al. 2019).

Table 4: Goals set for constraints to optimize the low-fat frozen yoghurt added with salt and pepper

Constraint	Goal	Lower limit	Upper limit
Maltodextrin, %	Maximize	4.00	8.00
S+P#, %	In range	0.50	1.00
S+E*, %	In range	0.25	0.75
Flavour	Maximize	35.14	40.57
Body & texture	Maximize	23.42	26.42
Melting characteristics	Maximize	3.28	4.25
Colour & appearance	Maximize	3.97	4.36
Total score	Maximize	81.94	89.74
Melting rate, %	Target – 50	43.75	59.23
Overrun, %	Target - 90	80.19	94.05

Salt and pepper blend; *Stabilizer-emulsifier blend

Table 5: Comparison of predicted values and observed values for low-fat frozen yoghurt added with salt and pepper

Attribute	Predicted value	Observed value	t-value
Flavour	39.96	39.93	NS
Body & texture	25.94	25.98	NS
Melting characteristics	3.98	4.00	NS
Colour & appearance	4.27	4.26	NS
Total score	89.16	89.17	NS
Melting rate, %	49.99	50.05	NS
Overrun, %	89.99	90.14	NS

Table 6: Comparison of experimental frozen yoghurt with control frozen yoghurt

Parameter	Control frozen yoghurt	Low-fat frozen yoghurt added with salt and pepper	t-value
Chemical composition			
Moisture, %	73.83±0.94	73.86±0.82	NS
Fat, %	2.33±0.12	2.29±0.09	NS
Protein, %	4.16±0.16	4.19±0.02	NS
Ash, %	1.89±0.02	1.91±0.02	NS
Carbohydrates, %	17.79±0.32	17.76±0.38	NS
Physical characteirstics			
Melting rate, %	53.19±0.55	50.07±0.17	NS
Overrun, %	92.61±0.87	90.11±0.21	NS
Sensory characteristics			
Flavour	39.45±0.97	39.93±0.83	NS
Body & texture	25.50±0.53	25.98±0.71	NS
Melting characteristics	3.90±0.18	4.00±0.16	NS
Colour & appearance	4.05±0.21	4.26±0.19	NS
Total Score*	87.90±1.33	89.17±1.54	NS
Microbial analysis			
APC (log_{10}cfu/g)	7.81±1.05	7.87±0.91	NS
Coliform	Absent in 1 g		
Y&M	Absent in 1 g		

*Score for bacteria (15) was added in the Total score

Optimization of variables for preparation of low-fat frozen yoghurt

The optimization of variables such as the rate of addition of maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture was carried out using numerical optimization technique. The criteria used for optimization are summarized in Table 4. Among the variables, the level of maltodextrin as well as salt and black pepper blend were maximized while stabilizer-emulsifier mixture was kept in range. Among the responses, sensory parameters were maximized while melting rate and overrun were set to target of 50 and 90 per cent respectively for the optimization process. RSM suggested the rate of addition of maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture to be 6.12, 0.65 and 0.45 per cent respectively with desirability of 0.86. The low-fat frozen yoghurt was prepared by adding maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture according to the suggestion of RSM. The predicted values for flavour, body & texture, melting characteristics, colour & appearance, total score, melting rate and overrun for the developed frozen yoghurt were 39.96, 25.94, 3.98, 4.27, 89.16, 49.99 and 89.99 respectively. The predicted values are not significantly ($P>0.05$) different from observed values for all the parameters (Table 5). Therefore, it was confirmed that the selected level of maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture is most suitable for preparation of low-fat frozen yoghurt with replacement of sugar with salt and black pepper.

Analysis of low-fat frozen yoghurt

The developed frozen yoghurt was analysed for its compositional parameters, physical characteristics, sensory attributes and microbiological quality and compared with control frozen yoghurt prepared using vanilla flavour. No significant ($P>0.05$) difference was observed between experimental and control sample for all the parameters (Table 6).

Conclusion

Low-fat frozen yoghurt was prepared by replacing sugar with salt & black pepper blend as well as maltodextrin. Stabilizer-emulsifier mixture was also modified accordingly. Maltodextrin, salt & black pepper blend and stabilizer-emulsifier mixture were optimized using response surface methodology. The optimized frozen yoghurt was highly acceptable.

References

Ateteallah H, Abd-Elkarim N, Hassan NA (2019) Effect of adding beetroot juice and carrot pulps on rheological, chemical, nutritional and organoleptic properties of ice cream. J Food Dairy Sci 10:175-179

Azari-Anpar M, Khomeiri M, Ghafouri-Oskuei H, Aghajani N (2017) Response surface optimization of low-fat ice cream production by using resistant starch and maltodextrin as a fat replacing agent. J Food Sci Technol 54:1175-1183

Blassy K, Osman M, Abbas F, Galal N (2019) Functional low-fat frozen yoghurt with carrot (Dascus Carota L.) puree. Ismailia J Dairy Sci Technol 6:19-34

Butt AY, Haq A, Aamir SH, Ashraf S, Ali R (2023) Development of functional ice cream by incorporation of oat milk and beetroot. J Agric Sci Food Res 14:1-6

Dean A, Voss D, Draguljić D (2017) Response Surface Methodology. In: Design and Analysis of Experiments. Springer Texts in Statistics. Springer, Cham.

Dharaiya CN, Jana A, Patel AM, Patel DH (2021) Comparison of natural Mozzarella cheese with acid casein-based Mozzarella cheese analogue. Indian J Dairy Sci 74:301-308

FSSAI (2015) Manual of Methods of Analysis of Foods: Milk and Milk Products. Food Safety and Standards Authority of India, New Delhi

Granato D, Santos JS, Salem RDS, Mortazavian AM, Rocha RS, Cruz AG (2018) Effects of herbal extracts on quality traits of yogurts, cheeses, fermented milks, and ice creams: a technological perspective. Curr Opin Food Sci 19:1-7

Himabindu D, Arunkumar H (2017) Effect of Black Pepper (*Piper Nigrum* L.) on the keeping quality of spiced cottage cheese. Res Rev: J Food Dairy Technol 5:30-36

Ilansuriyan P, Shanmugam M (2018) Rheological, physicochemical and sensory properties of no fat to high fat ice creams samples prepared using stabilizer/emulsifier blends created with liquid and powder polysorbate-80. Int Food Res J 25:2579-2584

Hussein HM, El-Kenany YM, Awad RA, El-Naga MYA (2023) Evaluation of frozen yoghurt produced with vegetable oils. Egypt J Food Sci 51:233-239

Jeong MY, Jang HM, Kim DH (2019) High-fat diet causes psychiatric disorders in mice by increasing Proteobacteria population. *Neurosci Lett*, 698:51-57

Kugashiya DS, Dharaiya CN, Mallik JM, Rathwa RB (2023) Application of carrot powder in preparation of low-fat frozen yoghurt. Indian J Dairy Sci 76:534-541

More TA, Shaikh Z, Ali A (2021) Artificial sweeteners and their health implications: A review. Biosci Biotechnol Res Asia 18:227-237

Shahein MR, Elkot WF, Albezrah NKA, Abdel-Hafez LJM, Alharbi MA, Massoud D, Elmahallawy EK (2022) Insights into the Microbiological and Physicochemical Properties of Bio-Frozen Yoghurt Made with Probiotic Strains in Combination with Jerusalem Artichoke Tubers Powder. Fermentation, 8:390-402

Sharif MK, Ejaz R, Pasha I (2018) Nutritional and therapeutic potential of spices. In *Therapeutic, Probiotic, and Unconventional Foods* (pp.181-199). Academic Press.

Skryplonek K, Henriques M, Viegas J, Fonseca C, Pereira C, Dmytrow I, Mituniewicz-Malek A (2019) Characteristics of lactose-free frozen yoghurt with κ-carrageenan and corn starch as stabilizers. J Dairy Sci 102:7838-7848

Solanki K, Rani R, Gaur GK (2023) The development and characterization of herbal kulfi (ice cream) using tulsi, ginger and clove. Indian J Dairy Sci 76:448-457

Sonwane RS, Hembade AS (2014) Sensorial quality of dietetic soft serve ice cream prepared by using different proportions of maltodextrin. Int J Curr Res Acad Rev 2:51-55

Swelam S, Zommara MA, El-Aziz MA, Elgammal NA, Baty RS, Elamhallawy EK (2021) Insights into chufa milk frozen yoghurt as cheap functional frozen yoghurt with high nutritional value. Fermentation, 7:255-264

Syed QA, Shah MSU (2016) Impact of stabilizers on ice cream quality characteristics. *MOJ Food Process Technol*, 3:246-252

Tawfek MAE (2021) Properties of low fat bio-frozen yoghurt fortified with extract and powder of pomegranate peel (Punica Ganatum L.) Egyptian J Food Sci 49:267-286

Trivedi V, Prajapati J, Pinto S, Darji V (2014) Use of basil (tulsi) as flavouring ingredient in the manufacture of ice cream. Amer Int J Contemp Res 3:28-43

WHO (2023) Report of the fourth meeting of the WHO technical advisory group on diabetes: hybrid meeting, 30 November-1 December 2022, Geneva

Indian J Dairy Sci 78(2): 134-139
https://doi.org/10.33785/IJDS.2025.v78i02.005

RESEARCH ARTICLE

Quality characteristics of apple fruit pulp and tulsi leaves powder incorporated goat milk shrikhand

Vivek Sahu[1], Vikas Pathak[2], Meena Goswami[3]✉ and Priya[4]

Received: 03 August 2023 / Accepted: 15 January 2024 / Published online: 23 April 2025

Abstract: The present study was conducted to evaluate the effect of apple fruit pulp and tulsi leaves powder on goat milk shrikhand. Preliminary trials were conducted to optimize the apple fruit pulp level in goat milk shrikhand, where 25% pulp was selected as the best treatment. This apple fruit pulp based goat milk shrikhand was further treated with different levels of tulsi leaves powder *i.e.* 0.4% (AT1), 0.6% (AT2) and 0.8% (AT3) and evaluated for various physico-chemical properties and sensory evaluation. The results revealed that pH and brix values decreased whereas ash content increased significantly ($P<0.05$) with increased level of tulsi leaves powder. There was no significant difference in titratable acidity, moisture, protein, fat content and water activity values between control and treatments. Among the textual and colour parameters, firmness, consistency, work of cohesiveness, lightness and yellowness values decreased significantly ($P<0.05$). Sensory scores decreased significantly ($P<0.05$) with incorporation of tulsi leaves powder, however AT1 had significantly ($P<0.05$) higher overall acceptability scores than AT2 and AT3. Therefore, AT1- goat milk shrikhand with 25% apple fruit pulp and 0.4% tulsi leaves powder was selected as the best treatment.

Keywords: Goat milk shrikhand, Apple fruit pulp, Textural and colour parameter, Sensory evaluation

[1,2,3]Department of Livestock Products Technology, College of Veterinary Sciences and Animal Husbandry, DUVASU, Mathura-281001
[4] Livestock Production and Management,College of Veterinary Science, Rampura Phul, Bhatinda, GADVASU, Punjab

Meena Goswami (✉)
Department of Livestock Products Technology
College of Veterinary Sciences and A.H.
DUVASU, Mathura, (U.P), India. Pin-281001
Email: dr.goswami2008@yahoo.co.in

Introduction

Milk production in the country has grown at a compound annual growth rate of about 6.2 % to reach 230.6 million tones (NDDB, 2023) due to advancement of technology, proper nutrition and appropriate managemental practices. Livestock contributes about 9.2% in gross value added (GVA) and 26.2 % in agriculture sector in India. The livestock population in India includes 302.3 million bovines, 74.3 million sheep, 148.9 million goats, about 9.1 million pigs and 851.8 million poultry. The rural and urban population of goat is 129.081 million and 6.092 million respectively in India. Total goat milk production in India is 7.61 million tones and it shares a contribution of 3.30% in the total milk production across the country (DAHD, 2023).. Goat milk production is a dynamic and growing industry that is fundamental to the wellbeing of millions people worldwide and is an important part of the economy in India. Goat milk is having better digestibility, alkalinity, buffering capacity and certain therapeutic values in medicine and human nutrition (Goswami et al. 2017) in comparison to cow's or human milk. The goat milk microbiota is also considered a good source of novel bacteriogenic *Lactic acid* bacteria (LAB) strains that can be exploited as an alternative for use as bio preservative in food (Perin and Nero, 2014). It is also rich source of amino acid, being 20-40 folds higher than cow milk (Mehaia and Al-Kanhal, 1992) which is involved in bile salt formation, osmoregulation, antioxidation, calcium transport and in the central nervous system (Redmond et al. 1998). Minerals content such as calcium, potassium, magnesium and chloride as well as vitamin A, B, C, D, thiamin and niacin content of goat milk is higher than that of cow milk. Goat milk is considered as —self-homogenized milk. Goat milk contains, water, protein, fat, sugar, minerals, and vitamins, which are essential for the maintenance of good health. Goat milk and its processed products are useful as functional foods, maintaining nourishment and health of young and elders (Singh et al. 2021). Goat milk also contains higher content of three characteristics fatty acids *i.e.* caproic acid, caprylic and capric acid which are having medicinal values for patients suffering from malabsorption, childhood epilepsy, cystic fibrosis and gallstones (Haenlin, 1992); however these are responsible for intense "goaty flavour" which limits the acceptability of goat milk products among the consumers.

Traditional dairy foods play a pivotal role in preservation of essential milk nutrients and promotion of its consumption among masses. Shrikhand is one of the widely relished indigenous milk product prepared by the fermentation of milk by using known strain of lactic acid bacteria. It is produced from chakka which in turn is obtained from dahi (curd) after draining off the whey. Shrikhand is a homogenous mass prepared from chakka with sugar, colour and flavor as basic ingredients (Sahu et al. 2021). The popularity of fermented dairy products from goats' milk has shown a gradual increase all over the world due to its better functional properties and health benefits. However, goat milk is also considered to be deficient in dietary fiber like milk from cattle and buffalo. The characteristic flavour is another constraint in acceptability of goat milk and products. The incorporation of fruit pulp in goat milk products like shrikhand will not only enhance the nutritional content especially in terms of dietary fiber but will also substantially mask the characteristic odour of shrikhand prepared by goat milk. Apple (*Malu sdomestica*) is one of leading fruits of Rosaceae family which is grown in temperate regions of various countries including India. The therapeutic value of apple is well known for different illnesses and is good for the treatment of anemia, dysentery, heart disease, kidney stones (Noure*t al.* 2010). It contains 85% water, 13% carbohydrate and 2.2% total dietary fiber. Incorporation of fruits pulp may enhance shrikhand's nutritive value making it more prone for physico-chemical and microbiological spoilage. Tulsi (*Ocimum tenuiflorum*) or holy basil is an aromatic shrub in family *Lamiaceae* that is thought to have originated in north central India and now grows native throughout the eastern world tropics. Tulsi is a sacred herb that has been used in Ayurveda and other traditional medical treatments in India for thousands of years. eugenol, camphor, flavonoids, nerol, and various terpenes. This rich blend of organic compounds *i.e.* eugenol, camphor, flavonoids, nerol present in tulsi delivers a number of health benefits and can help relieve acne, asthma, inflammation, respiratory issues, and lower your chances of heart diseases and atherosclerosis (Hanaa et al. 2016). There, the present study was conducted to evaluate the effect of tulsi leaves powder on physico-chemical properties and sensory evaluation of apple fruit pulp based goat milk shrikhand.

Materials and methods

The experiments were carried out in the Department of Livestock Products Technology, College of Veterinary Sciences and Animal Husbandry, U.P. Pt. Deen Dayal Upadhyaya Pashu ChikitsaVigyanVishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura, 281001 (UP), India. Starter culture (NCDC-159) was procured from NDRI, Karnal which contained mixed culture of *Lactococcus lactis, Lactococccus diacetylactis* and *Lactococcus cremoris.* The culture was activated to as per the standard method and the activated parent culture was maintained by sub culturing and stored under refrigeration. Clean crystalline sugar was procured from local market of Mathura. All the chemicals used in the study were of analytical grade and procured from Hi Media laboratories (P) Ltd, Mumbai.

Preparation of Shrikhand

The shrikhand was prepared as per method described by Gupta et al. (2018) with slight modifications. Fresh goat milk was filtered through muslin cloth and then fat content was standardized using Pearson square method. Then milk was subjected to heat treatment at 85 °C for 30 minutes followed by cooling at 37±2°C. Milk was inoculated with NCDC-159 @ 2.5 % by v/v of milk and incubated at 35-37 °C for 12-15 hours for proper curd setting. The curd thus obtained was transferred to clean muslin cloth and hanged for 16-18 hours in order to drain the whey to obtain chakka. The chakka was kneaded to have uniform consistency and then mixed with 30% ground sugar, apple fruit pulp and different level of tulsi leaves powder. Finally shrikhand was filled in pre sterilized thermo rigid polypropylene cups and stored at under refrigeration at 4±2°C. In present study, following abbreviations were used for present experiment: AT1- goat milk shrikhand prepared with 25% apple fruit pulp with 0.4% tulsi leaves powder, AT2- goat milk shrikhand prepared with 25% apple fruit pulp with 0.6% tulsi leaves powder and AT3- goat milk shrikhand prepared with 25% apple fruit pulp with 0.8% tulsi leaves powder**.**

Analytical methods

Physic-chemical properties

The pH of shrikhand was determined by using digital pH meter (WTW, Germany, model pH 330i) as per method given by Trout et al. (1992). Water activity of each sample was measured three times in duplicate using a water activity meter (AquaLab 3 TE, Inc. Pullman, WA) at Department of Goat Products Technology, CIRG, Makdhoom. Proximate parameters viz. moisture, protein, fat and ash content were estimated as per AOAC (1995).

Textural and colour parameters

The texture profile analysis of shrikhand was done with the help of instrumental texture profile analyser (TA HD Plus Texture analyser) for firmness, consistency, cohesiveness and work of cohesiveness (Bourne, 1978). Texture analyzer equipped with 5 kg load cell and back extrusion test (A/BE) using 35 mm cylinder probe was used where pre-test speed, test speed and post test speed was set at 1 mm/sec, 1mm/sec and10mm/sec respectively. The 30 mm distance was set with Auto (F) -10g Trigger type and 0.04903 N force. The colour parameters *i.e.* lightness (*L**), redness (*a**) and yellowness (*b**) of the shrikhand were measured using Hunter colourimeter of ColourTech PCM+ (Colour Tec Associates Inc. Clinton NJ, USA).

Sensory evaluation

Sensory evaluation was conducted by experienced semi trained panellists using 9-point descriptive scale (where 1= extremely disliked and 9= extremely liked) (Keeton, 1983) for colour and appearance, flavour, texture, sweetness, mouth coating and overall acceptability. Samples were served for sensory evaluation at around 7-9°C temperature in sensory evaluation room at late afternoon around 4:00 p.m. Sensory panellists were not allowed to communicate with each other and plain lukewarm water was given for mouth rinsing in between sensing two samples.

Statistical analysis

The data obtained in the study on various parameters were statistically analyzed on 'SPSS-16.0' software package as per standard methods of Snedecor and Cochran (1995). Duplicate samples were drawn for each parameter and the experiment was replicated thrice (n=6). Sensory evaluation was performed by a panel of seven member judges three times, so total observations being 21 (n=21) Data were subjected to one way analysis of variance, homogeneity test and Duncan's Multiple Range Test (DMRT) for comparing the means to find the effects between samples.

Results and Discussion

Several preliminary trials were conducted to standardize the processing technology of apple fruit based goat milk shrikhand on the basis of literature and preliminary trials. The final formulation of goat milk shrikhand was optimized following the method prescribed by Gupta et al.(2018) and 25% apple fruit pulp was finally selected on the basis of sensory evaluation.

Physico-chemical properties

The physico-chemical properties of goat milk shrikhand prepared with different levels of apple fruit pulp are presented in table 1. The pH and brix values decreased significantly (P<0.05) with increased level of tulsi leaves powder in treatments; however there was no significant difference between A3 and AT1 as well as between AT2 and AT3. Sahu et al. (2021) also reported significant (P<0.05) increase in brix values of goat milk shrikhand with increased level of starter culture. There was no significant difference in titratable acidity, moisture, protein and fat content as well as water activity values between control and treatments. Pramanick et al. (2017) also reported no significant change in protein and fat content of value added rasogulla prepared by incorporation of tulsi leaf extract. As per Sahu et al. (2022), protein and fat content decreased significantly (P<0.05) whereas ash content, water activity and brix values increased significantly (P<0.05) with the incorporation of papaya in goat milk shrikhand. Ash content increased significantly (P<0.05) with increased level of tulsi leaves powder in treatments, whereas ash content of AT2 was comparable to AT1 and AT3. Singh et al. (2023) reported that chemical composition of yogurt prepared by addition of (0.20 to 2.50) kiwi fruit pulp had 0.20% fat, 0.17% protein, 83.07% moisture, 16.70% total solids, 0.15% ash, 3.26 pH, 1.43% Titrable acidity and 14.67 % carbohydrate. Mehrotra et al. (2014) also observed significant (P<0.05) increase in ash content of yogurt incorporated with different levels of *Stevia* leaves powder. Vidhani et al. (2016) reported that ash content in tulsi leaves ranges between 0.90 to 0.96% which contained higher amount of minerals *i.e.* 61.75 pm Mn, 32.38ppm Zn, 0.62ppm K, 0.74ppm Na and 1.10 ppm P respectively. Kumar et al. (2023) also reported that goat milk yogurt contained 32.03±0.07 ppm Fe, 360.25±0.07 ppm Zn, 145.68±0.09 Na, 225.86±0.08 Ca and 285.27±0.05 Mg content.

Textural and colour parameters

The values of textural and colour parameters of goat milk shrikhand prepared with different levels of apple fruit pulp are presented in

Table 1: Physio-chemical properties (Mean±SE) of goat milk shrikhand prepared with different levels of apple fruit pulp

Parameters	A3	AT1	AT2	AT3	Treatment Mean
pH	4.21^{a}±0.03	4.18^{a}±0.02	4.15^{b}±0.03	4.11^{b}±0.03	4.16±0.03
Titratable acidity	0.54±0.01	0.55±0.02	0.56±0.02	0.56±0.01	0.55±0.02
Moisture (%)	47.24±0.24	47.45±0.36	47.50±0.25	47.63±0.31	47.45±0.35
Protein (%)	5.32±0.11	5.35±0.09	5.27±0.07	5.24±0.07	5.29±0.20
Fat (%)	7.22±0.07	7.25±0.12	7.23±0.11	7.18±0.11	7.23±0.12
Ash (%)	0.82^{c}±0.01	0.84^{b}±0.01	0.87^{ab}±0.02	0.90^{a}±0.02	0.85±0.01
Water activity	0.959±0.01	0.958±0.02	0.958±0.01	0.957±0.04	0.956±0.02
Brix value	33.57^{a}±0.12	33.30^{a}±0.08	32.49^{b}±0.05	32.26^{b}±0.10	32.90±0.14

- Overall means bearing different superscripts in a row (a, b, c, d.....) differ significantly (P<0.05)
- n=6
- A3- goat milk shrikhand with 25% apple fruit pulp and 0% tulsi leaves powder
- AT1- goat milk shrikhand with 25% apple fruit pulp and 0.4% tulsi leaves powder
- AT2- goat milk shrikhand with 25% apple fruit pulp and 0.6% tulsi leaves powder
- AT3- goat milk shrikhand with 25% apple fruit pulp and 0.8% tulsi leaves powder

table 2 & 3. Firmness, consistency and work of cohesiveness values of A3 and AT1 had significantly (P<0.05) higher than AT2 and AT3; however there was no significant difference between A3 and AT1.The decrease in textural parameters values was due to an increase in compactness on microstructure of shrikhand prepared using Tulsi extract (Rai et al. 2018)**.** There was no significant difference in cohesiveness values between control and treatments due to very less amount of tulsi leaves powder added in goat milk shrikhand. In contrast to present study, Fodaet al. (2007) reported significant (P<0.05) increase in firmness values of turmeric powder incorporated herbal milk.Lightness and yellowness values of A3 and AT1 were significantly (P<0.05) higher than AT2 and AT3; however there was no significant difference between A3 and AT1. Lower colour values at higher level

Table 2: Texture and colour parameters (Mean±SE) of goat milk shrikhand prepared with different levels of apple fruit pulp

Parameters	A3	AT1	AT2	AT3	Treatment Mean
Firmness	79.18^{a}±0.24	79.09^{a}±0.12	77.03^{b}±0.26	75.29^{c}±0.21	77.64±0.26
Consistency	60.24^{a}±0.25	60.18^{a}±0.20	58.88^{b}±0.32	57.74^{c}±0.26	59.26±0.29
Cohesiveness	47.31±0.25	47.45±0.31	47.76±0.34	48.12±0.26	47.58±0.28
Work of cohesiveness	41.51^{a}±0.27	41.60^{a}±0.18	39.87^{b}±0.19	37.64^{c}±0.13	40.15±0.18

Overall means bearing different superscripts in a row (a, b, c, d.....) differ significantly (P<0.05)
n=6
A3- goat milk shrikhand with 25% apple fruit pulp and 0% tulsi leaves powder
AT1- goat milk shrikhand with 25% apple fruit pulp and 0.4% tulsi leaves powder
AT2- goat milk shrikhand with 25% apple fruit pulp and 0.6% tulsi leaves powder
AT3- goat milk shrikhand with 25% apple fruit pulp and 0.8% tulsi leaves powder

Table 3: Colour estimation (Mean±SE) of goat milk shrikhand prepared with different levels of applefruit pulp

Parameters	A3	AT1	AT2	AT3	Treatment Mean
Lightness *(L*)*	71.85^{a}±0.37	71.29^{a}±0.35	69.54^{b}±0.20	67.29^{c}±0.35	69.99±0.42
Redness *(a*)*	10.22±0.11	10.66±0.09	10.89±0.09	11.41±0.15	10.79±0.51
Yellowness *(b*)*	11.58^{a}±0.11	11.51^{a}±0.09	10.04^{b}±0.04	9.54^{c}±0.04	10.66±0.19

Overall means bearing different superscripts in a row (a, b, c, d.....) differ significantly (P<0.05)
n=6
A3- goat milk shrikhand with 25% apple fruit pulp and 0% tulsi leaves powder
AT1- goat milk shrikhand with 25% apple fruit pulp and 0.4% tulsi leaves powder
AT2- goat milk shrikhand with 25% apple fruit pulp and 0.6% tulsi leaves powder
AT3- goat milk shrikhand with 25% apple fruit pulp and 0.8% tulsi leaves powder

Table 4: Sensory evaluation (Mean±SE) of goat milk shrikhand prepared with different levels of apple fruit pulp

Attributes	A3	AT1	AT2	AT3	Treatment Mean
Colour and appearance	7.27^{a}±0.03	7.13^{a}±0.06	6.70^{b}±0.09	6.46^{c}±0.10	6.89±0.08
Flavour	7.26^{a}±0.05	7.16^{b}±0.06	6.73^{c}±0.08	6.42^{c}±0.11	6.89±0.08
Texture	7.13^{a}±0.07	7.12^{b}±0.07	6.80^{c}±0.11	6.56^{c}±0.10	6.90±0.08
Sweetness	7.17^{a}±0.08	7.07^{ab}±0.05	6.96^{b}±0.08	6.46^{c}±0.12	6.91±0.07
Mouth coating	7.24^{a}±0.08	7.12^{b}±0.10	6.82^{c}±0.10	6.51^{c}±0.10	6.92±0.10
Overall acceptability	7.31^{a}±0.05	7.18^{b}±0.07	6.75^{c}±0.08	6.48^{c}±0.11	6.93±0.09

Overall means bearing different superscripts in a row (a, b, c, d.....) differ significantly (P<0.05)
n=21
A3- goat milk shrikhand with 25% apple fruit pulp and 0% tulsi leaves powder
AT1- goat milk shrikhand with 25% apple fruit pulp and 0.4% tulsi leaves powder
AT2- goat milk shrikhand with 25% apple fruit pulp and 0.6% tulsi leaves powder
AT3- goat milk shrikhand with 25% apple fruit pulp and 0.8% tulsi leaves powder

of tulsi incorporation in goat milk shrikhand might be due to dark green colour of tulsi leaves powder. Gaur et al. (2019) also reported that addition of tulsi leaves juice and turmeric powder imparted dark green colour to herbal flavoured milk. Merai et al. (2002) and Kumar et al. (2018) also observed similar results in tulsi leaves powder incorporated ghee and curry leaves powder incorporated herbal ice cream respectively. There was no significant difference in redness values between control and treatments.

Sensory evaluation

The sensory scores of goat milk shrikhand prepared with different levels of apple fruit pulp are presented in table 4. Sensory scores of all attributes decreased significantly ($P<0.05$) with increased level of tulsi leaves powder in apple fruit pulp incorporated goat milk shrikhand. Colour and appearance scores of A3 and AT1 were significantly ($P<0.05$) higher than AT2 and AT3; however there was no significant difference between A3 and AT1. Kumar et al. (2013) observed that colour and appearance scores of ice cream decreased significantly ($P<0.05$) with increased level of tulsi leaves powder due to dark green colour. However, Johri and Chauhan (2014) reported that color acceptability of herbal Tulsi doi was significantly ($P<0.05$) higher than control doi samples. The scores of sweetness of A3 were significantly ($P<0.05$) higher than AT2 and AT3; however scores of AT1 were comparable to A3 and AT2. Flavour, texture, mouth coating and overall acceptability scores of A3 were significantly ($P<0.05$) higher than AT1, AT2 and AT3. Kumar et al. (2018) also reported lower body and texture scores of herbal ice cream incorporated with 0.25-0.75% curry leaves and lemon grass powder due to perception of 'crumbly' texture and sometimes 'chewy' body in such ice cream. Among the treatments, AT1 had significantly ($P<0.05$) higher cores than AT2 and AT3; however there was no significant difference between AT2 and AT3. Therefore, AT1- goat milk shrikhand with 25% apple fruit pulp and 0.4% tulsi leaves powder was selected as the best treatment.

Conclusion

Fruits are essential parts of human life enriched with many macro and micronutrients also they add fiber to our food. Addition of apple fruit pulp and tulsi leaves powder did not only mask the goaty flavor but also improved the texture of goat milk shrikhand in terms of consistency, cohesiveness and work of cohesiveness upto0.4% level.Therefore it was concluded that goat milk shrikhand blended with 25% apple fruit pulp and 0.4% tulsi leaves powder was well acceptable in terms of flavor, consistency and sensory evaluation. Further studies may be carried out to evaluate the shelf life of tulsi leaves powder in terms of lipid oxidation and microbial count.

References

AOAC (1995) Official Methods of Analysis.17th edition Association of Official Analytical Chemists, Washington, D.C.

Babel P, Kumar AK, Singh V, Meena KK, Wadhawan N (2023) Studies on quality enhancement of Shrikhand using *Moringa oleifera* leaf extract. The Pharma Innovation J, SP-12(9): 540-545.

Blanda G, Cerretani L, Cardinali A, Barbieri S, Bendini A, Lercker G (2009) Osmotic dehydrofreezing of strawberries: Polyphenolic content, volatile profile and consumer acceptance. Food Sci Technol 42: 30–36

Bourne MC (1978) Texture Profile Analysis. Food Technol 32: 62-66

DAHD (2023) Basic animal husbandry & fisheries statistics (http://dahd.nic.in /Division/statistics/animal-husbandry-statistics-division)

Devi R, Argade A, Bhardwaj P K, Ahlawat SS (2018) Soy milk and fruit based shrikhand: A novel fermented milk product. *The Pharma Innovation J* 7(3):458-461

FAO Food outlook.2015.www.fao.org/3/a-i4581e.pdf.

Foda MI, Abd El-Aziz M, Awad AA (2007) Chemical, rheological and sensory evaluation of yoghurt supplemented with turmeric. Int J Dairy Sci 2(3): 252-259

Gaur G, RaniR, Bharti BK, Solanki K (2019) Development of herbal milk using tulsi, ginger and turmeric. Intern J Chem Stud 7(2): 1150-1157

Gahrui HH, Eskandari MH, Mesbahi G (2019) Development of functional yogurt fortified with wheat germ and strawberry as functional ingredients. Progress in Nutrition 21(1): 388-398.

García Pérez FJ, Lario Y, Fernández López J, Sayas E, Pérez Alvarez JA, Sendra E (2005) Effect of orange fiber addition on yogurt color during fermentation and cold storage. Color Research & Application: Endorsed by Inter Society Color Council, The Colour Group (Great Britain), Canadian Society for Color, Color Science Association of Japan, Dutch Society for the Study of Color, The Swedish Colour Centre Foundation, Colour Society of Australia, Centre Français de la Couleur, 30(6): 457-463

Ghule BK, Desale RJ, Gavhane MS, Khore MC (2015) Preparation of strawberry Lassi. Res J Animal Husbandry Dairy Sci 6(1): 22-26

Goswami M, Bharti SK, Tewari A, Sharma H, Karunakara KN, Khanam T (2017) Implication of functional ingredients of goat milk to develop functional foods. J Anim Feed Sci Technol 5:65-72

Gupta G, David J, Shukla G, Dubey S, Shukla, A (2018) Studies on quality of Shrikhand by blending papaya and banana pulp. The Pharma Inno J 7(8): 415-417

Hanaa AY, Edwin CP, Nitin M, Margaret AD (2016) Antimicrobial Activity of Tulsi (*Ocimumtenuiflorum*) Essential oil and heir major constituents against three species of Bacteria. Frontiers Microbiol 7: 681

Haenlein GFW (1992) March.Role of goat meat and milk in human nutrition.In Proceedings of the Fifth International Conference on Goats (Vol. 2, No. part II, pp. 575-580).Indian Council of Agricultural Research Publishers

Howard LR, Brownmiller C, Prior RL (2014) Improved color and anthocyan in retention in strawberry puree by oxygen exclusion. J Berry Res 4(2): 107-116

Isabel G, Deisy H (2011) By-Products from Plant Foods are Sources of Dietary Fibre and Antioxidants, Phytochemicals - Bioactivities and Impact on Health, Prof. Iraj Rasooli (Ed.), ISBN: 978-953-307-424-5.

Jaros D, Rohm H (2001) Identification of sensory color optima of strawberry yogurt. J Food Quality 24(1): 79-86

Johri S, Chauhan G (2014) Physico-chemical and organoleptic evaluation of Misthidoi prepared with different herbs. @inproceedings{Johri2014OriginalAP.

Kallio H, Hakala M, Pelkkikangas AM, Lapvetelainen A (2000) Sugars and acids of strawberry varieties. Euro Food Res Technol 212: 81–85

Kumar R, Atanu J, Dobariya A, Parmar S (2018) Suitability of type of herb and its form as flavoring in herbal ice cream. Int J Chem Stud 6(5): 1562-1567

Kumar S, Goswami M, Pathak V, Verma AK. Rajkumar V, Sharma B (2023) Comparative physico-chemical, textural, colour and sensory characteristics of yogurt prepared from indigenous goat and cow milk. Indian J Small Ruminants 29 (1): 109-112

Keeton JT (1983) Effect of fat and sodium chloride / phosphate levels on the chemical and sensory properties of pork patties. J Food Sci 48: 878-81

Kumar R, Bawa AS, KathiravanT, Nadanasabapathi S (2013) Thermal processing of mango nectar (*Mangiferaindica*) and its effect on chemical, microbiological and sensory quality characteristics. Int J Adv Res 1(8): 261-273

Lakshmi R, Ranganna B, Suresha KB (2013) Development of value rich jamun fruit shrikhand. Mysore J Agric Sci 47(2): 307-313

Mehaia MA, Al-Kanhal MA (1992) Taurine and other free amino-acids in milk of camel, goat, cow and man. Milchwissenschaft 47: 351–353

Merai M, BoghraVR , Sharma RS (2003) Extraction of antioxygenic principles from Tulsileaves and their effects on oxidative stability of ghee. J Food Sci Technol 40(1): 52-57

Mehrotra R, Singh D, Tiwari A (2014) Physico-chemical analysis of low calorie high protein shrikhand prepared using *Stevia* leaf powder. Innovare J Food Sci 2: 26-28

Nour V, Trandafir I and Ionica ME (2010) Compositional characteristics of fruits of several apple (*Malus domestica Borkh*.) cultivars. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 38: 228-233.

NDDB (2023) https://www.nddb.coop/information/stats/milkprodindia

Ozkurt H, Ozlem A (2018) Quality parameter levels of strawberry fruit inresponse to different sound waves at 1000 Hz with different dB Values (95, 100, 105 dB). Agronomy 8(127) doi:10.3390/ agronomy8070127

Park YW (1994) Hypo-allergenic and therapeutic significance of goat milk. Small Ruminant Res 14:151-159

Park YW, Chukwu HI (1989) Macro-mineral concentrations in milk of two goat breeds at different stages of lactation. Small Ruminant Res 1:157-166

Perin LM, Nero LA (2014) Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant lactococcuslactis. BMC Microbio 12:14-36

Rai HK, Rai DC (2018) To study the shelf life of Tulsi *(Ocimumtenuiflorum*) enriched herbal Shrikhand. The Pharma Innovation J 7(5): 611

Redmond HP, Stapelton PP, Neary P, Bouchier-Hayes D (1998) Immunonutrition: the role of taurine. Nutri 14: 599–604

Sahu V, Pathak V, Goswami M, Verma AK, Rajkumar V (2021) Optimization of fat content to develop goat milk shrikhand. Indian J Dairy Sci 74(6):1-7

Sahu V, Pathak V, Goswami M, Priya (2021) Optimization of starter culture to develop healthy goat milk shrikhand. The Pharma Innovation J SP-10(10): 1473-1477

Sahu V, Pathak V, Goswami M, Verma AK, Rajkumar V, Singh S, Priya (2022) Quality assessment of papaya pulp incorporated functional goat milk Shrikhand. Ruminant Sci, 11 (2): 425-430

Singh, AK, Kumar M, Singh R, Rai DC, Mishra K, Dikshit PKS (2021).Significance of goat milk and its products. Intern J Res Social Sci 11 (11): 163-182

Singh AK, Kumar M, Singh M, (2023) A study on physic-chemical properties of goat milk yoghurt incorporated with kiwi fruit (*Actinidia deliciosa*) pulp. Intern J Clinical Biochem Res, 10(1):71–76

Snedecor GW, Cochran WG (1995) Statistical Methods, 8th edition Pp.72-148. New Delhi: oxford and IBH Publishing Company

Trout ES, Hunt NC, Johnson DE, Claus JR, Kastner CL, Kropf DH, Stroda S (1992) Chemical, physical, and sensory characterization of ground beef containing 5 to 30 percent fat. J Food Sci 57:25–29

Vidhani SI, Vyas VG, Parmar HJ, Bhalani VM, Hassan MM, Gaber A, Golakiya BA (2016) Evaluation of some chemical composition, minerals fatty acid profiles, antioxidant and antimicrobial activities of Tulsi (*Ocimum sanctum*) from India. American J Food Sci Technol, 4(2): 52-57

Indian J Dairy Sci 78(2): 140-147
https://doi.org/10.33785/IJDS.2025.v78i02.006

RESEARCH ARTICLE

Estimation of production cost for Indian artisanal sour buttermilk powder

Subhadip Manik[1], Ganga Sahay Meena[1]✉, Yogesh Khetra[1], Ashish Kumar Singh[1], Richa Singh[2], Sumit Arora[2], Raghu H. V.[3]

Received:22 October 2024 / Accepted: 30 April 2025 / Published online: 23 April 2025

Abstract: The present investigation was aimed to estimate the production cost of Indian artisanal sour buttermilk powder that contains 53% protein. Additionally, it exhibits numerous techno-functional properties such as "instant" in wettability (3 s), excellent flowability (angle of repose=28.36º), water binding capacity (4.34 g/ g of protein) and excellent emulsification stability (80.70%) along with essential amino acids, fatty acids and antioxidants. This study aimed to estimate its production cost with a numerous assumption. Main assumption was production of 1260.12 tons of Indian artisanal sour buttermilk powder in 300 days. Numerous heads such as raw material, packaging material, land and building, manufacturing, utility, manpower, marketing and distribution etc. were taken as a crucial factor during estimation of total production cost of Indian artisanal sour buttermilk powder. The calculated cost for 500 g pack and 1 Kg pack of this powder was Rs. 211.52 and Rs. 423.04, respectively.

Keywords: Artisanal, sour buttermilk powder, techno-functional, production cost.

Introduction

India is global leader in milk production with the annual production of 230.58 million tonnes during year 2022-23 (PIB, 2024). Presently, about 46% of the total milk production in India is either consumed directly by producers (DAHD, 2021) or used to produce various dairy products such as curd (*dahi*), traditional butter (*makkhan*), clarified fat (*ghee*), artisanal buttermilk (*lassi* or *chhach*) for the household use (Halder et al., 2021). About 14% of total milk production is converted in fermented milk products by unorganized sector (MOFPI,2023). In rural households, excess whole milk is subjected to prolong heat treatment, cooled, inoculated using previous run *dahi* or *chhach* and incubated to produce *dahi* that is further churned to yield *makkhan* (an intermediate product) which is subsequently heated during *ghee* preparation. As per Aneja *et al.* (2002), production of 1 Kg of *ghee* (from *makkhan*) by tradition method produces 15 to 20 Kg of artisanal sour buttermilk. As per De (2004), artisanal sour buttermilk contains 3.8% of total solids (TS), 1.29% of protein, 0.8% of fat, 1.2% of lactose, 0.4% of ash, and 0.44% of lactic acid, respectively. It has a brownish color aroused from the prolonged heat treatment of milk before its inoculation. It contains numerous curd particles and non-homogeneous consistency which is prone to deposition of curdy stuff and separation of watery portion on its top owing to its higher acidity (Pal & Rajorhia,1985; Padghan et al., 2015). Additionally, the overall quality of the product varies greatly as function of differences in preparation techniques (Aneja et al., 2002). Recently, Manik et al. (2023) developed artisanal sour buttermilk powder via concentration of sour buttermilk up to 3.62× (12.86% of TS) using reverse osmosis (RO), a membrane-based process and subsequently converted its retentate / concentrate into artisanal sour buttermilk powder employing spray drying. The developed powder was "instant soluble (3 s wetting time)", exhibited free flowing (angle of repose=28.36º) characteristics, excellent emulsion stability (80.70%). This powder also contained essential amino acids, fatty acids along with sound antioxidative properties.

As per MOFPI (2023), the global market size of fermented milk was $264.77 billion in 2018 and is expected grow to $396.87 billion by 2026, with a CAGR of 5.1% during 2019 to 2026. Furthermore, the *lassi* market in India was INR 47.5 billion in 2023 and expected to reach INR 217.5 billion by 2032 with a CAGR of 17.9% during 2024-2032 (IMARC Group, 2024a). Moreover, the size of the worldwide milk powder market was US$ 34.6 billion in 2023 and

[1]Dairy Technology Division, [2]Dairy Chemistry Division, [3]Dairy Microbiology Division, ICAR-National Dairy Research Institute, Karnal-132 001, Haryana, India

Ganga Sahay Meena (✉)
Dairy Technology Division, ICAR-National Dairy Research Institute, Karnal, Haryana, India
Email: gsiitkgp@gmail.com; Contact No: +91- 9996129094.

will reach to US$ 57.2 billion with a CAGR of 5.6% from 2024 to 2032 (IMARC Group', 2024b). Hence, there is a huge market of Indian artisanal sour buttermilk powder in India and abroad. Although, technical feasibility and the cost effectiveness from Indian artisanal sour buttermilk by employing RO as a means for concentration is still unknown. Therefore, current study was undertaken to calculate the manufacturing cost of artisanal sour buttermilk powder.

Materials and Methods

Indian artisanal sour buttermilk

Freshly produced samples of Indian artisanal sour buttermilk were procured from nearby places of Karnal district (29.6857° N, 76.9905° E), Haryana in early morning and pooled. After that, the samples were subjected to thermization (63 ºC/ 15 s), cooled (to 40-45 ºC), defatted via cream separation followed by filtration through a muslin cloth to remove any curd particles or foreign materials.

Reverse osmosis plant

Reverse osmosis (RO) plant was procured from Peterson Candy International Ltd., Reading, UK. It contains polyamide, AFC 99 membrane with a total membrane area of 0.9 m^2 and having a length of 1500 mm, height of 800 mm, dept of 700 mm and weight of 70 kg. RO plant was furnished with tubular module of Type B1 (length-1.2 m). It equipped with a triple plunger pump that has a contact surface of SS, 4 kW of power consumed motor and a flow of 22 L per min. Total 70 bar pressure can be generated in this plant however, the concentration process was carried out at 35 bar as this membrane can withstand this pressure only. Beyond this pressure problem of leakage was observed. The initial flux, final flux and flux mean of this study were 24, 10.67, and 15.07 L/ m^2/h, respectively. Furthermore, it contains a hold up volume of 6.5 L and can be operated up to 70 °C. The feed can be used pH range 3-11 and can provide a flux range of 15–60 LMH (L/m^2/h).

Manufacturing of Indian artisanal sour buttermilk powder

Figure 1 shows different steps involved in processing and conversion of defatted Indian artisanal sour buttermilk into Indian artisanal sour buttermilk powder. The developed powder was further characterised in detail as reported by Manik et al. (2023).

Estimation of production cost for Indian artisanal sour buttermilk powder

As per Meena et al. (2017), techno-economic feasibility evaluation of the newly developed product is very much necessary before launching into market that's why estimation of manufacturing cost of this powder was performed. The cost estimation of manufacturing of Indian artisanal sour buttermilk powder was carried out as per the procedure mentioned by researchers (Chavan, 2019; Rani *e*t al., 2018; Meena et al., 2017and Kumar, 2011). Following assumptions were made to calculate the cost of manufacturing in a realistic way.

Basic assumptions for cost estimation of Indian artisanal sour buttermilk powder

Several assumptions were conducted for the estimation of total production cost of Indian artisanal sour buttermilk powder as mentioned hereunder.

a) The capacity for dairy plant to handle Indian artisanal sour buttermilk (0.72% fat and 3.41% SNF) was 1,00,000 Kg/ day. The price for Indian artisanal sour buttermilk was assumed to be Rs. 10/Kg. Cost for utilities such as water, steam, electricity and refrigeration were assumed as per current market price.

b) Plant will be operated in 3 shifts (8 h for each shift) for 300 days. Indian artisanal sour buttermilk will be defatted using cream separator and concentrated employing RO.

c) The RO plant composed of with AFC-polyamide membrane. The total operation for RO plant was 24 h which consist with a processing time of 18 h and cleaning time of 6 h.

d) Manufacturing of Indian artisanal sour buttermilk powder was performed as per Figure 1. The fat content recovered through defatting would be mixed with final concentrate.

e) The capacity of spray drier was 20 T/ day and would be used for operation in 2 shifts to manufacture Indian artisanal sour buttermilk powder. Developed Indian artisanal sour buttermilk powder will be packed in 500 g metalized-polyester LDPE pouches.

f) Total of 0.1% losses in Indian artisanal sour buttermilk powder sample and 0.5% losses in powder packaging material was assumed. Approximately 15% of overhead cost was assumed for manufacturing of Indian artisanal sour buttermilk powder including research and development and other expenses.

Cost of RO plant

- Concentration of 99264.5 Kg defatted Indian artisanal sour buttermilk (0.18% fat and 3.37% SNF) after removal of 735.5 kg cream containing 73.6% of fat and 8.2% of SNF up to 72.69% volume reduction ratio (VRR) employing RO.
- Quantity of permeate during production of Indian artisanal sour buttermilk powder sample=99264.5 Kg/ day *i.e.*, 72,155.36 Kg of permeate (72.69% VRR, Manik et al., 2023)

- Calculation of flux mean (FM) was done using following formula as per St-Gelais et al. (1992)

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

$$Where, IF = initial\ flux\ and\ FF = final\ flux\ values$$

- Total membrane area

$$= 99,264.5/15.07 = 6586.89\ m2/h \sim 6587\ m2/h$$

- During operation of RO plant for 18 h (2 shifts), requirement of membrane area=

$$6587\ (m2/h)/18\ (h) = 365.93\ m2 \sim 366\ m2$$

- Therefore, around 366 m^2 of membrane area required for effective handling of 99264.5 Kg of defatted Indian artisanal sour buttermilk per day. Fifteen percent of safety margin (54.89 m^2~55 m^2) in membrane area would be considered as per previously reported by Meena et al. (2017) and total membrane area required for RO plant installation would be 421 m^2 (366+55=421 m^2).

Cost of RO plant consisting with 421 m^2 of membrane area will be Rs. 2.45 crore (*i.e.*, assume cost for 1 m^2 membrane area would be Rs. 50000).

Detailed break-up of total production of Indian artisanal sour buttermilk powder

Capital requirements

During an annual production of 1260.12 tons Indian artisanal sour buttermilk powder *i.e.*, 4.20 tons/day, a capital investment estimation would be required and that mentioned in this section. Direct cost as well as indirect also was calculated on the basis of

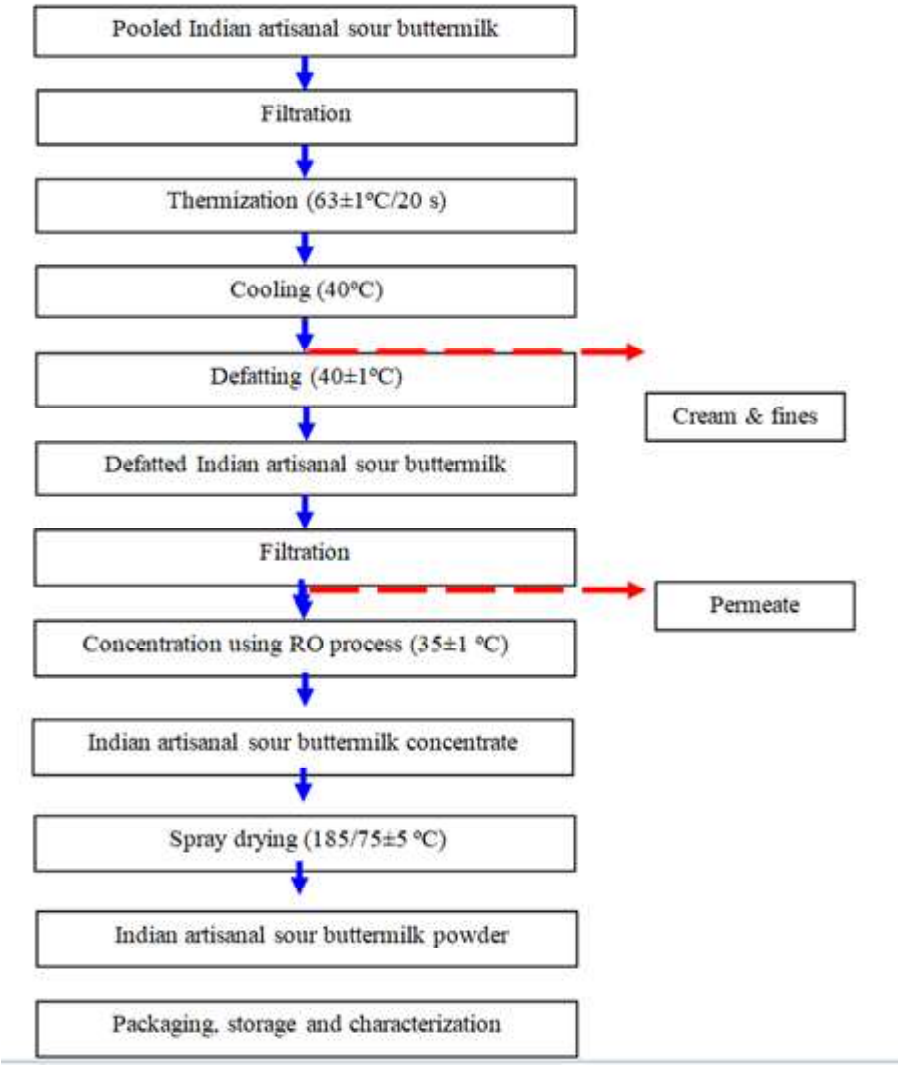


Fig. 1 Production process of Indian artisanal sour buttermilk powder

current scenario with an expectation in consistent. While, due to fluctuation in price and inflation, constant updating is highly recommended.

Cost of Land

The total land area for the manufacturing of Indian artisanal sour buttermilk powder was estimated around 4800 m^2. It would be around Rs. 1.32 crore (@ Rs. 2750/m^2 was assumed) and shown in Table 1. Meena *et al*. (2017) assumed the rate for land per m^2 was Rs. 2500.

Building

Estimated area for the construction of building was 5000 m^2 and construction rate was Rs. 5500/m^2 and shown in Table 1. Meena *et al*. (2017) assumed the rate of land per m^2 as Rs. 5500/ m^2.

Equipment

Equipment required for manufacturing of 1260.12 tons Indian artisanal sour buttermilk powder per annum was estimated with

Table 1. Capital investment for civil works of dairy plant

Description	Size(m^2)	Rate per m^2 (Rs)	Estimated cost (Rs in crores)
Cost of Land	4800	2750	1.32
Cost of civil Construction including production block with cold store, general store, laboratory, administrative block, road, hard park, internal electrification	5000	5500	2.75
Cost of Effluent Treatment Plant			0.50
Total Cost			4.57

Table 2: Capital investment for plant and machinery items (For Indian artisanal sour buttermilk powder

Item	Capacity	Quantity	Item	Capacity	Quantity
1. Buttermilk reception section			2. Buttermilk Processing Section		
Inline Strainer	STD	1	Buttermilk Transfer pump	10 KLPH	1
Buttermilk Pump	10 KLPH	1	Buttermilk pasteurizer	11 KLPH	1
Tanker Unloading hose pipe	50mm×5 m	1	Cream separator	12 KLPH	1
Tanker Unloading pump	10 KLPH	1	Multipurpose tank	10,000 L	3
Raw buttermilk storage tank	40,000	2	CIP equipment	2,000 L	1 set
Total Cost Rs. (Crores)		0.35	SS pipe and fittings for entire plant		1 set
			Total Cost Rs. (Crores)		3.07
3. Reverse osmosis membrane section			4. Buttermilk Powder plant section		
Membrane plant (Tubular module)	421 m^2	1	Buttermilk spray drying plant with nozzle atomizer	1100 Kg SMP/h	1 set
Reverse osmosis plant		1	Powder bag filling and stitching machine		1 set
			Weighing balanc e 0-30 Kg		1
Retentate storage tank	20,000 L	1	Stainless steel Table		1

Total Cost Rs. (Crores)		2.51	Nitrogen packing machine for the packaging of Indian artisanal sour buttermilk powder	1
			Total Cost Rs. (Crores)	2.50
5. Service equipment section			6. Miscellaneous equipment	
Refrigeration plant including cold store equipments/ Nitrogen gas for packaging machine		1 set	Laboratory equipment and glassware	1 set
Steam boiler (husk/ oil fired) and other accessories	5000 Kg/h	1 set	Fire fighting equipment	10 set
Electricals including LT panel, MCC, cables, Cable trays, earthing, generator set (150 KVA) with accessories		1 set	Steam water mixing batteries	4
Water supply equipment including tube well		1 set	Cans for plant use	50
Pipe and fittings (MS/GI) for		1 set	Furniture and fixtures, Computers Rs.	

depreciation cost and illustrated in Table 2. The calculated plant machinery cost and depreciation cost (Rs. 22.85 lakh at rate 5% of civil work+ Rs. 90.63 lakh at the rate of 7.5% for machinery and plant) was Rs. 12.08 and Rs. 1.13 crores, respectively.

Utility expenses

The assigned costs for various utility services, such as steam, water, and electricity, were taken into account when estimating the total direct expenses. Table 3 illustrated the individual utility service items that were determined as per Meena et al. (2017). The total estimated cost for utilities was Rs. 7.03 crores per annum as shown in Table 3.

Expenses on raw material and packaging material

The quantity of Indian sour buttermilk was required for manufacture of 1260.12 tons Indian artisanal sour buttermilk powder was estimated and presented in Table 2. As per current market situation, Rs. 10/- was assumed to provide cost for Indian artisanal sour buttermilk and that was calculated as Rs. 3.0 crores per annum. Additionally, the assumed total packaging cost per annum was estimated and that would be Rs. 1.26 crore (Table 4).

Expenses on manpower

During the manufacture of Indian artisanal sour buttermilk powder, the manpower will be work in three shifts and was calculated (Table 5). The estimated cost for total manpower would be Rs. 1.00 crores that includes labour expenses of Rs. 64.80 lakh, supervisor expenses of Rs. 10.8 lakh and administration expenses of Rs. 24.48 lakh as shown in Table 5.

Expenses on laboratory and cleaning and sanitization

Dairy machinery, equipment, tanks, and piping must be thoroughly cleaned and sanitized in order to produce safe Indian artisanal sour buttermilk powder sample, for which detergent and sanitizers are required. The required total expenses for laboratory were computed as Rs. 6.00 lakh as 0.2% of cost raw material and cleaning and sanitizing material were calculated as Rs. 3.00 lakh as 0.1% of cost of raw material (Table 6).

Expenses on marketing and distribution

As per Kumar (2011) and Meena *et al*. (2017), the total expenditure of marketing and distribution were calculated by assuming the rate of 10 and 15% of manufacturing cost. The calculated cost for plant overhead and marketing and distribution were Rs. 6.44 crores and Rs. 4.29 crores (Table 6).

Estimation of total manufacturing cost for Indian artisanal sour buttermilk powder

The total cost for annual production of 1260.12 tons Indian artisanal sour buttermilk powder was mentioned in this section. Cost related to direct and indirect ways were estimated and shown in Table 6. The cost required for annual production of 1260.12 tons of Indian artisanal sour buttermilk powder was Rs. 53.57 crores. Additionally, total manufacturing cost which includes the total sum of cost of the raw material, packaging material, utilities, manpower, laboratories and detergents is only 73.53% which was found less than the cost assumption for soluble MPC 70 (78.48%), reported by Meena *et al*. (2017). Moreover, major contributors for direct production cost were cost of raw material, packaging, manpower and utility. While, around Rs. 3.46 crores of total fixed charges have been computed which included fixed charges of Rs. 1.99 crores at 12% rate of total fixed capital and working capital, Rs. 1.13 crores of depreciation cost and Rs. 33.31 lakh of insurances and taxes as per 2% of capital investment as shown in Table 6.

Therefore, the calculated cost for 500 g of pack of Indian artisanal sour buttermilk powder was observed to be Rs. 211.52 and Rs. 423.04 for 1 Kg pack of Indian artisanal sour buttermilk powder by excluding applicable taxes. The estimated cost for

Table 3: Charges on power utilities (For Indian artisanal sour buttermilk powder)

Item	Requirement		Rate (Rs.)	Annual Cost (Rs.)
	Daily	Annual (300 Days)		
Steam (@2Kg/ Kg of powder) Indian artisanal sour buttermilk powder = 4200.40-42.00 (1% powder loss) =4158.39 Kg (From 27102.13 Kg Indian artisanal sour buttermilk concentrate [12.86% TS] + 735.5 Kg of cream = 27837.63 Kg concentrate solution)	10316.78	3095034	Rs. 2/Kg steam	Rs. 61.90 lakhs
Fuel oil [@ 1 L/ 2.5 Kg of powder	1663.356	499006.8	Rs. 95/ L	Rs. 4.74 Crores
Electricity (KWh)[@1 KW/3.5 Kg of powder+@1 KW/20 L milk+(@ 45 KWh/h in NF Plant]	7268.111429	2180433.429	Rs. 7/Unit	Rs. 1.52 Crores
RO Water for CIP in NF plant	4000	1200000	Rs. 1/L	Rs. 12 lakh
Refrigeration (1 TR=3.517KWh) 10 T for cream and retentate= 10 TR/ day	10	3000	Rs. 34 per TR	Rs. 1.02 lakh
Water (@1.5 L/Kg of powder+@1L/L of Indian artisanal sour buttermilk)	106237.585	31871275.5	Rs. 6/1000 L	Rs. 1.91 lakh
			Total Rs.	7.03 Crores

Table 4: Requirement and cost of raw material (For Indian artisanal sour buttermilk powder)

Sr. No.	Items	Requirement		Rate (Rs/Kg)	Annual Cost (Rs.)
		Daily	Annual (300 days)		
1	Sour buttermilk	100000	30000000	10	30.00 Crores
				Total cost Rs.	30.00 Crores
2 Annual cost of Packaging material Annual packaging material requirement= 4200.40 kg (daily Indian artisanal sour buttermilk powder production) *300(total plant run time) *2(500 g pack size) = 2520240+0.5% (12601.2) *i.e.* Total 2532841.2~2532842 units				Cost of pouch= Rs. 5 / pouch. Total cost= 2532842*5	Rs. 1.26 Crores

Table 5: Man Power Cost for the production of Indian artisanal sour buttermilk powder

Sr. No.	Staff	Number	Monthly Salary (Rs.)	Total Monthly Salary (Rs.)	Annual Cost (Rs . in lakhs)
1	Labourers (6×3)	18	9000	162000	19.44
2	Skilled Worker (3×3)	9	15000	135000	16.20
3	Mechanics/ electrician (2×3)	6	18000	108000	12.96
4	Boiler Attendants (1×3)	3	18000	54000	6.48
5	Lab analysts (1×3)	3	18000	54000	6.48
6	Lab attendants	3	9000	27000	3.24
Sub-total (A)				540000	64.80
	Operational Supervisors				
7	Shift Supervisors (1×3)	3	30000	90000	10.80
Sub-total (B)				90000	10.80
	Administrative Expenses				
1	Plant Manager (1×3)	1	70000	70000	8.40
2	Clerk-cum-Accountant (1×2)	2	20000	40000	4.80
3	Store Keeper (1×2)	2	20000	40000	4.80
4	Attendant (1×2)	2	9000	18000	2.16
5	Security staff (2×2)	4	9000	36000	4.32
Sub-total (C)				204000	24.48
Grand Total (A+B+C) Rs. (Crores)					1.00

Table 6. Detailed break-up cost of Indian artisanal sour buttermilk powder (Capacity 1260.12 Kg/day)

	Sr. No	Cost component	Rates	Per annum (Rs.)
	Manufacturing Cost			
	a	Direct product cost		
	i	Raw materials	Table 4	30.00 Crores
	ii	Annual packaging material requirement=4200.40 (daily Indian artisanal sour buttermilk powder production) * 300(total plant run time) *2(500 g pack size) = 2520240+0.5% (12601.2) i.e. Total 2532841.2~2532842 pouches	Rs.5 per pack, Table 4	1.26 Crores
A	iii	Manpower cost	Table 5	1.00 Crores
	iv	Power and utilities	Table 3	7.03 Crores
	v	Laboratory charges	0.2% of cost of raw material	6.00 lakhs
	vi	Cleaning and sanitizing material	0.1% of cost of raw material	3.00 lakhs
	Total			39.39 Crores
	b	Fixed charges		
	i	Interest on total fixed capital and working cost	12% of total fixed capital and working capital	1.99 Crores
	ii	Depreciation on capital investment		1.13 Crores
	iii	Insurance and taxes	2% of the capital investment	33.31 lakh
	Sub-total (b)			3.46 Crores
	Manufacturing cost (A=a+b)			42.85 Crores
	MARKETING AND DISTRIBUTION			
B	i	Plant overhead cost	15 % of manufacturing cost	6.42 Crores
	ii	Marketing and distribution	10% of manufacturing cost	4.29 Crores
Total (b)				10.71 Crores
Total cost (Rs.) Per annum (A+B)				53.57 Crores
Total cost of Indian artisanal sour buttermilk powder (per 500 g pack=2532842)				Rs. 211.52/-
The cost of Indian artisanal sour buttermilk powder (per Kg)				Rs. 423.04/-

manufacturing of Indian artisanal sour buttermilk powder was nominal and calculated as per current market situation.

Conclusion

Economic feasibility analysis revealed a production cost of Rs. 423.04 per kg for Indian artisanal sour buttermilk powder, demonstrating its affordability and viability for large-scale production. The process not only valorizes a traditional by-product but also develop a novel ingredient for the dairy and food industries, contributing to environmental sustainability and economic empowerment of rural farmers. Hence, the manufactured Indian artisanal sour buttermilk powder had potentiality for application as ready to serve instant Indian artisanal sour buttermilk powder powder and can be used as an ingredient in formulation of new processed foods.

Acknowledgement

Authors would to thank Director, ICAR-NDRI, Karnal and Haryana State Council for Science, Innovation and Technology (HSCSIT), Panchkula for providing fund and support.

Funding

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

Conflicts of Interest

The authors declare no conflict of interest.

References

Aneja RP, Mathur BN, Chandan RC, Banerjee AK (2002) Fat-rich Products. In Technology of Indian milk products: Handbook on process technology modernization for professionals, entrepreneurs and scientists (pp. 196). Dairy India Yearbook.

DAHD (2021) Annual Report 2021-22. Department of Animal Husbandry and Dairying, Government of India, 2021-22. Retrieved from https://dahd.nic.in/sites/default/filess/Annual%20Report%20English.pdf. (Accessed on 1 August 2024).

De, S. (2004). Indian Dairy Products In: Outlines of Dairy Technology, Oxford University Press, New Delhi, pp. 463-464.

Halder K, Sahu JK, Naik SN, Mandal S, Bag, SK (2021) Improvements in makkhan (traditional Indian cultured butter) production: a review. J Food Sci Technol 58(5):1640-1654.

IMARC Group (2024a) Lassi market in India size, share, growth and industry report [Data set]. Available online: https://www.imarcgroup.com/lassi-market-india (Accessed on 1 September, 2024).

IMARC Group (2024b) Milk powder market report by Product Type (whole milk powder, skimmed milk powder), function (emulsification, foaming, flavouring, thickening), application (infant Formula, Confectionery, Sports and Nutrition Foods, Bakery Products, Dry Mixes, Fermented Milk Products, Meat Products, and others), and region 2024-2032 [Data set].

Kumar S (2011) Studies on the preparation of Dairy Whitener employing Ultrafiltration Ph.D. Thesis. National Dairy Research Institute (Deemed University), Karnal, India.

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

Meena GS, Singh AK, Gupta VK, Jayswal D, Parmar PT, Gupta H R (2017) Estimating cost for production of soluble milk protein concentrate70 (MPC 70). Indian J Dairy Sci, 70(3):342-350.

MOFPI (2023) In Conversation Fermented Milk Special. PMFME E-Newsletter. Available online: https://pmfme.mofpi.gov.in/pmfme/newsletters/enewsnovember1.html (Accessed on 1 August 2024).

Padghan PV, Mann B, Rajeshkumar, Sharma R, Kumar A (2015) Studies on bio-functional activity of traditional lassi. Indian J Tradit Knowl 1:124–131.

Pal D, Rajorhia GS (1985) Buttermilk utilization in dairy Industry. Indian Dairyman 9:397-403.

PIB (2024) World Milk Day (June 01): Achievement of the department of animal husbandry and dairying (ministry of fisheries, animal husbandry and dairying). (n.d.). Gov.In. Retrieved July 20, 2024, from https://pib.gov.in/PressNoteDetails.aspx?NoteId=151889&ModuleId=3

St-Gelais D, Haché, S, Gros-Louis M (1992) Combined Effects of Temperature, Acidification, and Diafiltration on Composition of Skim Milk Retentate and Permeate. J Dairy Sci 75:1167–1172

Indian J Dairy Sci 78(2): 148-155
https://doi.org/10.33785/IJDS.2025.v78i02.007

RESEARCH ARTICLE

Quality characteristics of green gram blended instant sorghum porridge prepared from fermented and germinated grains

Tshiamo Seiphitlhile[a], Rekha[a], Rakesh Gehlot[a], Ameeta Salaria[b] and Shalini Arora[c] (✉)

Received: 17 November 2023 / Accepted: 23 April 2024 / Published online: 23 April 2025

Abstract: The present study aimed to develop nutritionally rich green gram blended sorghum porridge and evaluate the nutritional and organoleptic characteristics of the developed product. The raw, fermented, and germinated sorghum and green gram were dried and milled and the obtained grits were evaluated for nutritional parameters. The crude protein, phosphorus, and acidity significantly ($p<0.05$) increased in sorghum, while ash and carbohydrate content decreased ($p<0.05$) in both sorghum and green gram after fermentation and germination. Control, fermented grits, and germinated grains porridges were analyzed for organoleptic characteristics after reconstitution with water and 12% sugar. The fermented sorghum porridge with 20% fermented green gram received a significantly ($p<0.05$) higher acceptability score whilst germinated green gram could be blended up to 35% in germinated sorghum to obtain the highest sensorial scores. Using the best organoleptically accepted porridges when reconstituted with water, milk was used to reconstitute the porridges for further organoleptic characteristics analysis. The fermented blended sorghum porridge received significantly ($p<0.05$) highest acceptability scores, followed by germinated green gram sorghum porridge.

Key words: sorghum, green gram, fermented, germinated, reconstitution, nutritional composition

Introduction

Porridge is a popular grain-paste breakfast meal prepared primarily from whole or crushed cereal grains. Grains like rice, wheat, oat, maize, or sorghum are commonly used to prepare porridge. To get the desired consistency, the product is prepared by boiling whole, ground, crushed, or chopped grains in water or milk. Further, the grains can be either used as such (untreated grains) or fermented/germinated. Cooked porridge can be converted into a sweet cereal by adding sugar, honey, fruit, or syrup, or it can be made into a savory meal by adding salt and spices. Whole grain porridge is a good aid for diabetes patients because of its high level of dietary fiber and fructans. The high fiber content in whole-grain porridge helps with stool thickening and fullness, prevents coronary heart disease development, and regulates other physiological processes (Alahmari, 2024). Porridge nutrition is regulated by grain processing characteristics such as fermentation, germination, mixing, and heating. Porridge is a traditional and simple way to ingest grains like sorghum since it requires minimal processing, thus maintaining most of the nutritious value for users.

Among various grains, sorghum (*Sorghum bicolor*) is a hard-cereal grain that may be processed into various food products. It is the world's fifth most significant cereal. It's a low-fat, high-fiber grain and a good source of macro minerals, phosphorus, magnesium, potassium, and iron. The product prepared from sorghum does not impart colour, offers a neutral taste, or flavor, ~~a~~after consumption. Further, because of the current growth in celiac illness, this cereal can be used to substitute gluten-containing cereals such as wheat (Oghbaei and Prakash 2016).

The porridge prepared from sorghum is a staple cuisine in Africa, where it is primarily used as a supplement and weaning food for newborns (Adebo, 2020), and as dietary supplements for patients and elderly people. Sorghum can also be used to prepare fermented porridge that is sweetened and eaten by both newborns and children in tropical areas. Cereals generally lack lysine and tryptophan, but the germination of grains can be used to boost the lysine level. Further, in producing porridge, combining grains and legumes boosts the nutritious content and improves the protein quality. Supplementing cereal-based porridge with high-lysine meals like pulses further helps to

[a]Centre of Food Science and Technology, Chaudhary Charan Singh Haryana Agricultural University, Hisar, 125004, Haryana (India)

[b]Faculty of Dairy Technology ,Sher e Kashmir University of Agricultural Sciences and Technology, SKUAST-J,R.S.Pura, Jammu,181102, J&K, (India)

[c]Department of Dairy Technology, College of Dairy Science and Technology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, 125004, Haryana (India)

(✉)Email: shaliniarora.luvas@gmail.com
TEL.No. +91-7988425439

increase the digestibility of the product (Oghbaei and Prakash 2016).

Green gram *(Vigna radiata),* a legume that grows largely in tropical and subtropical climates, has a high lysine concentration. It is consumed as a whole, split, or in germinated form. It contains approx. 60% carbs, a small amount of fat, and high in fiber. The principal minerals present in green grams are calcium, phosphorus, iron, sodium, and potassium (Diatta et al. 2024).

Cereals and legumes are rich in macronutrients and micronutrients. Further, phytochemicals and anti-nutritional compounds are also present that obstruct the nutrient bioavailability by binding with the existing nutrients. Fermentation and germination of both cereals and legumes can disrupt these binding, by the activity of the enzymes that break down anti-nutritional components and break complicated macronutrients into simple ones and make them easy to digest (Nkhata et al. 2018). Keeping the above facts in view, the present study aimed to develop nutritionally rich sorghum-green gram-based porridge using fermentation and germination.

Materials and methods

Sorghum variety (HJ-541) and green gram variety (MH-421) used in the present study were procured from the Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar. Sugar and double-toned milk of the brand Amul were procured from the local market. Pure culture of *Lactobacillus casei* used for fermentation was procured from the Institute of Microbial Technology, Chandigarh, India. The culture was then maintained on slants and subcultured after every 30 days. All experiments were conducted in triplicates with analytical grade chemicals from CDH, Sigma-Aldrich, and Himedia.

Treatments of whole-grain grains, milling, and instantisation

The control sorghum porridge was prepared by pressure-cooking grits for 30 min with grains: water ratio, 1:1, followed by tray drying. Fermented porridge was prepared by inoculating a loop full of *Lactobacillus casei* (2%) culture in sorghum and green gram grits slurries (grits: water ratio 1:1) which were incubated at 37±2°C for 48 hours and stopped by cooking. To obtain germinated grains, sorghum and green gram grains were hydrated by soaking in distilled water (grain: water ratio1:5) then thinly spread on a wet jute bag and kept in the dark to germinate for 48 hours followed by cooking. The details of the preparation of green gram sorghum porridge are mentioned in fig. 1. About 50 g of the instant sorghum porridges ISP *i.e.,* control instant sorghum porridges CISP, fermented instant sorghum porridges FISP, and germinated instant sorghum porridge GISP were analyzed for various physicochemical and organoleptic analyses.

Estimation of proximate composition

The sample's moisture, crude protein, crude fat, crude fiber, and ash content were evaluated according to the AOAC method (2016). The difference method was used to calculate carbohydrate content. The energy was determined using the following formula:

Energy (kcal/100g) = 4.0 x Protein (%) + 4.0 x Carbohydrates (%) + 9.0 x Fat (%).

Estimation of acidity and pH

5 gm porridge sample was diluted with 50 ml distilled water and the pH of the sample were analysed using a pH meter (Systronics). The percent acidity of samples was estimated after dilution using the method given by AOAC (2016).

Estimation of mineral

The determination of phosphorus was carried out by the Vanadomolybdophosphoric yellow color method (Koenig and Johnson 1942). The method of Chopra and Kanwar (1990) was employed to determine the calcium and magnesium content. Available iron in the sample was extracted and estimated according to the procedure of Rao and Prabhavathi (1978).

Sensory evaluation

Various blends of (fermented and germinated grain) instant porridges (IPs) were reconstituted in boiling water (Instant Sorghum Porridge: water 1:5 w/v) and cooked for 5-7 minutes with continuous stirring on a slow flame before presenting for sensory evaluation. Sugar @ 12% initially standardized in the preliminary studies, was added during preparation. Similarly, the best combination of fermented and germinated grain instant porridge was reconstituted in boiling milk (Instant Sorghum Porridge: water 1:5 w/v) and cooked for 5-7 minutes with intermittent stirring. A panel of ten trained panellists carried out the sensory evaluation of hot-cooked porridge. The porridge was evaluated for colour and appearance, aroma, taste and consistency using a 9-point Hedonic scale as described by Ranganna (1986). Color and appearance (the visual appeal, presence of burnt particles, browning, or a dull appearance), flavour (sensory acidity and foreign flavours), texture (creaminess, mouthfeel, richness, and viscous behaviour on the palate and tongue), and overall acceptability were evaluated.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA), using OP Stat, Statistical analysis software developed by the Department of Mathematics and Statistics, CCS, HAU Hisar. The critical difference (CD) at a 5% level was used to compare different treatments. The results are expressed as a mean of three replications.

Results and Discussion

Physico-chemical properties of raw and treated sorghum and green gram grains

Proximate analysis

Fermentation and germination of grains affect their proximate composition, affecting the final product quality. Raw, fermented, and germinated sorghum and green gram grains were evaluated for proximate composition to determine the effect of different treatments on the nutritional composition of the grains (Table 1).

Fermented sorghum showed significantly ($p \leq 0.05$) higher moisture content (9.02 %), while the moisture content was comparable in raw and germinated sorghum. However, germinated green gram had significantly ($p<0.05$) higher moisture content (14.75 %) followed by fermented green grams. Shah et al. (2011); Khalil et al. (2007) observed an increase in the moisture content

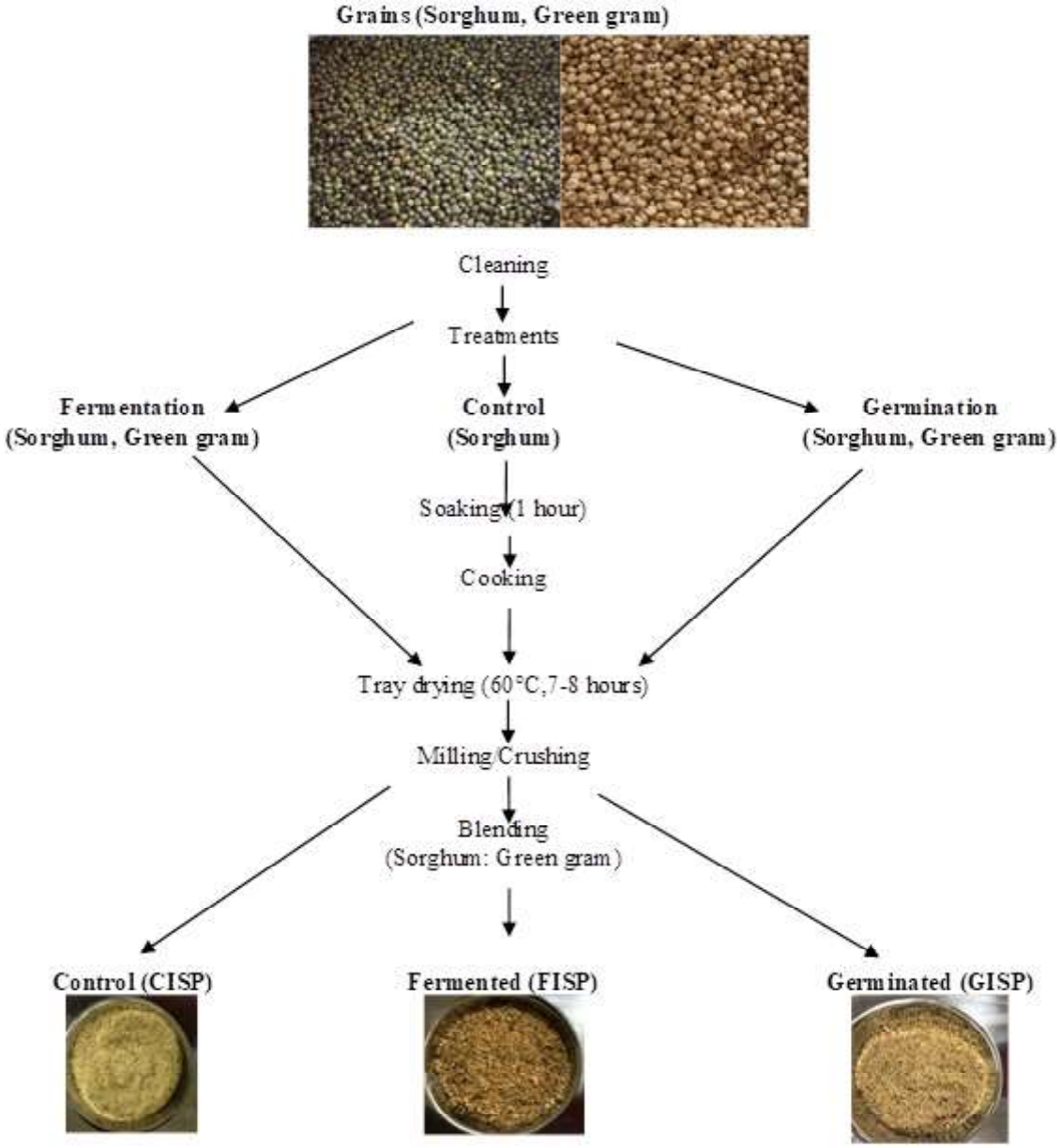


Fig. 1: Flow diagram for the development of green-gram blended instant sorghum porridge

after germination of chickpea, hence agreeing with the results obtained in the present study. Nonogaki et al. (2010) explained that the number of hydrated cells is a determining factor for the gained moisture after either fermentation or germination. Further, moisture content of developed product is directly dependent on the fermentation and germination duration. (Massod et al. 2014).

Fermentation and germination of sorghum grains resulted in a non-significant difference in crude fat content from raw sorghum grains, ranging from 1.39 to 1.55%. However, crude fat content was significantly ($p<0.05$) higher in raw green grams. On the other hand, fermented and germinated green grams had comparable fat content. The decrement in the fat content in fermented grains was because of the action of lactic acid bacteria using the fats for energy. During germination, the respiration process also requires energy derived from the fats. The results obtained agree with Adam et al. (2013), who studied the effect of fermentation on the nutritional composition of five different sorghum varieties. Adebo (2020) also shares similar results. Afify et al. (2012) studied three varieties of sorghum before and after germination, and the crude fat content decreased after germination. Warle et al. (2015) determined germination's effect on sorghum's nutritional quality, and a similar decrease in crude fat was observed. Oghbaei and Prakash (2016) observed that fermentation and germination processes decrease the crude fat content of green grams.

Germinated sorghum had significantly ($p<0.05$) higher crude protein content (10.91%) than fermented (10.50%) and raw (9.30%) grains. However, no significant difference was observed in raw and germinated green gram grains. Fermented green gram ($p<0.05$) grains had a significantly higher crude protein content of 22.30%. Bhathal and Kaur (2015) reported that the decrease of carbohydrates and crude fat led to increased proteins after fermentation and germination. The fermentation process increases protein content due to decreased dry matter as carbohydrates and fats are used for energy by microorganisms. Microbial fermentation increases the protein content, and lysine is produced during fermentation, thereby raising the protein level (Zhang et al. 2015).

Table 1: Proximate composition of raw, fermented and germinated sorghum and green gram –grains

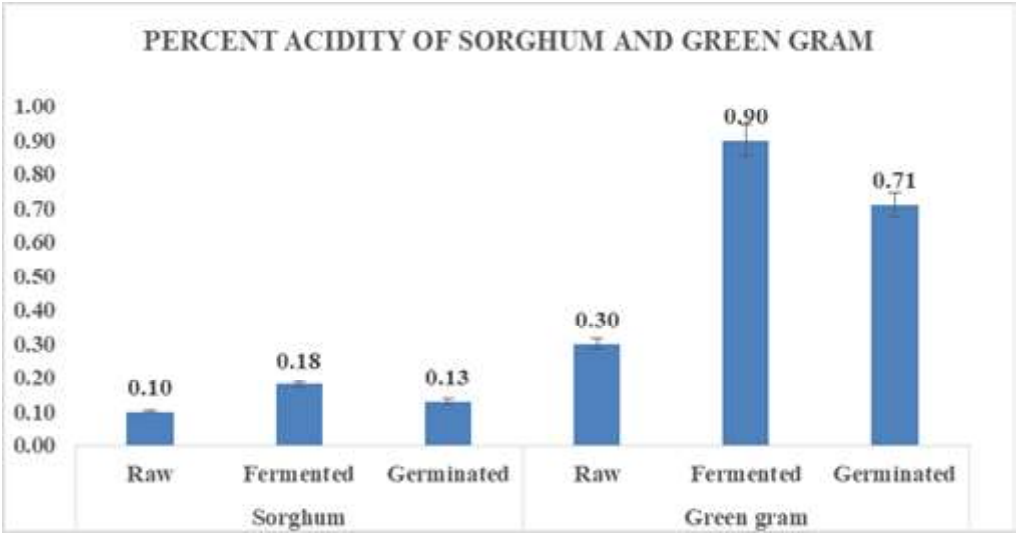
Parameters	Sorghum			Green gram		
	Raw	Fermented	Germinated	Raw	Fermented	Germinated
Moisture (%)	8.66 ± 0.47^{a}	9.02 ± 1.03^{b}	8.80 ± 0.81^{a}	10.22 ± 1.47^{x}	11.27 ± 1.01^{y}	14.75 ± 0.61^{z}
Crude fat (%)	1.55 ± 0.41^{ns}	1.42 ± 0.49^{ns}	1.39 ± 0.38^{ns}	2.45 ± 0.40^{y}	1.78 ± 0.21^{x}	1.74 ± 0.28^{x}
Crude protein (%)	9.30 ± 1.04^{a}	10.50 ± 1.34^{b}	10.91 ± 0.31^{c}	15.25 ± 0.10^{x}	22.30 ± 1.28^{y}	16.15 ± 2.05^{x}
Crude fibre (%)	2.27 ± 0.41^{b}	1.51 ± 0.30^{a}	1.99 ± 0.66^{b}	3.34 ± 0.56^{y}	1.42 ± 0.03^{x}	2.44 ± 0.15^{xy}
Ash (%)	3.44 ± 0.37^{c}	2.56 ± 0.31^{b}	1.77 ± 0.71^{a}	8.16 ± 0.22^{z}	5.02 ± 0.27^{x}	6.60 ± 0.66^{y}
Carbohydrates (%)	71.78 ± 1.26^{c}	69.12 ± 1.74^{a}	70.63 ± 1.23^{b}	64.33 ± 0.71^{z}	60.11 ± 0.78^{x}	61.60 ± 0.62^{y}
Energy (kcal)	352.70 ± 0.72^{c}	334.49 ± 1.51^{a}	342.55 ± 1.02^{b}	340.37 ± 1.64^{z}	313.43 ± 0.33^{x}	325.12 ± 0.43^{y}

Values are mean ± SD of three replicates
Means within rows with different superscripts, are significantly different ($p < 0.05$) from each other
ns= non-significant

Table 2: Mineral composition of raw, fermented and germinated sorghum and green gram grains

Parameters	Sorghum			Green gram		
	Raw	Fermented	Germinated	Raw	Fermented	Germinated
Phosphorus (mg/100g)	363.84 ± 2.28^{a}	473.65 ± 1.43^{b}	481.45 ± 1.32^{c}	214.27 ± 0.44^{x}	217.99 ± 0.38^{z}	216.45 ± 0.35^{y}
Magnesium (mg/100g)	250.16 ± 0.49^{c}	223.21 ± 0.30^{a}	246.38 ± 0.34^{b}	132.22 ± 0.58^{x}	134.21 ± 1.40^{x}	133.20 ± 1.53^{x}
Calcium (mg/100g)	198.55 ± 2.21^{c}	157.38 ± 0.30^{a}	164.44 ± 0.17^{b}	203.23 ± 0.46^{x}	210.36 ± 1.51^{y}	216.40 ± 1.83^{z}
Iron (mg/100g)	13.50 ± 2.12^{c}	13.00 ± 1.41^{b}	11.40 ± 0.14^{a}	18.28 ± 1.32^{x}	20.71 ± 0.74^{z}	19.27 ± 0.39^{y}

Values are mean ± SD of three replicates
Means within rows with different superscripts are significantly different ($p < 0.05$) from each other



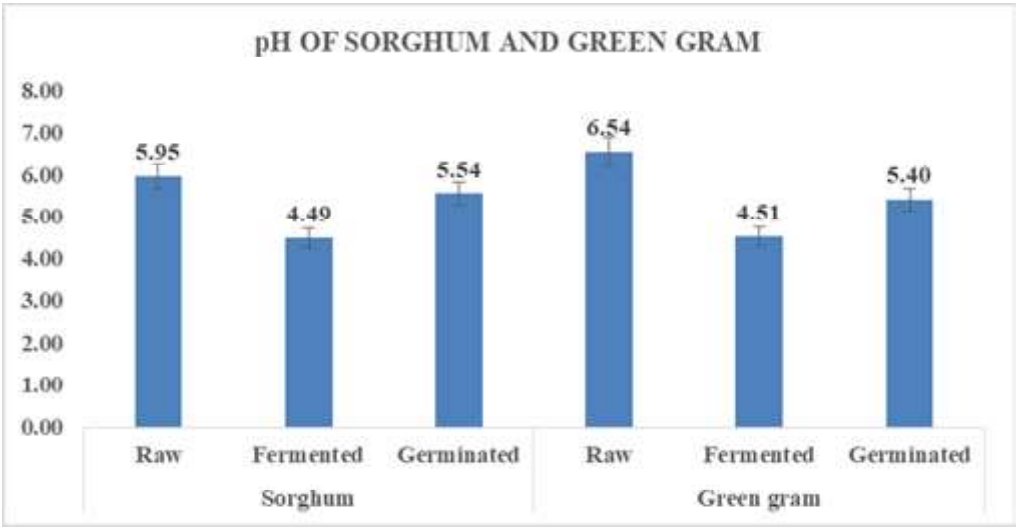


Fig. 2: Percent Acidity and pH of control, fermented and germinated Sorghum and green gram

a) Acidity b) pH

Fermentation of sorghum grains indicated a significant ($p<0.05$) decrease in the dietary fiber content to 1.51% from 2.27% of sorghum grains. However, there was no significant difference between raw and germinated grains. Similarly, fermented green gram showed a significantly ($p<0.05$) lower crude fiber content of 1.42% while germinated grains were neither significantly different from raw nor fermented grains. Determination of crude fiber measures the indigestible cellulose; lignin as well as other components found in the specific food, it also evaluates the milling efficiency to separate endosperm and bran (Fahey, 2019).

Ash content decreased significantly ($p<0.05$) from 3.44% in raw sorghum grains after fermentation to 2.56% and germination to 1.77%. Accordingly, the same trend of significant ($p<0.05$) decrease of ash content was observed after fermentation and germination of green gram grains. Warle et al. (2015) also indicated that ash content decreased due to a decrease in mineral content.

Carbohydrates (%) and Energy (kcal): Carbohydrates and energy significantly ($p<0.05$) decreased in fermented and germinated sorghum grains. Fermentation of grains showed a higher decrease in both sorghum and green gram grains. The glucose released during fermentation is a preferred substrate for microorganisms fermenting the food and could partly explain the decrease in total carbohydrates after 24 hr of fermentation. The sorghum grains results obtained are similar to those reported by Adebo (2020), Afify et al. (2012) and Warle et al. (2015). Carbohydrates are used for respiration during germination hence the decrement of carbohydrates content and energy consequently (Bhathal and Kaur 2015). Germination conditions allow enzymatic hydrolysis of starch to simple sugars leading to a decrement of carbohydrates after grain germination (Oghbaei and Prakash 2016).

Mineral composition

As depicted from Table 2, fermented and germinated sorghum grains indicated significantly ($p<0.05$) higher phosphorus content

than raw grains, however Mg, Ca and Fe content were significantly ($p<0.05$) low in grain. Green gram showed no significant ($p<0.05$) difference in Mg content of raw, fermented, and germinated lentils whilst Ca and Fe content were significantly ($p<0.05$) higher after fermentation and germination. A study by Makokha et al. (2002) agrees that fermentation and germination of Kenyan varieties of sorghum grains increased mineral content with a higher increase in germinated grains. An effect of germination on the mineral content of horse gram and green gram malt was determined by Sadawarte et al. (2018), germination was found to decrease phosphorus, magnesium, calcium, and iron from 321.68 to 256, 184 to 136.5, 70.86 to 11.6 and 5.6 to 4.03 mg/100g simultaneously. Cereals and legumes contain minerals that are complexed with non-digestible materials like polysaccharides and phytate making their bioavailability low. Fermentation and germination free the complexed minerals in the grains (Oghbaei and Prakash 2016). The phytase enzyme contained in legumes and cereals is activated during germination leading to the destruction of phytate for the release of bound minerals. Mineral content, therefore, is increased post-fermentation and germination (Ogbonna et al. 2012).

Acidity and pH of Sorghum and green gram

Acidity of sorghum grains significantly ($p<0.05$) increased after fermentation from an initial 0.10 % to 0.18 % and to 0.13 % after germination (Fig. 2a). pH decreased after both fermentation and germination (Fig. 2b). Green gram grain similarly showed a significant ($p<0.05$) increase in acidity and decrease in pH. Jood et al. (2012) studied the effect of germination and probiotic fermentation on pH and titratable acidity of sorghum-based food mixtures, the pH was 6.23 and the titrable acidity was 1.71 g lactic

Table 3: Effects of fermentation and germination on sensory characteristics of water reconstituted* instant sorghum porridges

Porridge (Sorghum:GG)	Colour & Appearance	Texture	Taste	Flavour	Overall acceptability
Fermented sorghum porridge (incorporated with green gram)					
Control (T_o)	7.93±0.43^{cd}	7.98±0.27^{a}	7.80±0.76^{a}	6.60±0.55^{b}	7.69±0.75^{a}
100:0 (T_1)	8.00±0.79^{d}	8.00±0.33^{a}	8.31±0.53^{c}	6.51±0.44^{b}	7.65±0.66^{b}
80:20 (T_2)	7.56±0.32^{c}	8.38±0.27^{b}	8.56±0.33^{c}	7.31±0.79^{d}	7.88±0.78^{b}
75:25 (T_3)	7.00±0.33^{b}	8.50±0.41^{bc}	7.74±0.62^{b}	6.97±0.63^{c}	6.75±0.34^{a}
70:30 (T_4)	6.79±0.54^{ab}	8.66±0.11^{bc}	7.51±0.22^{a}	6.91±0.42^{c}	6.62±0.66^{a}
65:35 (T_5)	6.51±0.22^{a}	8.77±0.29^{c}	7.33±0.29^{a}	6.24±0.34^{a}	6.38±0.21$^{a\backslash}$
Germinated sorghum porridge (incorporated with green gram)					
Control (T_o)	8.03±0.43^{a}	7.98±0.27^{b}	7.80±0.76^{a}	6.90±0.55^{a}	7.69±0.75^{a}
100: 0 (T_6)	8.51±0.33^{b}	6.91±0.76^{a}	7.32±0.42^{a}	7.68±0.38^{a}	7.03±0.55^{a}
80: 20 (T_7)	7.50±0.61^{a}	7.01±0.32^{a}	7.51±0.22^{a}	7.81±0.33^{a}	7.24±0.33^{a}
75: 25 (T_8)	7.38±0.82^{a}	7.88±0.43^{a}	7.54±0.24^{a}	8.43±0.67^{b}	7.48±0.77^{a}
70: 30 (T_9)	7.30±0.22^{a}	7.95±0.91^{b}	7.67±0.38^{b}	8.64±0.66^{b}	7.56±0.92^{a}
65:35 (T_{10})	7.20±0.91^{a}	8.32±0.36^{c}	8.78±0.11^{c}	8.75±0.43^{b}	8.26±0.33^{b}

Values are mean ± SD of three replicates
Means within columns with different superscripts, are significantly different ($p < 0.05$) from each other
*After reconstitution (ISP: Water = 1:5)

Table 4: Effects of fermentation and germination on sensory characteristics of milk reconstituted* instant sorghum porridges

Instant Sorghum Porridge	Colour & Appearance	Texture	Taste	Flavour	Overall acceptability
Control	8.10±0.45^{a}	7.96±0.27^{b}	7.88±0.25^{a}	6.99±0.58^{a}	7.69±0.20^{a}
Fermented 80:20 (T_2)	8.49±0.55^{b}	8.10±0.33^{b}	8.58±0.45^{b}	7.08±0.35^{a}	8.08±0.25^{b}
Germinated 65:35 (T_{10})	7.96±0.32^{a}	6.53±0.77^{a}	8.10±0.36^{b}	8.20±0.41^{b}	7.78±0.30^{a}

Values are mean ± SD of three replicates
Means within columns with different superscripts, are significantly different ($p < 0.05$) from each other
*After reconstitution (ISP: milk = 1:5)

acid/ml. Onwurafor et al. (2014) studied the effect of fermentation methods on chemical and microbial properties of green gram (*Vigna radiata*) flour; an increase in acidity from 0.009 % to 0.011 % and decrease in pH from 6.24 to 3.68 simultaneously was reported. The fermentation process leads to decreased pH and increased acidity because of lactic acid accumulated due to microbial activity. Lowered pH inhibits the growth of spoilage microbes in grains resulting in high storage quality. Fermentation of legumes resulted in lower pH values because their high protein content buffers acids involved in the process (Nkhata et al. 2018).

Effects of fermentation and germination on sensory scores of water reconstituted Instant sorghum porridge (ISPs)

Fermentation and germination of sorghum and green gram were done to add value to sorghum porridge and instantization provides convenience by reduction of cooking time. Table 3 shows the effect of fermented and germinated instant sorghum porridges on the sensory scores after reconstitution.

Appearance is an important factor in food. Fermented sorghum porridge (T_1) and germinated sorghum porridge (T_6) had colour & appearance scores of 8.00 and 8.51 respectively. Results obtained by (Onweluzo and Nnamuchi (2009) on a study of sorghum porridge agree with the present findings depicting that sorghum fermented for 48 hours had significantly ($p<0.05$) higher colour and appearance score. Fermentation and germination reduce the tannins thereby lightening cereals. The longer the process, the lighter the cereals become (Olamiti et al. 2020). The addition of green gram significantly ($p<0.05$) decreased the colour & appearance scores of both fermented sorghum porridge and germinated sorghum porridge since green gram is darker in appearance than sorghum and judges preferred lighter colour and appearance in the porridges. Adebo et al. (2020) indicated in the sensory evaluation of sorghum porridge that the dark brown colour in porridge is unattractive. The texture score of fermented sorghum porridge was significantly ($p<0.05$) lower in T_1 whilst in germinated porridges (T_6, T_7 and T_8) the scores were not significantly different. Osungbaro (1990) contradicts the texture sensorial results associated with fermented maize porridge as in their study, the fermentation process improved the textural characteristics in terms of consistency, gelling tendency, and starch stability. It is probable that different cereal grains behave differently due to their distinct kernel structure. Fermented sorghum porridge taste score was significantly ($p<0.05$) higher in T_1 (8.31) and T_2 (8.56) whereas in germinated sorghum (T_{10}) it was 8.78. Despite Osungbaro (1990) indicating that fermented porridge can have low taste and flavour scores due to high acids and other flavors development, it can be argued that the period of fermentation process may also have an impact on these sensory scores. Taste of fermented sorghum porridge decreased with higher green gram proportion whilst germinated sorghum porridge taste increased with higher green gram proportion; this maybe because of the undesirable smell and taste from fermented green gram. Fermented sorghum porridges had the flavour scores ranging from 6.24 to 7.31 whereas in germinated porridge the range was from 7.68 to 8.75. As mentioned above, the undesirable flavour of fermented green gram led to lower sensory scores hence the scores reduced as the fermented green gram proportion increased. Overall acceptability of fermented sorghum porridge was not significantly ($p<0.05$) different between T_1 (7.65) and T_2 (7.88) while GISP (T_{10}) with 8.26 overall acceptability scores being the most preferred sorghum porridge. Porridge made from germinated grains was found to be more acceptable overall with the increased green gram proportion having a high overall acceptability score. This is due to the flavonoids activated in green gram during the germination process. It is the opposite for fermented green gram which produces an undesirable flavour and taste. Subsequently increased proportion of fermented green gram improved porridge texture.

Effects of fermentation and germination on sensory scores of milk reconstituted Instant sorghum porridge (ISPs)

To determine the effect of fermentation and germination on sensory scores of milk reconstituted instant sorghum porridges, T_2 and T_{10} were used since they had the best overall acceptability according to Table 4. It was observed that colour and appearance score was significantly ($p<0.05$) higher in T_2 whilst T_{10} and control were not significantly ($p<0.05$) different. Evidently, the light colour in fermented porridge gave attractive appearance hence the best sensory score in this accord. Germination process led to a development of sprouts which contains radicles in their structure. Since these radicles were not removed during processing, they probably gave an unattractive appearance in the porridge hence the low colour & appearance and textural scores were obtained. T_{10} obtained a significantly ($p<0.05$) lower textural score for the reason mentioned above as the dried rootlets present were felt in the in mouth during sensory evaluation of porridge. Taste scores were significantly ($p<0.05$) higher in fermented porridge (8.58) and germinated porridge (8.10) as compared to control (7.88). Processes of fermentation and germination improved taste in the porridges. Because taste plays a significant role in food selection and consumption, a developed food product with high sensory taste ratings is likely to be well-received and palatable. Hutkins (2006) indicated that fermented foods are highly valued because of rich and complex taste and odour. It can be argued that fermentation generates flavour. The results on the other hand displayed no significant ($p<0.05$) difference between control (6.99) and fermented (7.08) porridges. T_{10} on the other hand had a significantly ($p<0.05$) higher flavour score of 8.20 and this might be due to the reactions occurring during germination that activate the flavour components to release desirable flavours in green gram grains. Overall acceptability score of 8.08 of T_2 was not significantly ($p<0.05$) different from that of T_{10} (7.78). Even though T_2 overall acceptability (8.08) was significantly ($p<0.05$) higher than control porridge (7.69), T_{10} (7.78) was not significantly

different from the control. Overall, the fermented porridge had the best sensorial qualities.

Conclusion

Fermentation and germination processes were desirable for the development of sorghum based instant blended porridge. The processes added value through the improvement of the nutritional and sensory properties of sorghum porridge. Fermentation of sorghum was preferred and produced the best sensory properties. Both fermented and germinated porridges significantly increased the nutritional and physico-chemical properties and sensory characteristics of instant blended porridge compared to untreated sorghum porridge and the product goes well with water and milk after reconstitution. Future studies may be carried on vitamin and amino-acid profiling of the developed product to explore and validate the developed product.

References

Adam GOA, Hu Y, Chamba MVM, Gasmalla MAA (2013) Functional properties and in vitro protein digestibility of fermented sorghum and broad bean (*Visia faba* L. Major) blended flour. Pakistan J Food Sci 23(1):10-16

Adebo, OA (2020) African sorghum-based fermented foods: past, current and future prospects. Nutrients 12(4):1111-1119.

Afify AMR, El-Beltagi HS, Abd El-Salam SM, Omran AA (2012) Protein solubility, digestibility and fractionation after germination of sorghum varieties. Plos One 7(2):e31154

Alahmari LA (2024) Dietary fiber influence on overall health, with an emphasis on CVD, diabetes, obesity, colon cancer, and inflammation. Frontiers in Nutrition 11:1510564

AOAC (2016) Official Method of Analysis 12th ed., Association of Official Chemists.Washington D.C., USA

Bhathal S, Kaur N (2015) Effect of germination on nutrient composition of gluten free Quinoa (*Chenopodium quinoa*). Int J Scientific Res 4(10):423-425

Chopra SL, Kanwar JS (1990) Influence of immigration with pulp and paper mill effluent on soil chemical and microbiological properties. Biol Fertility Soils 10(3):197-207

Diatta AA, Abaye O, Battaglia ML, Leme JF, Seleiman M, Babur E, Thomason WE (2024) Mungbean [Vigna radiata (L.) Wilczek] and its potential for crop diversification and sustainable food production in Sub-Saharan Africa: a review. Technol Agronomy 4(1):e031.

Fahey GC, Novotny L, Layton B, Mertens DR (2019) Critical factors in determining fiber content of feeds and foods and their ingredients. J AOAC Int 102(1):52-62.

Hutkins R (2006) Microbiology and technology of fermented foods. Ames: Blackwell Publishing

Jood S, Khetarpaul N, Goyal R (2012) Effect of germination and probiotic fermentation on pH, titratable acidity, dietary fibre, β-glucan and vitamin content of sorghum-based food mixtures. J Nutrition Food Sci 2(9):164-168.

Khalil AW, Zeb A, Mahmood F, Tariq S, Khattak AB, Shah H (2007) Impact of germination time on comparative sprout quality characteristics of desi and Kabuli type chickpea cultivars (*Cicer arietinum* L). LWT-Food Sci Technol 40(6):937-945.

Koenig RA, Johnson CR (1942) Colometric determination of phosphorus in biological materials. Industrial and Engineering Chemistry Analytical edition 14(2):155-156.

Makokha AO, Oniango RK, Njoroge SM, Kamar OK (2002) Effect of traditional fermentation and malting on phytic acid and mineral availability from sorghum (*Sorghum bicolour*) and finger millet (*Eleusine corocana*) grain varieties grown in Kenya. Food Nut Bull 23: 241-245

Massod T, Shah HU, Zeb A (2014) Effect of sprouting time on proximate composition and ascorbic acid level of mung bean (*Vigna radiate* L.) and chickpea (*Cicer arietinum* L.) seeds. J Animal Plant Sci 24(3):850-859.

Nkhata SG, Ayua E, Kamau EH, Shingiro JB (2018) Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. Food Sci Nut 6:2446–2458.

Nonogaki H, Bassel GW Bewley JW (2010). Germination-still a mystery. Plant Sci 179(6): 574-581

Ogbonna AC, Abuajah CI, Ide EO, Udofia US (2012) Effect of malting conditions on the nutritional and anti nutritional factors of sorghum grist. Food Technol 36:64–72

Oghbaei M, Prakash J (2016) Effect of primary processing of cereals and legumes on its nutritional quality, A comprehensive review. Cogent Food and Agri 2(1):1447-1474.

Olamiti G, Takalani TK, Beswa D, Jideani AIO (2020) Effect of malting and fermentation on colour, thermal properties, functional groups and crystallinity level of flours from pearl millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*). Heliyon 6(12) e05467

Onwurafor EU, Onweluzo JC, & Ezeoke AM (2014) Effect of fermentation methods on chemical and microbial properties of mung bean (*Vigna radiata*) flour. Nigerian Food J 32(1), 89-96

Osungbaro, TO (1990) Effect of fermentation period on amylose content and textural characteristics of "Ogi" (a fermented maize porridge). J Ferm and Bioeng 70(1): 22-25

Ranganna S (1986) Handbook of Analysis and Quality Control for Fruit & Vegetable Products. Tata McGraw Hills Publication Co. Ltd., New Delhi

Rao NBS, Prabhavathi R (1978) An in vitro method for predicting the bioavailability of iron from foods. The American J Clinical Nut 31(1):169-175

Sadawarte SK, Pawar VS, Sawate AR, Thorat PP, Shere PD Surendar J (2018) Effect of germination on vitamin and mineral content of horse gram and green gram malt. Int J Chem Stud 6(3):1761-1764

Shah SA, Zeb A, Masood T, Noreen N, Abbas S J, Samiullah M, Alim MA Muhammad A (2011) Effect of sprouting time on biochemical and nutritional qualities of mung bean varieties. African J Agricul Res 6(22):5091-5098

Warle BM, Riar CS, Gaikwa, SS, Mane VA, Sakhale BK (2015) Effect of germination on the nutritional quality of sorghum. Int J Current Res 7(05):16029-16033

Zhang G, Xu Z, Gao Y, Huang X, Yang T (2015) Effects of germination on the nutritional properties, phenolic profiles, and antioxidant activities of buckwheat. J Food Sci 80(5): H1111–H1119

Indian J Dairy Sci 78(2): 156-164
https://doi.org/10.33785/IJDS.2025.v78i02.008

RESEARCH ARTICLE

Detection of palmolein oil adulteration in milk fat using ATR-FTIR Spectroscopy and Chemometrics

Vivek Sonvanshi[1], Kamal Gandhi[1] (✉), Akshay Ramani[1], Rajan Sharma[1], Raman Seth[1] and Bimlesh Mann[2]

Received: 14 January 2024 / Accepted: 21 July 2024 / Published online: 23 April 2025

Abstract: Clarified milk fat (ghee), a household staple in domestic India faces the threat of adulteration due to its economic value. Examination of the purity of milk fat by chemical analysis is complex and time-consuming. The presence of palmolein oil in clarified milk fat was investigated using ATR-FTIR spectroscopy in combination with chemometric techniques. Spectral data within the wavenumber range of 4000-500 cm{ ¹ were obtained for pure ghee, palmolein oil, and spiked ghee samples at various palmolein oil concentrations (1, 3, 5, 10, 15, and 20%). PCA identified distinct spectral clustering patterns at specific wavenumbers (1167-1137 cm^{-1}) at 5% significance level, effectively separating pure ghee from adulterated samples. The SIMCA method yielded a remarkable 100% classification efficiency for both pure ghee and palmolein oil samples. Additionally, developed PLS and PCR models exhibited strong predictive accuracy, with high R^2 values (0.96), enabling the detection of palmolein oil adulteration in ghee, even at concentrations as low as 1%. This research showcases the potential of ATR-FTIR with chemometrics analysis in ghee adulteration, offering a faster and more accurate routine analysis method.

Keyword: Pure ghee, Palmolein oil, Fatty acid analysis, ATR-FTIR

Introduction

Ghee is a type of clarified butter prepared by heating butter to separate the butterfat from the milk solids, resulting in a clear, golden liquid with a nutty flavor. Its unique flavor and versatility make it popular in Indian cooking. In India, approximately 28% of the milk supply is transformed into ghee (Atbhaiya et al. 2022), and the per capita consumption of ghee in the country stands at around 7 grams per person per day, constituting approximately 27.5% of all dairy product consumption (Gandhi et al. 2023). Ghee plays a vital role in the dairy industry; it serves as a valuable product in milk processing and contributes to the overall sustainability and profitability of dairy operations. Beyond its economic importance, ghee is revered for its health benefits, which is attributed to the better digestibility and anti-cancerous properties due to short chain fatty acids (Atbhaiya et al. 2023). Due to its rich taste and perceived health benefits, ghee has been gaining popularity outside India (Wani et al. 2022). Ghee's chemical composition is quite intricate, as it contains 70% saturated fatty acids and 30% unsaturated fatty acids. It also contains bioactive compounds, including butanoic acid ($C_{4:0}$), conjugated linoleic acid (CLA), *cis* and *trans* palmitoleic acid, and á-linolenic acid (ALA), all of which offer significant health benefits. Studies suggest that butanoic acid ($C_{4:0}$) and cis-9, 12, 15 octadecatrienoic acid ($C_{18:3}$) have potential cancer-preventive properties. CLA is associated with reducing the risk of type 2 diabetes and cardiovascular disease, improving vision, and displaying antithrombotic effects. Furthermore, CLA has been found to enhance immune function, support weight management and the development of lean muscle mass, and inhibit the formation of cancerous cells (Gautam et al. 2022). Ghee contains a significant amount of essential fatty acids and fat-soluble vitamins. It plays a vital role in the preparation of various ayurvedic and umami medicines.

Ghee stands out in the edible fats market due to its exceptional nutritional value and distinctive flavor, making it considerably more expensive than other common fats, often priced at 3-4 times their cost. During the lean season, however, with high demand and limited supply of ghee, unscrupulous traders adulterated ghee with less expensive vegetable oil. This adulteration practice involves replacing milk fat with more economical vegetable oils to increase profits, making it a prevalent issue in India (Atbhaiya et al. 2022). Various foreign fats, including vegetable oils, animal body fat and hydrogenated fats, are frequently used as adulterants (Rani et al. 2015; Upadhyay et al. 2016; Antony et al. 2018; Ramani et al. 2019). Palmolein oil is a very common adulterant used for ghee adulteration in India (Ramani et al. 2018). Palm oil, a natural vegetable oil derived from the mesocarp of palm fruits, comprises 50% saturated fatty acids, 40% monounsaturated fatty

Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal, Haryana, India
ICAR, New Delhi, India

(✉)Kamal Gandhi
Email: kamalgandhindri@gmail.com
Mob No: +91 9729134444

acids, and 10% polyunsaturated fatty acids. Palm oil, a primary edible oil in India, is mainly imported from Malaysia and Indonesia due to its limited domestic production (Tandra et al. 2022). India's palm oil imports account for about 18% of the global total, with a significant volume of 8.4 million metric tonnes. Due to similar physical characteristics and low cost, palmolein oil is the primary source of ghee adulteration. This practice poses significant risks to both consumer health and product authenticity. Detection of palmolein oil adulteration in ghee is a challenging task for the dairy industry. Numerous techniques have been employed to check the authenticity of ghee, *i.e.* physicochemical constants, fatty acid profile, sterol test, and colorimetric tests, but all of these tests have some drawbacks. No quick, simple, and effective tests are available for detecting the presence palmolein oil in ghee. With a rise in fraudulent activities in the dairy sector, more sophisticated instruments, like HPLC and GCMS have been used globally to detect non-dairy substances in dairy products but are quite expensive and time-consuming. Therefore, a rapid, precise, and cost-effective method with minimal sample pre-processing is needed for detecting palmolein oil in ghee.

Fourier transform infrared spectroscopy (FTIR) has emerged as a valuable alternative to traditional examination techniques. Attenuated total reflectance (ATR)-FTIR spectroscopy is very suitable because of its high speed, and simplicity, avoids toxic reagents considered a green analytical tool. Mid-infrared (4000-400 cm^{-1}) spectroscopy is most commonly used because it provides information based on fundamental vibrations and rotational vibration of the functional groups in the sample (Gandhi et al. 2022), on the other hand NIR (Near-infrared spectroscopy) spectra provide information from the complex overtone and high-frequency combinations at the shorter wavelengths (Ozaki & Morisawa, 2021). Attenuated total reflectance Fourier transform mid-infrared spectroscopy (ATR-FT-MIR) is used to analyze samples by examining the chemical composition of their surface by applying principles of total internal reflection (da Silva Bruni et al. 2021). FTIR combined with the chemometrics technique is frequently used for quality monitoring of milk and dairy products (Saji et al. 2024). Recent advancements in FTIR spectroscopy have made the FTIR technique a strong tool for ascertaining the purity of dairy products. In a very short time, this approach can produce accurate outcomes with an identical degree of accuracy and precision as conventional techniques. In spectral quantification, chemometrics approaches are widely utilized.

Chemometric technique applied at wavenumbers in which maximum variation in absorbance peak is found, is the most efficient approach for extracting qualitative and quantitative data from FTIR spectra and eliminating food scams (Mendes et al. 2021). Principal component regression (PCR), partial least squares (PLS), and soft independent modelling of class analogies (SIMCA) are most widely used for this purpose. Using PLS and PCR regression, the relationship between actual and predicted values for both calibration and validation sets can be established. SIMCA is a classification modeling technique wherein samples are independently categorized into their respective classes without dependence on other samples. This approach is versatile, allowing its application across various strategies, ranging from one-class to multi-class classifications (Burmistrova et al. 2021). Numerous literatures are available that indicate the effective use of multivariate analysis along with ATR-FTIR to examine the foods. ATR-FTIR along with multivariate analysis was used to detect pork in beef meatball (Rohman et al. 2010), lard in vegetable oils (Rohman et al. 2011), chicken fat in butter (Nurrulhidayah et al. 2013), cow and buffalo milk adulterated with soy milk (Jaiswal et al. 2015), ghee adulterated with goat and pig body fat in ghee (Upadhyay et al. 2016, 2018), coconut oil in ghee (Gandhi et al. 2022), mineral oil in milk fat (Gandhi et al. 2023), vanaspati ghee (hydrogenated vegetable oil) in milk fat (Sonvanshi et al. 2024) and flunixin residues in milk (Saji et al. 2024). These research findings highlight the efficacy of combining FTIR with chemometric techniques, establishing it as a potent tool for ensuring the quality and authenticity of dairy products.

To identify pamolein oil adulteration in clarified milk fat, this work employed ATR-FTIR spectroscopy in conjunction with chemometric techniques such as principal component analysis (PCA), SIMCA, PLS, and PCR. The effectiveness of PLS and PCR regression methods to detect pamolein oil in clarified milk fat was also studied.

Material and Methods

Sample collection

The ghee sample was prepared using milk obtained from cow and buffalo, housed at the Livestock Research Centre, NDRI, Karnal. Palmolein oil from five reputable brands in Karnal was used to prepare adulterated samples for testing and model development.

Sample preparation

Cow and buffalo ghee were individually prepared using the creamery butter method (De, 2012). A pure mixed ghee (PMG) sample was prepared by blending cow and buffalo ghee in 1:1 ratio. Each type of sample was prepared 15 times and divided into two to have a total of 30 samples. Following the experimental design, pure mixed ghee was then adulterated with 1%, 3%, 5%, 10%, 15%, and 20% w/w palmolein oil (PO) to simulate various levels of adulteration.

Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared and fatty acid analysis by GC as per ISO 15884 (2002) to correlate FT-MIR spectral data of ghee, palmolein oil and adulterated ghee samples with their fatty acid composition.

Spectral acquisition

The samples were analyzed using FTIR (IR-Affinity-01, Shimadzu, Tokyo, Japan) with a diamond crystal cell ATR and integrated IR-Solution software, operating at a resolution of 4 cm^{-1}. The data collection process was conducted at a constant temperature of 40°C for both control and adulterated samples to maintain uniformity. To ensure reliability, each sample was scanned forty times in the mid-infrared region (4000-500 cm^{-1}) against an air background to collect absorption spectra. After each scan, the ATR crystal was carefully cleaned with ethanol and wiped to remove contaminants, using soft tissue paper.

Spectral analysis

The reflectance mode was used to acquire the absorption spectra in the mid-infrared range. The samples included a control sample and a range of pamolein oil spiked ghee samples (1%, 3%, 5%, 10%, 15% and 20% w/w). A comprehensive analysis was performed on the acquired spectra to identify the specific regions where the increased concentration of PO in the adulterated samples had the most notable impact on the intensities and positions of the peaks.

Chemometric analysis

In this study, chemometric analysis was performed using the Unscrambler software (version 10.2; CAMO AS, Trondheim, Norway). The spectral data were partitioned into a training set (calibration set) and a testing set (validation set) to facilitate thorough model assessment. Both sets were balanced, including PMG as control samples and (PO in PMG) as spiked samples, to maintain the integrity of the data. Subsequently, a detailed examination of spectral absorption regions was performed to identify the variables required for PCA, PLS, and PCR modeling. The deliberate selection of these spectral variables served as the basis for a thorough chemometric analysis, leading to the development of predictive models aligned with the research objectives. Specific regions 1167-1137 cm^{-1} were identified through peak analysis in the raw spectra. PCA was employed to explore clustering patterns within these regions, identifying a few outliers within the selected wavenumber range. The importance of choosing a spectral range that accurately represents changes in analyte concentration.

Developing models

For this study, PLS and PCR models were applied to the sample sets, with a focus on the spectral region of 1167-1137 cm^{-1}. The Unscrambler software's cross-validation feature was employed to enhance the robustness of the model. The principal objective was to establish predictive models for accurately quantifying PO content in PMG. Model selection criteria encompassed the optimization of the coefficient of determination (R^2) to achieve the highest precision while concurrently minimizing bias, SEC, and SEP values. This method aligns with the established methodology of previous researchers (Upadhyay et al. 2016, 2018; Jha et al. 2015).

Application of SIMCA to the derived models

A comprehensive model was established through the calibration set, which included both control and spiked samples. Subsequent to this, specific class models were developed to discern the levels of PO added to ghee. The SIMCA method was applied for the classification of each sample based on its PO content. The validation dataset was used to assess class memberships at a 5% significance level. Test samples were then assigned to predefined class models based on their closest resemblance. Efficiency metrics for classification, including true positive, true negative, false positive, and false negative samples, were introduced. True positives represent samples correctly classified within their designated class, true negatives signify accurately rejected samples, false positives involve misclassifying samples into an incorrect class, and false negatives represent samples that do not align with the designated class model (Balan et al. 2020)

Results and discussion

Fatty acid analysis

Milk fat is the most complex of all fats, containing over 400 different types of fatty acids, including short-chain, medium-chain, and long-chain fatty acids (Fox et al. 2015). The fatty acid composition of PMG, PO, and ghee adulterated with different concentrations of PO is shown in Table 1. Palmitic acid was the most prominent fatty acid in PO followed by oleic, stearic, and linoleic acid. Our findings were similar to the earlier results reported by Jeyarani et al. (2005) and Dorni et al. (2018). As the level of spiking increased, the concentration of palmitic acid in PMG also increased. Butyric acid, a characteristic milk fatty acid, was absent in PO, and its level decreased with increased spiking levels. Similarly, short-chain fatty acids like $C_{6:0}$, $C_{8:0}$, and $C_{10:0}$ are absent in PO and exhibit reduced concentrations with increasing spiking. Medium-chain FAs, such as $C_{12:0}$, $C_{14:1}$, and $C_{15:0}$, were also absent in PO and displayed a decrease in concentration with higher spiking levels. Gandhi (2015) reported that pure cow/buffalo ghee exhibited a lower concentration of linoleic acid ($C_{18:2}$) compared to PO, serving as a marker for detecting ghee adulteration up to 5% level. The concentrations of linoleic acid in PMG and PO, 1.22% and 9.78% respectively, were consistent with values reported by Gandhi (2015). Kumar (2015) also employed linoleic acid as a reference FA to identify vegetable oil adulteration in ghee and found that the concentration of oleic acid was significantly higher in PO compared to pure buffalo and cow ghee. In this study, a similar concentration of oleic acid (25.48%) was observed in PMG. Furthermore, they reported that as the concentration of PO increased in ghee, the levels of stearic,

linolenic, and arachidic acid significantly decreased, while oleic, linoleic, and palmitic acid concentrations increased.

Examination of FTIR spectra

It can be seen from Fig. 1 that distinct differences were observed in the absorbance peak of both the PMG and PO samples. In the mid-infrared region, 12 absorbance peaks were observed, which was mainly due to the presence of various bonds and functional groups showing stretching and bending movement after striking IR light to the PMG and PO sample. The absorbance peak shown at a wavenumber of 2922.16 cm^{-1} was mainly due to the asymmetric stretching occurring between C-H of functional groups like CH_2 and CH_3. Similarly, the peak shown at the wavenumber of 2852.72 cm^{-1} was due to the symmetric stretching that occurs between C-H of the same functional group. Free fatty acids having carbonyl functional group and stretching occurs in this functional group showing characteristic absorbance peak at a wavenumber of 1743.65 cm^{-1}. Similarly, another peak obtained at a wavenumber of 1465.90 cm^{-1} was due to the bending that occurs between the H-C-H of the CH_2 and CH_3 functional groups. Similarly, the absorbance peak shown at a different wavenumber of 1236.37 cm^{-1} was due to the stretching between C-O in O-C(=O)-CH_2 of ester. Absorbance peak at a wavenumber of 1161.15,1112.93, 1099.43, 966.34, and 721.38 cm^{-1} are due to the stretching between

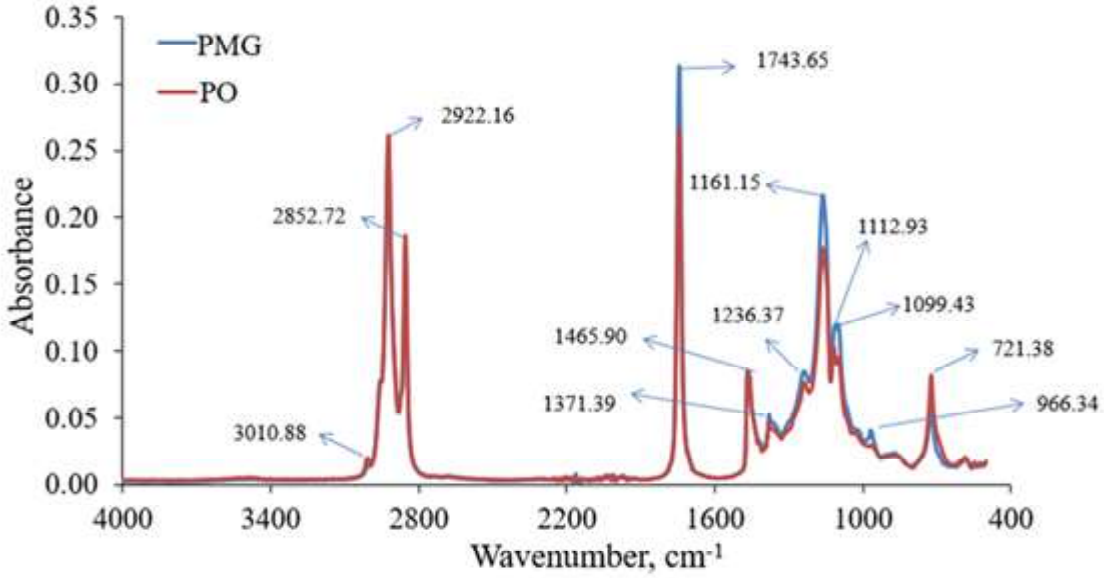


Fig. 1 ATR-FTMIR spectra of pure mixed ghee and palmolein oil

Table 1 Fatty acid compositions of PMG, PO and ghee spiked with different level of PO

FATTY ACIDS	PMG	PO	1% PO	3% PO	5% PO	10% PO	15% PO	20% PO
$C_{4:0}$	3.86 ± 0.518	-	3.84 ± 0.005	3.80 ± 0.006	3.62 ± 0.019	3.40 ± 0.006	3.40 ± 0.002	3.08 ± 0.004
$C_{6:0}$	2.02 ± 0.005	-	1.82 ± 0.059	1.82 ± 0.065	1.82 ± 0.009	1.69 ± 0.007	1.64 ± 0.004	1.46 ± 0.005
$C_{8:0}$	1.18 ± 0.005	-	1.11 ± 0.018	1.09 ± 0.006	1.07± 0.008	0.98 ± 0.010	0.94 ± 0.010	0.87 ± 0.013
$C_{10:0}$	2.33 ± 0.003	-	2.26 ± 0.024	2.20 ± 0.004	2.11 ± 0.023	1.96 ± 0.013	1.88 ± 0.019	1.78 ± 0.001
$C_{12:0}$	3.14 ± 0.003	-	2.92 ± 0.007	2.84 ± 0.004	2.82 ± 0.018	2.57 ± 0.017	2.49 ± 0.019	2.30 ± 0.038
$C_{14:0}$	11.57 ± 0.002	1.61 ± 0.005	11.38 ± 0.039	11.00 ± 0.031	10.77 ± 0.041	10.07 ± 0.043	9.66 ± 0.016	9.11 ± 0.010
$C_{14:1}$	0.80 ± 0.004	-	0.77 ± 0.005	0.77 ± 0.001	0.75 ± 0.012	0.67 ± 0.005	0.65 ± 0.005	0.63 ± 0.004
$C_{15:0}$	1.06 ± 0.004	-	0.99 ± 0.004	0.96 ± 0.013	0.91 ± 0.005	0.89 ± 0.026	0.85 ± 0.008	0.80 ± 0.004
$C_{16:0}$	37.71 ± 0.432	40.51 ± 0.022	38.23 ± 0.021	38.24 ± 0.038	38.61 ± 0.019	38.68 ± 0.094	39.01 ± 0.021	39.05 ± 0.023
$C_{16:1}$	1.55 ± 0.027	-	1.42 ± 0.009	1.41 ± 0.006	1.40 ± 0.002	1.36 ± 0.042	1.24 ± 0.007	1.18 ± 0.004
$C_{17:0}$	0.67 ± 0.00	-	0.67 ± 0.009	0.64 ± 0.012	0.55 ± 0.020	0.54 ± 0.025	0.49 ± 0.012	0.45 ± 0.005
$C_{18:0}$	11.17 ± 0.011	4.82 ± 0.015	11.12 ± 0.013	10.97 ± 0.012	10.94 ± 0.021	10.24 ± 0.002	10.24 ± 0.004	9.69 ± 0.024
$C_{18:1}$	20.48 ± 0.075	42.72 ± 0.091	20.92 ± 0.157	21.57 ± 0.160	21.73 ± 0.045	23.54 ± 0.063	23.93 ± 0.024	25.53 ± 0.013
$C_{18:2}$	1.22 ± 0.046	9.78 ± 0.099	1.34 ± 0.029	1.53 ± 0.029	1.81 ± 0.008	2.36 ± 0.014	2.59 ± 0.016	3.09 ± 0.047
$C_{18:3}$	0.79 ± 0.010	0.19 ± 0.004	0.77 ± 0.004	0.73 ± 0.008	0.70 ± 0.004	0.67 ± 0.005	0.63 ± 0.007	0.61 ± 0.006
$C_{20:0}$	0.37 ± 0.00	0.33 ± 0.00	0.36 ± 0.002	0.35 ± 0.008	0.32 ± 0.005	0.31 ± 0.00	0.30 ± 0.003	0.29 ± 0.008
Total SFA	75.12 ± 0.073	47.29 ± 0.004	74.75 ± 0.120	73.96 ± 0.134	73.58 ± 0.034	71.38 ± 0.024	70.93 ± 0.020	68.93 ± 0.045
Total USFA	24.87 ± 0.072	52.70 ± 0.004	25.24 ± 0.118	26.03 ± 0.133	26.41 ± 0.034	28.61 ± 0.024	29.06 ± 0.021	31.06 ± 0.045

All experiments were conducted in triplicate (n=3). Data were presented as mean ± SD

C-O in HC-O-(C=O) of ester, C-C link of the hydrocarbon chain, bending between C-H in trans double bond and rocking movement that occurs between C-H of CH_2 functional group and cis double bonds, respectively. The responsible bonds, functional groups and various types of vibrational movements were the same as described by Antony et al. (2017). The specific bond and functional group responsible for the characteristic absorbance peak of the sample were presented in Table 2.

The study further revealed that the level of spiking of PO increased from 1 to 15% then maximum variations in absorbance peak occurred in the wavenumber range of 1167-1137 cm^{-1} as shown in Fig. 2 and this region was useful for the detection of PO in PMG. Variations in the concentration of FA in PMG, PO, and spiked samples might be the reason for visual variation in FTIR spectra, in the wavenumber region of 1167-1137 cm^{-1}. GC analysis revealed that the concentration of palmitic, oleic, and linoleic acid was increased as the level of spiking of PO increased. Similarly, stearic, linolenic acid concentration decreased with increased spiking level. Oleic acid was responsible for a distinct absorbance peak at a wavenumber of 3010.88 cm^{-1}. Its concentration was higher in PO as compared to that in PMG so an intense peak was observed in FTIR spectra of PO. Another intense peak was observed at a wavenumber of 1741.72 cm^{-1} which was due to the carbonyl groups of fatty acids present in ghee. Deshwal et al. (2021) reported that a characteristic peak was observed at a wavenumber of 1735 cm^{-1} which was mainly due to the presence of conjugated linoleic acid in ghee.

Chemometrics analysis

Visual changes in absorbance peaks were noted in the wavenumber range of 1167-1137 cm^{-1} as the level of palmolein oil

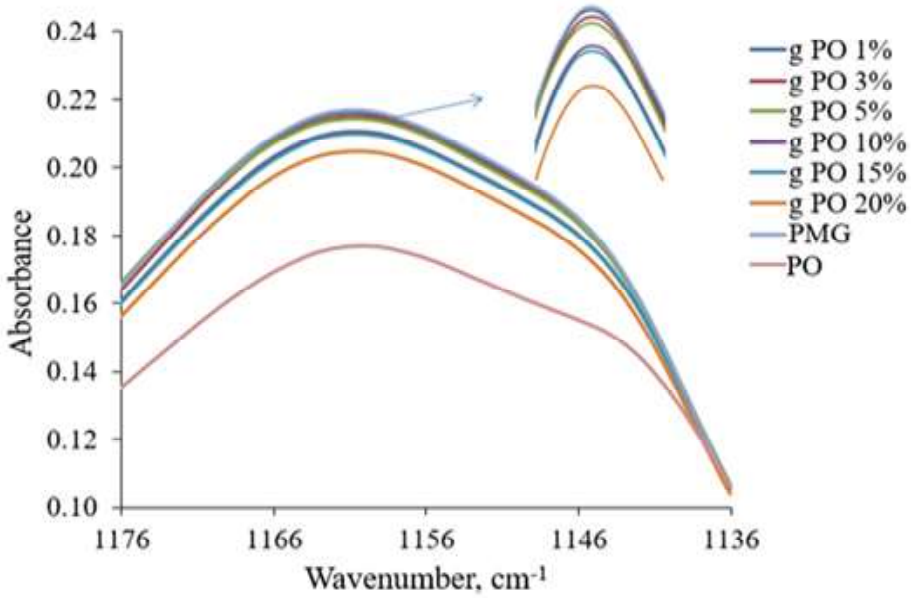


Fig. 2 Spectra of pure mixed ghee and ghee adulterated with different concentrations of palmolein oil at selected 1167-1137 cm{ [1] wavenumber range

po- palmolein oil, g 1%po, g 3% po, g 5% po, g 10% po, g 15% po and g 20%po - ghee spiked with 1, 3, 5, 10, 15 and 20% palmolein oil.

Table 2 Wavenumbers, corresponding functional groups and the types of vibrations associated with them

Wavenumber (cm^{-1})	Functional Groups	Type of Vibration/Phenomena
3010.88	C–H asymmetric stretching of CH_2 and CH_3	C–H Stretching
2922.16	C-H symmetric stretching C–H (CH_2)	C-H stretching
2852.72	C–H symmetric stretching of CH_2 and CH_3	C–H Stretching
1743.65	C=H stretching of esters and FFA	C–H Stretching
1465.90	H–C–H bending of CH_2 and CH_3	C–H Bending
1371.39	H–C– H symmetric bending of CH_2	H–C–H Symmetric Bending
1236.37	C–O stretching in O–C(=O)–CH_2 of ester	C–O Stretching
1161.15	C–O stretching in HC–O–(C=O) of ester	C–O Stretching
1112.93	C–O stretching in O–C–C of ester	C–O Stretching
1099.43	C–C links of a hydrocarbon chain	C–C Stretching
966.34	C–H bending of a *trans* double bond	C–H Bending
721.38	C–H rocking of CH_2 and *cis* double bond	C–H Bending

(PO) spiking increased in ghee. This specific region was employed for chemometrics analysis.

Principal Component Analysis (PCA)

PCA was applied in the region where maximum variation in absorbance peak was observed at 1167-1137 cm^{-1}. It was observed that the PO and PMG samples gave separate clusters after applying PCA and as the level of spiking of PO increased clusters shifted toward the PO (Fig. 3). Remarkably, samples spiked with the lowest PO (1%) formed separate clusters distinct from PMG samples. These findings highlight the crucial role of FTIR in accurately detecting PO within PMG. Principal Components 1 and 2 explained cumulative variations of 100% (99% by PC1 and 1% by PC2) in the spectral region 1167-1137 cm^{-1} at a 5% significance level (Fig. 3). These results align with the study conducted by Upadhyay et al. (2018), where PCA effectively detected pig body fat in ghee within the spectral range of 3030-2785 cm{†¹, with PC1 and PC2 account for variations of 98% and 2%, respectively. Similarly, the work by Gandhi et al. (2023) reported the successful detection of mineral oil in milk fat using PCA within the specific spectral range (1350-950 and 1800-1600 cm{†¹), with PC1 and PC2 explaining variances for both 99% and 1%, respectively.

Partial least square (PLS) and Principal component regression (PCR) Models

The PLS and PCR method was used to generate calibration and validation models in the selected wavenumber region and the impact of different spectral windows on the spectral modelling. PMG and ghee spiked with PO at different levels (120 samples)

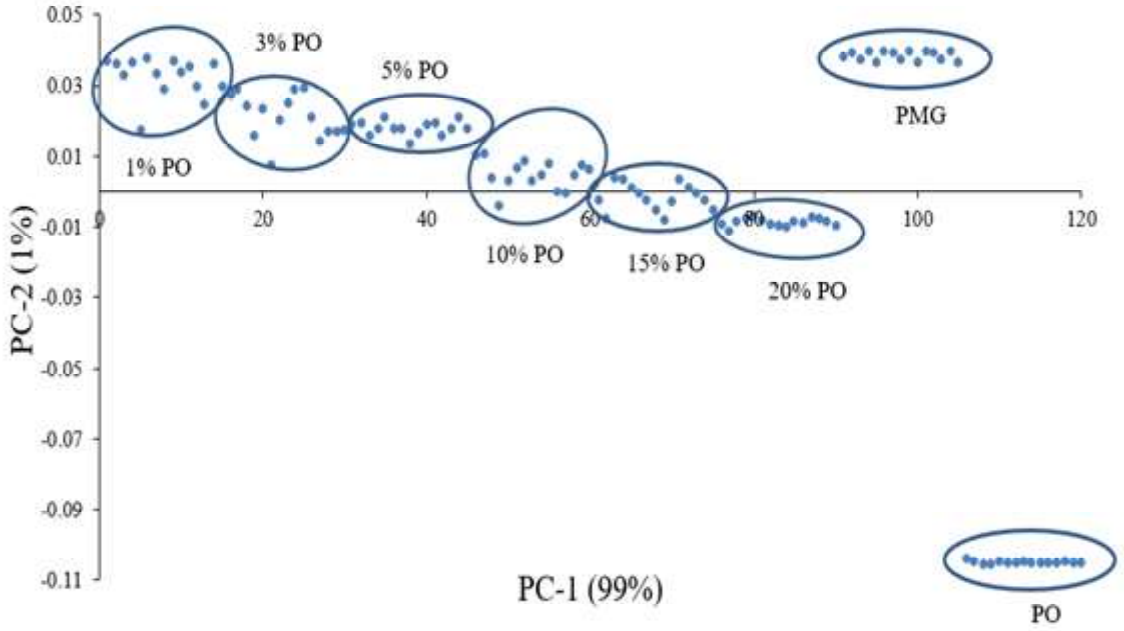


Fig. 3 PCA plot showing the clusters of pure mixed ghee and ghee adulterated with different concentrations of Vanaspati ghee at selected 1167-1137 cm{ ¹ wavenumber range

po- palmolein oil, g 1%po, g 3% po, g 5% po, g 10% po, g 15% po and g 20%po - ghee spiked with 1, 3, 5, 10, 15 and 20% palmolein oil.

Table 3 Comparison between PLS and PCR regression models applied in the wavenumber region of 1167-1137cm^{-1}

Regression model	Wavenumber range (cm^{-1})	Calibration			Validation			Difference between RMSEC and RMSEV value
		R^2	RMSEC	Bias	R^2	RMSEV	Bias	
PLS	1167-1137	0.9654	5.8050	0.665	0.9644	5.9410	0.686	0.136
PCR	1167-1137	0.9652	5.8156	0.668	0.9646	5.9174	0.663	0.1018

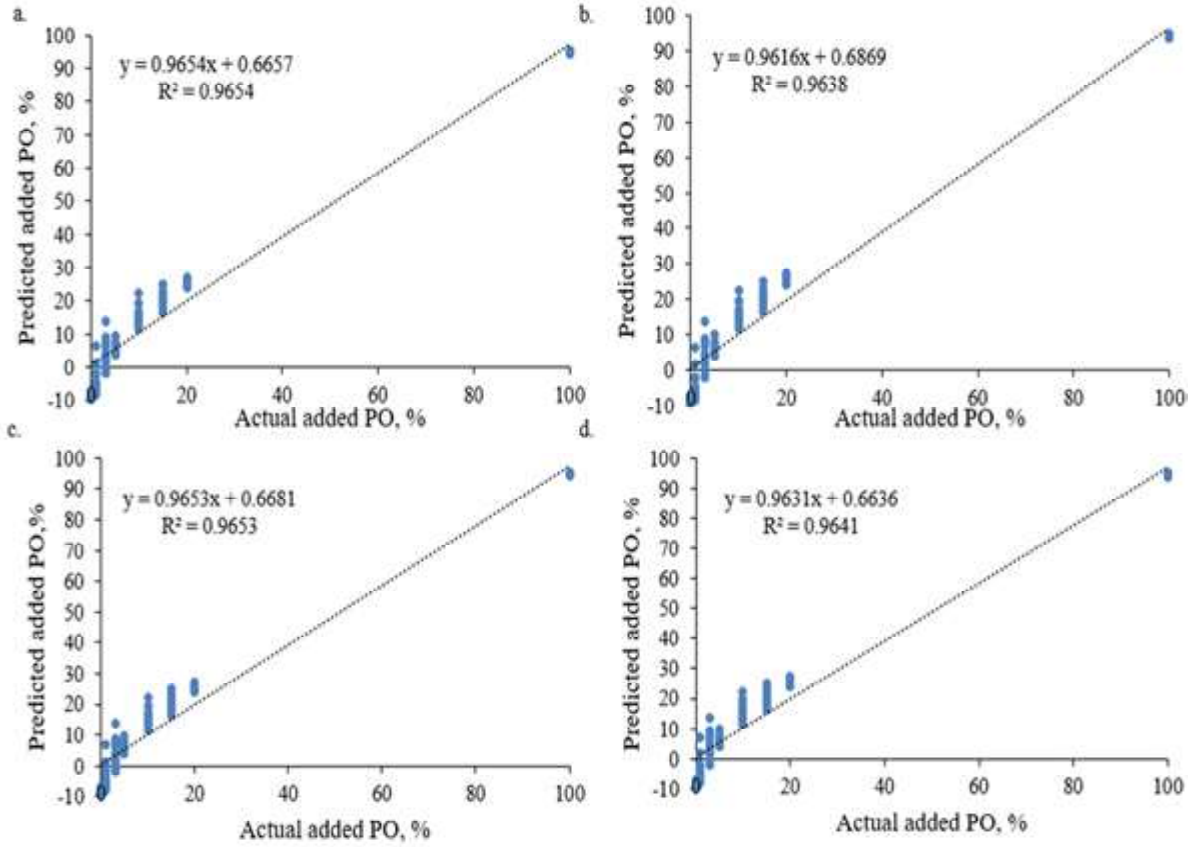


Fig. 4 Relationship between the actual and predicted levels of palmolein oil in ghee using PLS regression *(a) Calibration - Wavenumber Range: 1167-1137 cm{ ¹ (b) Validation - Wavenumber Range: 1167-1137 cm{ ¹*

were used to establish a calibration model using complete cross-validation. The best models were selected based on their higher R^2, and lower RMSEC and RMSEV values. PLS and PCR were applied in the same regions where maximum variations in absorbance peaks were observed *i.e.*, 1167-1137 cm^{-1} and their calibration curves are shown in Fig. 3. It was found that there was a linear relationship between the actual and predicted value of PO because value of coefficient of determination (R2) was near to the one *i.e.*, 0.9654 and 0.9652 for the PLS and PCR models, respectively in the wavenumber region of 1167-1137 cm^{-1}. This suggested that the actual and predicted PO contents in ghee were in agreement. The difference between RMSEC and RMSEV values for developed PLS and PCR models in the specified wavenumber was less, showing that the models were well constructed for detecting the PO in ghee. The difference between both values was about 0.136 and 0.1018 for PLS and PCR models, respectively in the wavenumber region of 1167-1137 cm^{-1}, respectively (Table 3). Earlier researchers employed both PLS and PCR methodologies in their respective studies, revealing the efficiency of the developed models in successfully detecting various adulterants, such as goat body fat and coconut oil, in ghee (Upadhyay et al. 2016; Gandhi et al. 2022).

Classification of samples using SIMCA

The study utilized the soft independent modeling of class analogy (SIMCA) method to evaluate class probabilities for control, PO, and spiked samples. This analysis was performed within the specific spectral range of 1167-1137 cm^{-1}, employing established PLS models. The SIMCA analysis consistently demonstrated precise classification of control and PO, achieving 100% classification accuracy in both spectral regions. Among adulterated ghee samples, those in the region of 1167-1137 cm^{-1} were all correctly classified, except for one sample at 1% level and two samples each at 3% and 10% levels. Table 4 revealed that only five out of 120 samples (4.16%) were misclassified. The classification efficiency for all samples in both regions exceeded 90%, with only five samples (4.16%) misclassified. No spiked sample was classified in PMG and vice versa. These findings underscore the effectiveness of SIMCA in detecting PO with high sensitivity, even at lower levels like 1% in PMG. The PLS model's ability to accurately detect PO adulteration across diverse sample types is supported by the summary in Table 4. These results demonstrate the reliability and accuracy of SIMCA for identifying PO in ghee. The study aligns with Gandhi et al. (2022), who applied the SIMCA approach to detect coconut oil

Table 4 SIMCA applied in the wavenumber region of 1167-1137 cm^{-1}

Wavenumber (cm^{-1})	% PO in PMG	Total no. of sample	Number of selected classes								Misclassified	Classification efficiency (%)
			0	1	3	5	10	15	20	100		
1167-1137	0	15	15	–	–	–	–	–	–	–	0	100
	1	15	–	14	–	–	–	–	–	–	1	93.33
	3	15	–	–	12	–	–	–	–	–	2	86.66
	5	15	–	–	–	15	–	–	–		0	100
	10	15	–	–	–	–	13	–	–	–	2	86.66
	15	15	–	–	–	–	–	15	–	–	0	100
	20	15	–	–	–	–	–	–	15	–	0	100
	100	15	–	–	–	–	–	–	–	15	0	100

adulteration in ghee up to the level of 2%. Previous studies by Upadhyay et al. (2016, 2018) also successfully identified goat body fat at 1% and pig body fat at 3% using the SIMCA approach in ghee.

Conclusion

ATR-FTIR spectroscopy and advanced chemometric techniques were employed to identify and measure the adulteration of pure ghee with palmolein oil. Spectral analysis uncovered unique absorption patterns within the 1167-1137 cm^{-1} spectral ranges for pure ghee, palmolein oil, and ghee samples spiked with palmolein oil. PCA demonstrated distinct clusters, even for ghee samples spiked at a 1% level. The PLS and PCR models in specific spectral regions showed excellent performance, with high accuracy and precision. The addition of the SIMCA method improved sample classification to 100% accuracy. This research highlights the potential of these methods to identify 1% palmolein oil adulteration in ghee. This research not only demonstrates the potential of these methods for adulteration detection but also promotes a safer and environmentally friendly analytical approach, reducing reliance on toxic solvents and contributing to the integrity of dairy products and consumer safety.

Acknowledgment

The authors are thankful to the Director of the ICAR-National Dairy Research Institute, Karnal for granting access to the facilities necessary to conduct the research work.

Conflict of Interest

The authors declare no conflict of interest in the presented research work.

Funding- Not applicable

Abbreviation

FT	Fourier transform
ATR	Attenuated total reflectance
FTIR	Fourier transform infrared spectroscopy
SIMCA	Soft-independent modeling of class-analogies
MIR	Mid-infrared
NIR	Near-infrared spectroscopy
PCA	Principal component analysis
PLS	Partial least squares
PCR	Principal component regression
PGM	Pure mixed ghee
PO	Palmolein oil
R^2	Coefficient of determination
SEC	Standard-error of calibration
SEP	Standard-error of prediction
PC	Principal component
RMSEC	Root mean square error of calibration
RMSEV	Root mean square error of validation
FTMIR	Fourier transform mid-infrared spectroscopy

Reference

Antony B, Mehta B M, Sharma S, Ratnam K, Aparnathi K D (2018) Comparative appraisal of ghee and common vegetable oils for spectral characteristics in FT-MIR reflectance spectroscopy. J Food Sci Technol 55(9): 3632-3639

Antony B, Sharma S, Mehta B M, Ratnam K, Aparnathi K D (2017) Study on FT-MIR spectra of ghee (anhydrous milk fat). Br Food J 119(1): 181-189

Atbhaiya Y, Sharma R, Gandhi K, Mann B, & Gautam P B (2022) Methods to differentiate between cotton tract area ghee and cotton seed oil adulterated ghee. J Food Sci Technol 59(12): 4782-4793

Atbhaiya Y, Sharma R, Gandhi K, Mann B, Gautam P B (2023) Detection of cotton seed oil in cow ghee using triglyceride profiling. Indian J Dairy Sci 76(6): 515-521

Balan B, Dhaulaniya A S, Jamwal R, Sodhi K K, Kelly S, Cannavan A, Singh D K (2020) Application of Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy coupled with chemometrics for detection and quantification of formalin in cow milk. Vib Spectrosc 107

Bhavaniramya S, Vishnupriya S, Vijayarani K, Baskaran D (2018) A review on understanding the subterranean insights in nature of South Indian

ghee with its biological and physiochemical properties. Int J Food Sci Nutr 3(6): 257-262

Burmistrova N A, Soboleva P M, & Monakhova Y B (2021) Is infrared spectroscopy combined with multivariate analysis a promising tool for heparin authentication. J Pharmaceutical Biomedical Analysis 194: 113811

Cozzolino D (2014) An overview of the use of infrared spectroscopy and chemometrics in authenticity and traceability of cereals. Food Res Int 60: 262-265

De S (2012) Outlines of Dairy Technology. Oxford University Press, New Delhi, 382–466

da Silva Bruni A R, de Oliveira V M A T, Fernandez A S T, Sakai O , Març P H, & Valderrama P (2021) Attenuated total reflectance Fourier transform (ATR-FTIR) spectroscopy and chemometrics for organic cinnamon evaluation. Food Chemistry 365: 130466.

Deshwal G K, Singh R, Singh A K, Kumar D, Sharma H (2022) Comparative characterisation of ghee from Indian camel breeds using GC†MS and FTIR techniques. Int J Dairy Technol 75(1): 182-193

Dorni C, Sharma P, Saikia G, Longvah T (2018) Fatty acid profile of edible oils and fats consumed in India. Food Chem 238: 9-15

Dupuy N, Duponchel L, Huvenne J P, Sombret B, Legrand P (1996) Classification of edible fats and oils by principal component analysis of Fourier transform infrared spectra. Food Chem 57(2): 245-251

Fox P F, Uniacke-Lowe T, McSweeney P L H, O'Mahony J A, Fox P F, Uniacke-Lowe T, O'Mahony J A (2015) Milk proteins. Dairy Chem Biochem 145-239

Gandhi K, Sharma R, Seth R, Mann B (2022) Detection of coconut oil in ghee using ATR-FTIR and chemometrics. Appl Food Res 2(1): 100035

Gandhi K, Sharma N, Gautam P B, Sharma R, Mann B, & Pandey V (2022) Infrared (IR) Spectroscopy. In Advanced Analytical Techniques in Dairy Chemistry (pp. 177-198). New York, NY: Springer US.

Gautam P B, Gandhi K, Sharma R, Sharma N, & Mann B (2022) Conjugated Linoleic Acid: Synthesis, Physiological, and Functional Aspects. In Functional Dairy Ingredients and Nutraceuticals (pp. 145-169). Apple Academic Press

Gandhi K, Sharma R, Seth R, Ramani A, Mann B (2023) Mineral oil detection in ghee using attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) in conjunction with chemometrics. Food Humanity 1: 1523-1530

ISO 15884 (2002)/IDF 182: 2002 Milk fat- Preparation of fatty acid methyl esters. International Organization for Standardization, Geneva

Jeyarani T, Reddy S Y (2005) Physicochemical evaluation of vanaspati marketed in India. J food lipids 12(3): 232-242

Mendes E, Duarte N (2021) Mid-infrared spectroscopy as a valuable tool to tackle food analysis: a literature review on coffee, dairies, honey, olive oil and wine. Foods 10(2): 477

Nurrulhidayah A F, Rohman A, Amin I, Shuhaimi M, Khatib A (2013) Analysis of chicken fat as adulterant in butter using Fourier transform infrared spectroscopy and chemometrics. Grasas y Aceites 64(4): 349-355

Ozaki Y, Morisawa Y (2021) Principles and characteristics of NIR spectroscopy. Near-Infrared Spectroscopy: Theory, Spectral Analysis, Instrumentation, and Applications, 11-35

Ramani A, Hazra T., Parmar M P, Sindhav R G, Ramani V M (2019) A simple rapid technique for detection of palm oil in ghee. Indian J Dairy Sci 72(4): 441-444

Ramani A, Hazra T, Sudheendra C V, Hariyani A S, Prasad S, & Ramani V M (2018) Comparative appraisal of ghee and palm oil adulterated ghee on the basis of chromogenic test. Int J Curr Microbiol App Sci 7(12): 623-627

Rohman A, Man Y C (2010) Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. Food Res Int 43(3): 886-892

Rohman A, Erwanto Y, Man Y B C (2011) Analysis of pork adulteration in beef meatball using Fourier transform infrared (FTIR) spectroscopy. Meat Sci 88(1): 91-95

Sonvanshi V, Gandhi K, Ramani A, Sharma R, & Seth R (2024) ATR-FTIR coupled with chemometric techniques to detect vanaspati ghee (hydrogenated vegetable oil) adulteration in milk fat. Results in Chemistry 7: 101343

Saji R, Ramani A, Gandhi K, Seth R, & Sharma R (2024) Application of FTIR spectroscopy in dairy products: a systematic review. Food and Humanity 100239

Saji R, Gandhi K, Sharma R, Bajaj R, Mann B, & Ramani A (2024) Detection of flunixin residues in milk using ATR-FTIR spectroscopy coupled with chemometrics. Journal of Food Measurement and Characterization 1-11

Tandra H, Suros A I, Syaukat Y, & Najib M (2022) The determinants of competitiveness in global palm oil trade. Economies 10(6): 132

Upadhyay N, Jaiswal P, Jha S N (2016) Detection of goat body fat adulteration in pure ghee using ATR-FTIR spectroscopy coupled with chemometric strategy. J Food Sci Technol 53(10): 3752–3760

Upadhyay N, Jaiswal P, Jha S N (2018) Application of attenuated total reflectance Fourier Transform Infrared spectroscopy (ATR–FTIR) in MIR range coupled with chemometrics for detection of pig body fat in pure ghee (heat clarified milk fat). J Mol Struct 1153: 275-281

Wani A D, Prasad W, Khamrui K, & Jamb S (2022) A review on quality attributes and utilization of ghee residue, an under-utilized dairy by-product. Future Foods 5, 100131

Indian J Dairy Sci 78(2): 165-172
https://doi.org/10.33785/IJDS.2025.v78i02.009

RESEARCH ARTICLE

Analysis of sensory, textural and compositional attributes of protein-rich dairy spread using response surface methodology

D RPrajapati[1], AM Patel[2] (✉), Smitha Balakrishnan[3], J M Mallik[1], C N Dharaiya[1] and D H Patel[2]

Received: 15 August 2024 / Accepted: 25 October 2024 / Published online: 23 April 2025

Abstract: A protein-rich dairy spread was formulated using white butter, WPC-80, and Greek yoghurt. Response surface methodology (RSM) was used to optimize the levels of white butter and WPC-80. The optimization considered sensory attributes like flavour, body and texture, colour and appearance, spreadability, and overall acceptability, as well as hardness, fat, and protein content. RSM suggested the optimal levels to be 38.10 per cent white butter and 18.10 per cent WPC-80.The experimental protein-rich dairy spread, prepared based on RSM suggestions, was compared to a control spread. The experimental spread showed statistical similarity to the control in terms of compositional parameters, except had a significantly higher protein content (14.85%) and lower carbohydrate content (5.54%) compared to the control spread. The acidity (% LA) and tyrosine value were also significantly higher in the protein-rich dairy spread. The experimental sample was also superior to the control sample in sensory attributes. Microbiologically, both the experimental and control samples were free from aerobic plate count, coliform count and yeast and mould count.

Key words: Protein-rich dairy spread, white butter, WPC-80, response surface methodology

Introduction

A dairy spread is a product consisting of an aqueous phase and a fat phase derived from milk fat, with additional ingredients that enhance its spreadability at refrigeration temperature. These spreads can be categorized based on their type of fat into dairy spreads, such as butter spread, cheese spread, ghee spread, Channa spread, paneer spread etc. (Hirpara et al. 2016; Yadav et al. 2019); and plant-based spreads, such as nut or fruit spreads, chocolate spreads etc. (Kumari & Sharma, 2022). Margarine, a cost-effective alternative to butter, is primarily composed of vegetable fats, which results in a distinct lack of the characteristic flavor, body and texture of butter (Galindo-Cuspinera et al. 2017). Whereas butter has poor spreadability when refrigerated. Thus, new form of butter with improved spreadability emerged as spreads (Prajapati et al. 1991).Low-fat dairy spreads are spreadable products with less fat than butter or margarine, making them an alternative, particularly in regions where butter is expensive (Deshmukh et al. 2002).

These spreads not only enhance the flavor of food but also offer functional benefits due to the inclusion of proteins. Proteins in spreads contribute to the viscosity and water-holding capacity of the aqueous phase, thereby improving the stability of the emulsion during processing and storage (Mishra et al. 2019). Whey protein concentrate (WPC) is commonly utilized in such formulations due to its excellent functional properties, including solubility, emulsification, and water binding (Suthar et al. 2017). WPC also boasts a high biological value and a rich profile of essential amino acids, making it an ideal ingredient for enhancing the nutritional profile of low-fat dairy products (Smithers, 2008).

Greek yoghurt, also known as *labneh*, is a concentrated dairy product with higher protein and fat content compared to regular yoghurt. It is produced by allowing natural yoghurt to drain for a period, resulting in a thicker consistency (Kathiriya et al. 2018). This concentrated yoghurt enhances the texture and creaminess of dairy products, making it a valuable ingredient for improving the sensory and nutritional attributes of dairy spreads.

The current study focuses on optimizing the use of white butter and WPC-80 in the formulation of a protein-rich dairy spread using response surface methodology (RSM). The primary objective is to determine the optimal levels of white butter and WPC-80 to enhance sensory attributes while also improving the spreadability and nutritional content of the spread. This optimization aims to address the growing consumer demand for

[1]Department of Dairy Technology, SMC College of Dairy Science, Kamdhenu University, Anand-388110, Gujarat, India
[2]Department of Dairy Processing and Operations, SMC College of Dairy Science, Kamdhenu University, Anand-388110, Gujarat, India
[3]Department of Dairy Chemistry, SMC College of Dairy Science, Kamdhenu University, Anand-388110, Gujarat, India

AM Patel (✉)
Department of Dairy Processing and Operations, SMC College of Dairy Science, Kamdhenu University, Anand-388110, Gujarat, India
Email: amitmpatel@kamdhenuuni.edu.in
Mobile #: +91 93761 32364

healthier, higher-protein options. By utilizing white butter, WPC-80 and Greek yoghurt, the study intends to produce a spread that excels in sensory and functional qualities and also meets the nutritional criteria for a high-protein product.

Materials and Methods

The protein-rich dairy spread was formulated using white butter (82% fat) from Vidya Dairy, Anand, WPC-80 from a local dealer, and skim milk (0.5% fat, 8.7% SNF) for Greek yoghurt from Vidya Dairy. Skim milk powder for the control spread was also obtained from Vidya Dairy. Non-dairy ingredients included a starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*) from DSM Food Specialties, Netherland; stabilizer Carboxy methyl cellulose (CMC) from Molychem, Mumbai; emulsifier Lecithin soya (30%) from HiMedia laboratories Pvt. Ltd., Mumbai and emulsifying salt Disodium hydrogen orthophosphate (Na_2HPO_4) from Central Drug House Pvt. Ltd., New Delhi. Common salt was purchased from Tata Chemicals, Mumbai. Annatto butter colour formulated within department. The spread was packaged in polypropylene cups (125 mL) with thickness of 0.52 mm purchased from an authorized dealer.

Preparation of Greek yoghurt: For the preparation of Greek yoghurt, skim milk was heated to 90! for 15 minutes. Milk was cooled to 42±2! followed by inoculation with Direct-to-Vat Starter (DVS) culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*) and incubation at that temperature for 3-4 h (or until acidity of 0.6% lactic acid was obtained). Thereafter, the curd was hung in a cloth bag (at refrigeration temperature) and allowed to drain the whey until it reached the desired Greek yoghurt consistency.

Preparation of protein-rich dairy spread: Potable water was initially heated to 60°C. Dry ingredients, including lecithin (0.5%), CMC (0.25%) and common salt (0.5%), were then added to the water, which was subsequently heated to 90°C (without a holding period). Following this, disodium hydrogen orthophosphate (0.5%) and Greek yoghurt (10%) were incorporated into the mixture. WPC-80was then added and blended until a smooth texture was achieved. Then white butter tempered overnight at 20°C was added. Once the butter had fully dissolved, 0.2 per cent annatto colour was incorporated. The product was then heated to 70°C without a holding period and hot filled into polypropylene cups at 50°C. The filled cups were stored at 7±2°C.

Analysis of protein-rich dairy spread: Greek yoghurt was analyzed for total solids, fat, protein, ash, lactose and titratable acidity. Titratable acidity and total solids were determined by method FSSAI (2022). Fat content was analyzed using IS: 2785-1979 (1992). Protein was determined by the Block Digestion/Steam Distillation method (ISO 8968-2:2001). Ash content was analyzed using AOAC, (1981) method. Lactose was calculated by difference. Butter was analyzed for fat, moisture, and curd content using the method given in FSSAI (2022).In spread moisture was determined as per the method for butter. Fat, Protein and ash were analyzed using the method for Greek yogurt. Total carbohydrates were calculated by difference. Hardness was measured by the cone penetrometer method (Verma, 1996). Titratable acidity was determined following the FSSAI (2022), free fatty acids (FFA) using the procedure by Thomas et al. (1954), and peroxide value by the AOAC (1981) method. Protein breakdown, assessed by measuring tyrosine content, followed Hull (1947) procedure, while water activity was measured with a water activity meter. Aerobic plate count, coliform count, and yeast and mould count were determined using FSSAI (2023), IS:5401 Part I (2002), and IS:5403 (1999) methods respectively.

Sensory evaluation of protein-rich dairy spread: The sensory evaluation of protein rich dairy spread was done by panel of 7 judges using a 9-point hedonic scale. The panellist included scientists, technical officers/assistants and students of the institute. Each panellist was asked to taste the samples and evaluate the sensory parameters on a 9-point hedonic scale. They were asked a series of questions pertaining to flavour, body and texture, colour and appearance, spreadability and overall acceptability of each sample. Panellists were requested to give the scores and comments on a sensory evaluation score card. Saline water was provided to rinse the palate before and after tasting the sample. Sensory responses were evaluated based on a 9-point hedonic scale (Meilgaard et al. 1999). Mean score was calculated from the responses of panellists for each set of samples.

Statistical analysis: Design Expert software is to be used for all statistical work including the selection of the number of trials, range of parameters to be studied, number of replications and final analysis of the data generated. An advanced statistical software programmed named RSM Design Expert 13.0.1.0 was employed in the study. The experiment was conducted using various combination of treatment with some range of the parameters under study for manufacture of an acceptable quality of protein-rich dairy spread. RSM design expert 13.0.1.0 was used to optimize two selected variables namely white butter and WPC-80 in the study. On the basis of preliminary trials, the range of variables was obtained. RSM with faced centered rotatable design (FCRD) for two variables at five levels and six replicates at central point was adopted to optimize the quality of protein-rich dairy spread with respect to selected sensory responses. The result of the 13 trials (Table 1) formed the base for an optimized level of white butter and WPC-80 which were suggested by software. The optimized level of ingredients was then replicated seven times and actual values of sensory analysis was compared with predicated value.

Results and Discussion

The optimization of white butter and WPC-80 was conducted based on sensory properties such as flavour, body and texture, colour and appearance, spreadability and overall acceptability, as well as other

attributes like hardness, fat, and protein. Successive regression analysis produced quadratic models for each response, with the coefficient of determination (R^2) ranging from 0.76 to 0.97 (Table 2). The model F-value for all responses was significant, indicating a strong fit. The adequacy of the models in predicting response variables for any combination of variables within the range was confirmed by a non-significant lack of fit. These indicate that the obtained quadratic model fitted the data strongly. Adequate precision value (APV), a measure of signal-to-noise ratio, exceeded the threshold of 4, with APVs ranging from 7.12 to 19.64. These results suggest that the developed model is reliable for optimizing the formulation of the protein-rich dairy spread.

Effect on flavour scores: Flavour is a critical parameter in food quality assessment, primarily determined by taste and smell, and greatly affects consumer acceptability. In protein-rich dairy spread samples, the flavour score ranged from 7.86 to 8.43 on a 9-point hedonic scale (Table 1). The spread with 40.00 per cent white butter and 17.50 per cent WPC-80 was rated best, while that with 35.00 per cent white butter and 20.00 per cent WPC-80 scored the lowest. The R^2 value of 0.88 indicates a good model fit (Table 2), with an APV of 10.54 suggesting reliability. White butter had a significant positive linear effect on flavour ($p<0.05$), while WPC-80 had a non-significant negative effect. Interactive effects showed non-significant results, but quadratic effects of white butter were significantly positive ($p<0.05$) and WPC-80 significantly negative. Kumar (2014) found that in the chocolate spread with added butter fat, olive oil, and WPC, WPC did not significantly affect the flavour score of the chocolate spread ($P>0.05$). Popalia (2018) found that in the development of a value-added milk-cereal-based product with added MPC-85 and white butter, butter had a significant positive effect on the flavour score of the milk-cereal product (P d”0.05), while interaction had non-significant effect ($P>0.05$). Hamid (2023) investigated the effect of total fat, omega fat, and diacetyl on the flavour of omega fatty acids enriched fat spread. The study found that total fat had a significant positive effect on flavour (P d”0.1).

Effect on body and texture scores: Body and texture reflect the physical feel and structure of a food product, including attributes like firmness, smoothness, and consistency, which impact the sensory experience and mouthfeel. The body and texture score for protein-rich dairy spread samples ranged from 7.29 to 8.39 on a 9-point hedonic scale (Table 1). The spread with 37.50 per cent white butter and 17.50 per cent WPC-80 received the highest rating, while that with 35.00 per cent white butter and 20.00 per cent WPC-80 scored the lowest. The R^2 value of 0.90 (Table 2) and an APV of 12.47 indicate a good model fit and reliability. White butter had a significant positive linear effect on body and texture ($p<0.05$), whereas WPC-80 had a non-significant negative effect. Interactive effects showed a significant positive impact, while quadratic effects of white butter were non-significant and WPC-80 had a significant negative effect ($p<0.05$). Kumar (2014) found that in the chocolate spread with added butter fat, olive oil, and WPC, WPC did not significantly affect the body and texture score of the chocolate spread ($P>0.05$). Chaudhari et al. (2023) demonstrated that the body and texture of low-fat paneer were significantly influenced by the rate of WPC addition. Hamid (2023) examined the influence of total fat, omega fat, and diacetyl on the body and texture of omega fatty acids enriched fat spread. The results showed that total fat had a significant negative effect on body and texture (P d”0.05).

Effect on colour and appearance scores: Colour and appearance refer to the visual attributes of a food product, including hue, brightness, and uniformity, which influence consumer perception and initial acceptability. The colour and appearance score of protein-rich dairy spread ranged from 7.80 to 8.43 on a 9-point hedonic scale (Table 1). The spread with 35.00 per cent white butter and 20.00 per cent WPC-80 was rated highest, while that with 35.00 per cent white butter and 15.00 per cent WPC-80 scored the lowest. The R^2 value of 0.82 (Table 2) and an APV of 9.68 indicate a good model fit. WPC-80 had a significant positive effect ($p<0.05$) on colour and appearance, while white butter’s effect was non-significant. The interaction effect of white butter and WPC-80 was significantly negative ($p<0.05$), while quadratic effects were non-significant for both variables ($p<0.05$). Kumar (2014) found that in the chocolate spread with added butter fat, olive oil, and WPC, WPC did not significantly affect the color and appearance score of the chocolate spread ($P>0.05$).Popalia (2018) noted that in the development of a value-added milk-cereal-based product with added MPC-85 and white butter, butter had a significant positive effect on the colour and appearance score (P d”0.05). Hamid (2023) analyzed the effect of total fat, omega fat, and diacetyl on the color and appearance of omega fatty acids enriched fat spread. The study revealed that none of the variables total fat, omega fat, or diacetyl had a significant effect on color and appearance ($P>0.05$).

Effect on spreadability scores: Spreadability measures the ease with which a product can be spread, assessing its smoothness and consistency. The spreadability scores for protein-rich dairy spread ranged from 7.88 to 8.44 on a 9-point hedonic scale (Table 1). The spread with 37.50 per cent white butter and 17.50 per cent WPC-80 was rated highest, while the spread with 37.50 per cent white butter and 15.00 per cent WPC-80 scored the lowest. The coefficient of determination (R^2) was 0.81 (Table 2), with an APV of 7.54, indicating a good fit of the model. White butter had a significant positive effect on spreadability at the linear level ($p<0.05$), whereas WPC-80 had a non-significant positive effect. The interactive effect of white butter and WPC-80 on spreadability was non-significant, while a significant negative effect of WPC-80 at quadratic terms was observed ($p<0.05$). Kumar (2014) found that in the chocolate spread with added butter fat, olive oil, and WPC, WPC did not significantly affect the spreadability score of the chocolate spread ($P>0.05$). In contrast, Hamid (2023) found total fat had significant negative effect on spreadability (P d”0.05) in omega fatty acids enriched fat spread.

Effect on overall acceptability scores: Overall acceptability is a crucial sensory attribute that reflects the consumer's overall judgment of the product, integrating aspects such as flavour, body and texture, colour and appearance and spreadability. The scores for overall acceptability of the protein-rich dairy spread ranged from 7.68 to 8.42 on a 9-point hedonic scale (Table 1). The spread with 40.00 per cent white butter and 17.50 per cent WPC-80 was rated highest, while the spread with 35.00 per cent white butter and 20.00 per cent WPC-80 scored the lowest. The coefficient of determination (R^2) was 0.91 (Table 2), with an APV of 12.38, indicating a good fit of the model. White butter had a significant positive effect on overall acceptability at the linear level ($p<0.05$), whereas WPC-80 had a non-significant negative effect. The interactive effect of white butter and WPC-80 was non-significant, while a significant negative effect of WPC-80 at quadratic terms was observed ($p<0.05$). The impact of different variables on overall acceptability is shown in Figure 1. In contrast Chaudhari et al. (2023) illustrated that the overall acceptability of low-fat paneer was significantly affected by the rate of WPC addition.Hamid (2023) explored the effect of total fat, omega fat, and diacetyl on the overall acceptability of omega fatty acids-enriched fat spread. Diacetyl had a significant positive effect on overall acceptability (P d"0.1), whereas total fat and omega fat did not significantly affect overall acceptability (P>0.05).

Effect on hardness: Hardness describes the resistance to deformation in food products, influencing the firmness and overall texture experienced during consumption. The hardness of protein-rich dairy spread samples ranged from 0.98 g/cm² to 1.46 g/cm² (Table 1). The spread with 35.00 per cent white butter and 20.00 per cent WPC-80 exhibited the highest hardness, while the one with 35.00 per cent white butter and 15.00 per cent WPC-80 had the lowest. The coefficient of determination (R^2) was 0.76 (Table 2), with an APV of 7.12, suggesting a good model fit. White butter and WPC-80 had non-significant positive effects on hardness at the linear level. The interactive effect of white butter and WPC-80 was significantly negative, while non-significant effects were noted for quadratic terms of white butter and WPC-80 ($p<0.05$). The impact of different variables on hardness is shown in Figure 1.Radoèaj et al. (2011) found that the stabilizer and hemp oil had significant positive effect on the hardness of the spread (P d"0.01). The quadratic effect of hemp oil was also significant positive (P d"0.05), while the interaction between the stabilizer and hemp oil did not significantly affect hardness ($P > 0.05$). Patel et al.(2016) observed that increasing *fada* (germinated and dried wheat semolina) significantly increased hardness (Pd"0.01) in Halvasan, while increasing gluten significantly decreased hardness (Pd"0.05).

Effect on fat: Fat content is crucial for determining the nutritional profile and sensory characteristics such as flavour, mouthfeel and spreadability. The fat content of protein-rich dairy spread samples ranged from 26.92 per cent to 33.60 per cent (Table 1). The spread with 35.00 per cent white butter and 20.00 per cent WPC-80 had the lowest fat content, while the one with 40.00 per cent white butter and 15.00 per cent WPC-80 had the highest. The coefficient of determination (R^2) was 0.95 (Table 2), indicating an excellent model fit. The APV of 19.64, well above the minimum desirable APV (4.00), supports the use of this response for design. Statistical analysis showed a significant positive effect of white butter and a significant negative effect of WPC-80 on fat content at the linear level ($p<0.05$). The interactive effect had a non-significant positive effect, while quadratic terms for white butter and WPC-80 had non-significant

Table 1 Experimental design matrix, sensory, hardness and compositional attributes of protein-rich dairy spread

Run No.	White butter (%)	WPC-80 (%)	Sensory scores					Hardness (g/cm^2)	Fat (%)	Protein (%)
			Flavour	Body &texture	Colour & appearance	Spreadability	Overall acceptability			
1	35.00	17.50	8.08	7.78	8.03	7.89	7.83	1.09	29.30	15.01
2	35.00	15.00	7.92	7.67	7.80	7.92	7.73	0.98	29.88	10.80
3	37.50	17.50	8.17	8.39	8.22	8.44	8.31	1.27	30.83	14.24
4	37.50	15.00	8.00	7.81	8.00	7.88	7.94	1.31	31.81	11.70
5	40.00	17.50	8.43	8.31	8.13	8.43	8.42	1.22	33.00	14.35
6	37.50	20.00	7.89	7.60	8.35	8.09	7.90	1.38	30.19	16.01
7	40.00	15.00	8.43	7.86	8.36	8.07	8.25	1.41	33.60	11.45
8	37.50	17.50	8.19	8.06	8.38	8.34	8.29	1.14	30.83	14.15
9	40.00	20.00	8.29	8.21	8.29	8.37	8.33	1.17	30.77	16.40
10	35.00	20.00	7.86	7.29	8.43	8.00	7.68	1.46	26.92	16.25
11	37.50	17.50	8.14	8.29	8.21	8.29	8.21	1.43	30.42	14.50
12	37.50	17.50	7.93	8.04	8.24	8.29	8.04	1.43	30.83	13.80
13	37.50	17.50	8.16	8.23	8.34	8.37	8.26	1.27	30.83	14.15

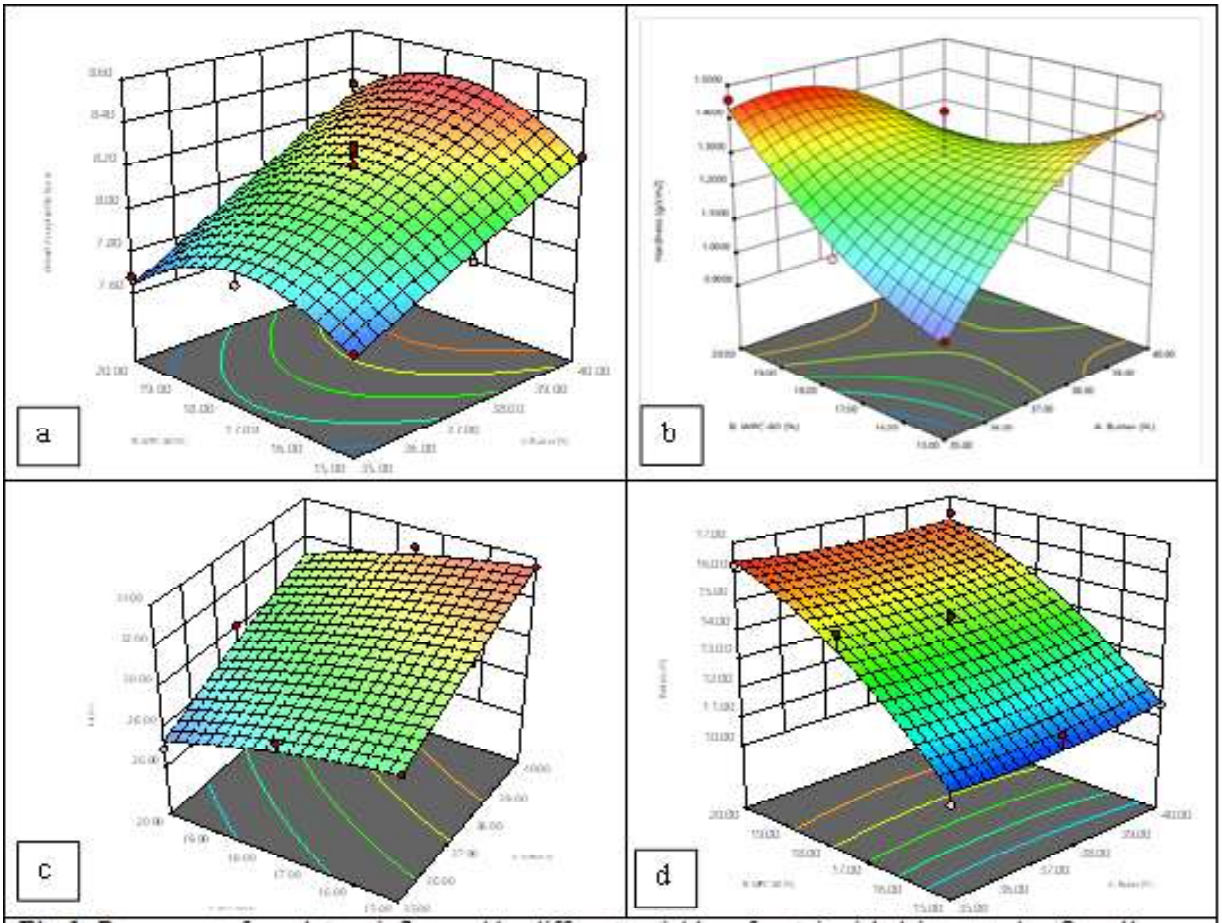


Fig. 1: Response surface plots as influenced by different variables of protein-rich dairy spread. a: Overall acceptability, b:Hardness, c: %Fat, d: %Protein

negative effects. The impact of different variables on fat is shown in Figure 1.

Effect on protein: Protein content is crucial for determining the nutritional profile and functionality of the spread, impacting body and texture, and overall nutritional value. The protein content of protein-rich dairy spread samples ranged from 10.80 per cent to 16.40 per cent (Table 1). The spread with 35.00 per cent white butter and 15.00 per cent WPC-80 had the lowest protein content, while the one with 40.00 per cent white butter and 20.00 per cent WPC-80 had the highest. The coefficient of determination (R^2) was 0.97 (Table 2), indicating an excellent model fit. The APV of 18.73, well above the minimum desirable APV (4.00), supports using this response for design. Statistical analysis showed a significant positive effect of WPC-80 and a non-significant positive effect of white butter on protein content at the linear level ($p<0.05$). The interactive effect had a non-significant negative effect, while white butter had a non-significant positive effect and WPC-80 had a significant negative effect at the quadratic level ($p<0.05$). The impact of different variables on protein is shown in Figure 1.

Optimization of product formulation for protein-rich dairy spread

The optimization of protein-rich dairy spread was performed using numerical optimization techniques, as summarized in Table 3. The goal was to optimize various parameters including white butter and WPC-80 percentages while maximizing sensory attributes. In the optimization process, white butter and WPC-80 were maintained within their specified ranges (35-40% and 15-20% respectively). The sensory attributes, hardness, fat and protein were also kept in range. The RSM suggested optimal levels of 38.10 per cent white butter and 18.10 per cent WPC-80, achieving a desirability of 1.00. Protein-rich dairy spread was prepared by adding white butter andWPC-80 as suggested by RSM. The predicted values for flavour, body and texture, colour and appearance, spreadability, overall acceptability, hardness,

fat, and protein were 8.15, 8.21, 8.29, 8.36, 8.25, 1.31 g/cm², 31.03 per cent and 14.82 per cent respectively. The observed values for these parameters were not significantly different from the predicted values (Table 4), confirming that the selected levels of white butter and WPC-80 are optimal for achieving desirable sensory, hardness, fat and protein in the protein-rich dairy spread.

Analysis of protein-rich dairy spread

The protein-rich dairy spread (T_2) was analysed and compared with the control spread (T_1) for its proximate composition, sensory attributes, and hardness, with results statistically analysed using a t-test as shown in Table 5. The moisture content of T_2 was significantly ($P<0.05$) lower than T_1, while its protein content was significantly ($P<0.05$) higher, being twice that of T_1. This increase in protein is due to the inclusion of WPC-80 in T_2,

Table 2: Partial coefficients of regression equations of suggested models for sensory, hardness and compositional attributes of protein-rich dairy spread

Terms	Sensory scores (9-point hedonics scale)					Hardness (g/cm^2)	Fat (%)	Protein (%)
	Flavour score	Body & texture score	Colour & appearance score	Spreadability score	Overall acceptability score			
Intercept	8.11	8.17	8.24	8.31	8.20	1.30	30.90	14.26
A: White Butter	0.215*	0.275*	0.085	0.178*	0.294*	0.045	1.878*	0.023
B: WPC-80	-0.051	-0.039	0.151*	0.099	-0.001	0.051	-1.234*	2.452*
AB	-0.020	0.183*	-0.175*	0.054	0.033	-0.179*	0.032	-0.125
A^2	0.158*	-0.056	-0.083	-0.045	-0.012	-0.119	-0.133	0.202
B^2	-0.153*	-0.394*	0.014	-0.222*	-0.218*	0.067	-0.283	-0.623*
R^2	0.88	0.90	0.82	0.81	0.91	0.76	0.95	0.97
Model F-Value	10.63	12.28	6.57	6.10	13.69	4.35	28.22	44.43
APV	10.54	12.47	9.68	7.54	12.38	7.12	19.64	18.73
Suggested Model	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic

*: $p < 0.05$; APV= Adequate Precision Value, R^2 = Coefficient of determination

Table 3: Criteria/responses chosen for optimization of protein-rich dairy spread

Sr No.	Parameter	Units	Goal	Lower Limit	Upper Limit
1.	A: Whit butter	%	In range	35	40
2.	B: WPC-80	%	In range	15	20
3.	Flavour	Out of 9	In range	7.85	8.43
4.	Body & texture	Out of 9	In range	7.29	8.39
5.	Colour & appearance	Out of 9	In range	7.80	8.43
6.	Spreadability	Out of 9	In range	7.87	8.44
7.	Overall acceptability	Out of 9	In range	7.67	8.42
8.	Hardness	g/cm^2	In range	0.98	1.46
9.	Fat	%	In range	26.92	33.60
10.	Protein	%	In range	10.80	16.40

which has a higher protein content (~78%) compared to SMP used in T_1 (~35%). The total solids content of T_2 was significantly higher due to the incorporation of Greek yogurt. Carbohydrate content in T_2 was significantly ($P<0.05$) lower, attributed to the lower lactose content of WPC-80 (10.8%) compared to the higher lactose content of SMP (52%) in T_1. Sensory evaluation showed T_2 had significantly ($P<0.05$) better scores for flavour, body and texture, spreadability and overall acceptability. Hardness was slightly higher in T_2 but not significantly ($P>0.05$) different from T_1. Protein-rich dairy spread showed significantly higher acidity, FFA and tyrosine values than the control spread, primarily due to the addition of Greek yogurt. While peroxide value and water activity remained similar between the two spreads. Microbiologically, both the experimental and control samples were free from aerobic plate count, coliform count and yeast and mould count.

Conclusion

Table 4: Comparison of predicted v/s actual values of responses used for optimization of protein-rich dairy spread

Response	P Value	Predicted Value *	Actual Value @	Cal. t-Value #	Level of Significance
Flavour	0.30	8.15	8.18	1.12	NS
Body & Texture	0.08	8.21	8.28	2.10	NS
Colour & Appearance	0.25	8.29	8.24	1.27	NS
Spreadability	0.73	8.36	8.35	0.35	NS
Overall Acceptability	0.83	8.25	8.26	0.22	NS
Hardness	0.08	1.31	1.24	2.05	NS
Fat	0.41	31.03	31.00	0.87	NS
Protein	0.48	14.82	14.84	0.75	NS

* Predicted values of Design Expert 13.0.1.0 package
@ Actual values are average of seven trials for optimized product
# t-values at 5 per cent level of significance
NS = non-significant
Tabulated t-value = 2.447 (cal. t-value less than tabulated value)

Table 5 : Comparison of protein-rich dairy spread with control spread (n=3)

Parameter	Control spread	Protein-rich dairy spread	CD (0.05)
Chemical composition			
Moisture, %	47.97±0.11	46.89±0.14	0.19
Fat, %	31.08±0.04	31.05±0.03	NS
Protein, %	7.81±0.13	14.85±0.11	0.17
Ash, %	1.65±0.01	1.67±0.01	0.01
Carbohydrates, %	11.49±0.10	5.54±0.17	0.2
Rheological attribute			
Hardness (g/cm^2)	1.19±0.01	1.22±0.09	NS
Physico-chemical properties			
Acidity (% LA)	0.320±0.01	0.536±0.01	0.015
FFA (% oleic acid)	0.251±0.01	0.338±0.01	0.008
Peroxide value (meq/kg fat)	0.077±0.01	0.078±0.01	NS
Tyrosine value (µg tyrosine/ 5 ml filtrate)	11.96±0.18	20.68±0.18	0.26
Water activity	0.925±0.01	0.919±0.01	NS
Sensory attributes			
Flavour	8.06±0.07	8.19±0.08	0.11
Body & texture	8.10±0.08	8.28±0.10	0.13
Colour & appearance	8.40±0.09	8.25±0.12	NS
Spreadability	8.26±0.10	8.38±0.06	0.12
Overall acceptability	8.09±0.05	8.28±0.11	0.12
Microbial analysis			
APC (cfu/g)	Absent/g		
Coliform	Absent/g		
Y&M	Absent/g		

A protein-rich dairy spread was developed using response surface methodology (RSM), optimizing the proportions of white butter and WPC-80 to achieve a sensorially acceptable product. At the linear level, the addition of WPC-80 significantly improved the protein content due to its high protein concentration while white butter also contributed to the desired fat content. At the quadratic level, white butter and WPC-80 showed non-significant effects on sensory attributes. Additionally, the hardness of the spread was not significantly impacted by the quadratic levels of these ingredients. Based on these outcomes, RSM suggested preparing the protein-rich dairy spread using 38.10 per cent white butter and 18.10 per cent WPC-80. The predicted values for sensory attributes, hardness, fat and protein content were closely aligned with actual values, confirming the reliability of the optimization. In conclusion, a protein-rich dairy spread with superior sensory attributes and enhanced nutritional value can be successfully developed through the optimization process using RSM.

References

AOAC (1981). Methods of Analysis (Method 920.117 & 965.33), Association of Official Analytical Chemists

Deshmukh MS, Patil GR, SontakkeAT, MitkariKR (2002) Development of low-fat spread from safflower milk blended with buffalo milk. Indian J Dairy Biosci 13:60-64

ChaudhariMP, PintoSV, DharaiyaCN, Patel SM (2023). Application of response surface methodology in preparation of low-fat paneer from recombined milk. Indian J Dairy Sci 76:231–237 doi:10.33785/ijds.2023.v76i03.004

FSSAI (2022). Manual of methods of analysis of foods: Dairy and Dairy Products. Food Safety and Standard Authority of India, New Delhi

FSSAI (2023) Methods of analysis microbiological examination of food and water. Food Safety and Standard Authority of India, New Delhi

Galindo-Cuspinera V, Valen a de Sousa J, Knoop M (2017). Sensory and analytical characterization of the “cool-melting” perception of commercial spreads. J Texture Stud 48:302–312

Hamid AQ (2023). Development of technology for omega enriched fat spread. M. Tech. Thesis, Kamdhenu University, Gandhinagar

Hirpara K, Patel H, Gokhale A, Patel A, (2016). Effect of level of fat on compositional, physico-chemical, rheological and sensory attributes of processed cream cheese based (PCCB) spread. Indian J Dairy Sci 69:1–7

Hull M (1947). Studies on Milk Proteins. II. Colorimetric determination of the partial hydrolysis of the proteins in milk. Journal of Dairy Sci 30:881–884. https://doi.org/10.3168/jds.s0022-0302(47)92412-0

IS: 2785-1979. (1992). Natural Cheese (Hard Variety), Processed Cheese, Processed Cheese Spread and Soft Cheese. Bureau of Indian Standards, New Delhi

IS: 5401 (Part I). (2002). Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for the Detection and Enumeration of Coliforms, Part 1: Colony Count Technique. Bureau of Indian Standards, New Delhi

IS: 5403 (1999). Yeast and Mold Count of Foodstuffs and Animal feeds (first revision). Bureau of Indian Standards, New Delhi, 209

ISO 8968-5/IDF 020-5:2001 - Milk - Determination of nitrogen content - Part 5: Determination of protein-nitrogen content. International Organization for Standardization, Geneva, Switzerland

Kathiriya MR, SreejaV, Prajapati JB, Vekariya Y(2020). Optimization of process parameters for pickle masala flavored probiotic Greek yoghurt. Indian J Dairy Sci 73:425-433. doi:10.33785/IJDS.2020.v73i05.006

Kumar P (2014). Process optimization for the preparation of chocolate spread incorporating whey protein concentrate, cocoa powder, olive oil and butterfat using response surface methodology. J Food Processing Preserv 39:745–757 doi:10.1111/jfpp.12284

Kumari K, Sharma D (2022). Development of different types of dairy and plant-based spreads: A review. Pharma Innovation11:2244–2250. doi:10.22271/tpi.2022.v11.i6sab.13435

Meilgaard MC, CarrBT, CivilleGV (1999). Sensory evaluation techniques. CRC press

Mishra VK, DavidJ, Rani R, Bharti BK, Dixit NK (2019). Storage study of filled milk chhana spread. J Pharmacognosy and Phytochemy 8:1567-1571

Patel AM, Modha HM, Dharaiya CN, Patel DH, Patel HG (2016). Analysis of rheological properties of Halvasan as function of ingredients using response surface methodology. Indian J Dairy Sci 69:634-640

Popalia (2018). Development of value added milk-cereal based product. M. Tech. Thesis, Anand Agricultural university, Anand

PrajapatiPS, Gupta SK, Patel AA, Patil GR (1991). Processing of low-fat butter flavoured spread. J Food Sci Technol 28:208–211

Radoèaj O, DimiæE, Diosady LL, Vujasinoviæ V (2011). Optimizing the texture attributes of a fat based spread using instrumental measurements. J Texture Stud 42:394–403. doi:10.1111/j.1745-4603.2011.00300.x

SmithersGW (2008). Whey and whey proteins—From ‘gutter-to-gold’. Int Dairy J18:695-704.doi:10.1016/j.idairyj.2008.03.008

Suthar J, JanaA, Balakrishnan S (2017). High protein milk ingredients-A tool for value-addition to dairy and food products. J Dairy, Vet Anim Res 6:259-265.doi:10.15406/jdvar.2017.06.00171

Thomas W, Harper W, Gould I (1954). Free fatty acid content of fresh milk as related to portions of milk drawn. Journal of Dairy Science 37:717–723. https://doi.org/10.3168/jds.s0022-0302(54)91317-x

Verma (1996). A study on technological aspects for development of low fat butter spread. M.Sc. Thesis, Anand Agricultural university, Anand

Yadav S, Rani R, Singh B, Thompkinson DK. (2019) Low fat channa spread from filled milk. Indian J Dairy Sci 72:445–448 doi:10.33785/ijds.2019.v72i04.016

Indian J Dairy Sci 78(2): 173-181
https://doi.org/10.33785/IJDS.2025.v78i02.010

RESEARCH ARTICLE

Prevalence of *Enterococcus faecalis* and *Pseudomonas aeruginosa* from mastitis milk and their antimicrobial resistance

Aishvarya Borkar[1], Mudit Chandra[1] (✉), Deepti Narang[1], D.K. Gupta[2], A. K. Arora[1]

Received: 08 September 2023 / Accepted: 25 February 2024 / Published online: 23 April 2025

Abstract: Mastitis an inflammatory condition of mammary glands is a multi-factorial disease of dairy animals. It is characterized by physical, chemical and bacteriological changes in the milk. In the present study prevalence of *Enterococcus faecalis* (*E. faecalis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were studied along with their antibiotic resistance pattern. One hundred and ten milk samples were screened from mastitis cattle and buffaloes in and around Ludhiana from December 2020 till June 2021. The prevalence of *E. faecalis* and *P. aeruginosa* was 7.05% and 4.70% respectively. The isolates of *E. faecalis* were resistant to penicillin G (100%), vancomycin, erythromycin, and tetracycline (83.33%) and sensitive to nalidixic acid (100%), streptomycin (83.33%), oxacillin, gentamicin, ciprofloxacin and ceftriaxone (66.66%) whereas isolates of *P. aeruginosa* were resistant to ampicillin, ciprofloxacin, ceftriaxone, penicillin G, streptomycin, amikacin, vancomycin (100%), tetracycline and teicoplanin (75%) and sensitive to cefuroxime, gentamicin, and oxacillin (100%). In *E. faecalis, vanA, vanB* (50%), *tetL* (83.33%) and *mrsA/B* (100%) antibiotic genes were amplified whereas in *P. aeruginosa*, *aadA, DHAM* (100%), *sulI, sulII* (100%), *gyrA, gyrB* (100%) and *tetC* (50%) antibiotic genes were amplified. Thus, it is concluded that there is an evolving prevalence of environmental pathogens such as *E. faecalis* and *P. aeruginosa* in mastitis which is alarming and thus necessary action and plans should be followed for controlling the antibiotic resistance in these pathogens as these are of zoonotic significance.

[1]Department of Veterinary Microbiology, [2]Department of Clinical Veterinary Medicine, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004

(✉) drmuditchandra@rediffmail.com

Keywords: Mastitis, Prevalence, Antibiotic Resistance, *Enterococcus faecalis*, *Pseudomonas aeruginosa*

Introduction

Mastitis is a multifactorial complex disease characterized by inflammation of the parenchyma of the mammary gland, as demonstrated by physical, chemical, and bacteriological changes in milk, as well as pathological abnormalities in glandular tissues (Constable et al. 2017). It has mainly two forms clinical and subclinical. In most dairy herds, subclinical mastitis is 3 to 40 times more frequent than the clinical mastitis and overall it generates the biggest losses to the farmers (Bachaya et al. 2011, Singh et al. 2018). *Enterococcus* spp. and *Pseudomonas aeruginosa* are one of the important environmental pathogens causing mastitis. *Enterococcus* spp. is an environmental causative agent of mastitis and seen in the gut flora of healthy humans and animals. *Enterococcus* spp. coexisted since long but has been highlighted in recent years causing diseases like bacteraemia, endocarditis, meningitis, urinary tract infections, soft tissue infections and bovine mastitis. There are many species of *Enterococcus* such as *E. fecalis*, *E. faecium*, *E. durans*, *E. avium*, *and E. gallinarum*. Among these species *E. faecalis* (80%) and *E. faecium* (10%-15%) are the most commonly found bacteria that cause mastitis (Różańska et al. 2019). Enterococci are important because of their ability to harbour antimicrobial resistance genes (Klare et al. 2003). *Enterococcus* spp. are known to as indicator organisms for antimicrobial resistance development (Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP, 2003) as they provide accurate information on the animals prior antibiotic treatment (Centre for Disease Control and Prevention) (CDC, 2002).

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram negative bacterium, ubiquitous, and has a wide host range with metabolic versatility. They multiply rapidly in various environmental conditions and milk being one of them. *P. aeruginosa* has been associated with sub-clinical mastitis. The isolates of *P. aeruginosa* are strong biofilm producer which decreases the potency of antibiotics and leads to chronic mastitis (Melchior et al. 2006). In human and veterinary medicine,

antibiotic resistance is a global problem and it generally occurs due to extensive use of antibiotics. Bovine mastitis is the single most common cause for antimicrobial use in lactating cattle worldwide and there is a variety of antimicrobials that are used to prevent for the cure of mastitis. The antimicrobial therapy for enterococci infections is complicated because of the inherent resistance exhibited. *P. aeruginosa* too poses a serious therapeutic challenge for treatment because of its ability to develop resistance to multiple classes of antibacterial agents quickly (Lambert, 2002).Thus, identification of the mastitis-causing pathogens along with their antibiotic resistance assumes great importance for the effective control of mastitis. Therefore, the present study was planned with an objective to isolate and identify *Enterococcus* spp. and *Pseudomonas* spp. from mastitis milk along with their antimicrobial resistance pattern for the purpose of gaining a better understanding of the importance of this infection in dairy animals.

Material and methods

Sample collection

A total of 110 milk samples were collected from mastitis animals from Teaching Veterinary Clinical Complex (TVCC) GADVASU (66), Haibowal dairies (25), Noor Mahal farm (7), and from an organized dairy farm (12) at Ludhiana. All the samples were tested by SLS test for initial screening and all those positive were transferred immediately to laboratory on ice for the isolation of bacteria.

Isolation and identification of bacteria

The milk samples brought to the laboratory were thoroughly mixed and inoculated on basal media like Brain Heart Infusion (BHI) Agar and Nutrient Agar. These plates were incubated at 37°C for 12-24 h to observe the bacterial growth. The bacteria from both the media were further inoculated on bile esculin agar, enterococcus agar base and cetrimide agar. *Enterococococcus* isolates on bile esculin agar produced brown colonies with black discolouration due to hydrolysis of esculin and they tolereated 6.5% NaCl concentration. *Pseudomonas* isolates produced pyocyanin and pyoverdin on cetrimide agar. Further, they were subjected to biochemical tests like indole test, methyl red test, voges proskauer's test, citrate utilization, oxidase test, catalase tests, urease, nitrate reduction, esculin hydrolysis, 6.5% NaCl tolerance, triple sugar iron test, fermentation of various sugars viz. glucose, lactose, sucrose, sorbitol and maltose for confirmation. All the isolates were confirmed by MALDI-TOF (Bruker daltonics, GmBH).

Confirmation of *E. faecalis* strains

These isolates when grow on bile esculin gives brown coloured colonies with black discolouration due to hydrolysis of esculin and they can tolerate bile salts. They have ability to grow on 6.5% NaCl concentration (Table 1).

Confirmation of *P. aeruginosa* strains

These isolates produce pigments such as pyocyanin and pyoverdin which was examined using the "fluorescent technique," which involved growing the isolates on cetrimide and then exposing them to UV light illumination (Table 2).

Antibiotic Sensitivity Testing

All the isolates were tested for antibiotic sensitivity using Bauer et al. (1966) disc's diffusion method using twenty one antibiotics viz., amikacin (30 mcg), amoxycillin (10 mcg), ampicillin (10 mcg), ampicillin/sulbactam (10/10 mcg), cefoperazone (75 mcg), ceftriaxone (30 mcg), cefuroxime (30 mcg), cephalothin (30 mcg), ciprofloxacin (5 mcg), co-trimoxazole (25 mcg), enrofloxacin (10 mcg), erythromycin (15 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), oxacillin (1 mcg), penicillin G (10 mcg), sparfloxacin (5 mcg), streptomycin (10 mcg), teicoplanin (30 mcg), tetracycline (30 mcg) and vancomycin (30 mcg). In brief, the individual bacterium were grown overnight (10-12 h) in BHI broth at 37°C and was spread uniformly on Muller Hinton Agar with the help of sterilized cotton swab. Antibiotic discs were placed equidistantly under sterile conditions and the plates were then incubated for 12-24 h at 37°C. The zones of sensitivity was measured and were classified as sensitive, intermediate or resistant on the basis of zone of inhibition as per the standard guidelines of CLSI Standards (CLSI, 2018) (Table 3).

DNA Extraction

The DNA of *Enterococcus* spp. and *P. aeruginosa* isolates was extracted using NucleoSpin® Microbial DNA kit as per the manufacturer's instructions.

Polymerase Chain Reaction

PCRs were carried out for *Enterococcus* spp. and *Pseudomonas* spp. using genus specific primers (Table 4). A 25 µl PCR reaction mixture was formulated using 12.5 µl of master mix (2X Go Taq Green Master mix, (Promega, WI USA), 0.5 µl of 20 pmol/ul of each forward and reverse primers (IDT, USA), 1.0 µl of template DNA and finally the reaction volume was made up to 25 µl using NFW (NEB Labs, USA). PCR was performed using thermocycler (Veriti, Applied Biosystem, USA) with the following conditions; an initial denaturation at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes for *Enterococcus* spp. and initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes for *P. aeruginosa*.

Detection of antibiotic resistance genes in *E. faecalis*

All the *E. faecalis* isolates were tested for the presence of *vanA, vanB, vanC1, tetL, tetk* and *msrA/B* (Table 5). A 25µl reaction mixture was formulated by using 12.5µl (2X Go Taq Green Master Mix) (Promega, WI USA), 1µl of 20 pmol/ul of each forward and reverse primers for each of the antibiotic resistant genes (IDT, USA), 2µl of template DNA and 8.5µl of nuclease free water. PCR was performed using thermocycler (Veriti, Applied Biosystem, USA). For *vanA* and *vanB* genes, initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. For *vanC1* gene, an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. For *tetK, tetL* genes, an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds and extension at 72°C for 1 minute 30 seconds followed by a final extension 72°C for 5 minutes. For *msrA/B* gene, an initial denaturation at 94C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 120 seconds and extension at 72°C for 1 minute 30 seconds followed by a final extension at 72°C for 10 minutes.

Detection of antibiotic resistance genes in *P. aeruginosa*

All the *P. aeruginosa* isolates were tested for the presence of *blaTEM, blaSHV, sulI, sulII, aadA, DHAM, MOXM, tetA, tetB, tetC, oxa-1, blaCTX-M, gyrA* and *gyrB* (Table 6). A 25µl reaction mixture by adding 12.5µl master mix (2X Go Green Taq Master Mix) (Promega, WI USA), 1µl of 20 pmol/ul of each forward and reverse primers for each of the antibiotic resistant genes (IDT, USA), 2µl of template DNA and 8.5µl of nuclease free water. PCR was performed on a thermocycler (Veriti, Applied Biosystem, USA). For *blaTEM, blaSHV, Sul1, SulII* and *tetC* genes an initial denaturation at 94°C for 5minutes, 30 cycles 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1.5 minutes followed by a final extension at 72°C for 10 minutes. For *tetA, tetB, DHAM, MOXM* and *aadA* an initial denaturation at 95°C for 5minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. For *blaCTX-M,* an initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 95°C for 20 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds followed by a final extension at 72°C for 3 minutes. For *oxa-1*, an initial denaturation at 96°C for 5 minutes, 35 cycles of denaturation at 96°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. For *gyr*A, an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 45 seconds, annealing for 57°C for 45 seconds, extension at 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. For *gyr*B, an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 45 seconds, annealing for 40°C for 45 seconds, extension at 72°C for 45 seconds followed by a final extension at 72°C for 5 minutes.

Gel Electrophoresis and documentation

The PCR products were run on 1.5% agarose along with 100bp DNA molecular weight marker (New England Biolabs, USA) at 80V/cm and visualized using a gel documentation system (AlphaImager, Alpha Innotech, USA).

Results and Discussion

Prevalence

Out of 110 milk samples screened using SLS test, 108 (98.1%) milk samples showed positive reaction. Out of 108 SLS positive samples 85 yielded bacterial growth (78.7%). Out of these six (7.05%) were *E. faecalis* and four (4.70%) were *P. aeruginosa*. These organisms were identified on the basis of cultural characters, gram's staining, biochemical tests and MALDI-ToF. *E. faecalis* prevalence was found to be 7.05% which was in accordance with the study conducted by Ali et al. (2011) where they reported prevalence of *E. faecalis* from dairy buffaloes to be 3.17%. In another study Yang et al. (2019) observed *E. faecalis* prevalence of 4.5% from subclinical bovine mastitis and 6.36% from clinical mastitis (Awandkar et al. 2022). These findings were similar to the present study findings where a prevalence of less than 10% was observed. Since, *E. faecalis*, a major environmental mastitis-causing pathogen (Elhadidy and Elsayyad, 2013) has the ability to produce biofilm which causes inherent resistance for many antibiotics, thus its prevalence as well as antibiotic resistance profile needs to be examined on the regular basis.

P. aeruginosa prevalence was found to be 4.70 % which was in tandem with the findings of Sekhri et al. (2021) where they observed *P. aeruginosa* prevalence as 5.15%. Various studies too indicated prevalence of *P. aeruginosa* between 1-5% (Sharma and Sindhu, 2007, Banerjee et al, 2017, Yadav et al, 2020, Awandkar et al, 2022) similar to the findings of the present study.

Antibiotic sensitivity test

E. faecalis isolates showed sensitivity towards nalidixic acid (100%), streptomycin (83.33%), cepalothin, ciprofloxacin, co-trimaxazole, ceftriaxone, cefuroxime, gentamicin, oxacillin and vancomycin (66.66%) and resistance against penicillin G (100%), erythromycin, tetracycline (83.33%) and ampicillin/salbactam, ampicillin (66.66%). Similar results were observed by Frazzon et al. (2010) and Yang et al. (2019) where they reported that the isolates of *Enterococcus* spp. showed higher resistance against tetracycline (87.7%) and erythromycin (79.0%) which was similar to the present study findings as high resistance was observed against penicillin G (100%), erythromycin and tetracycline

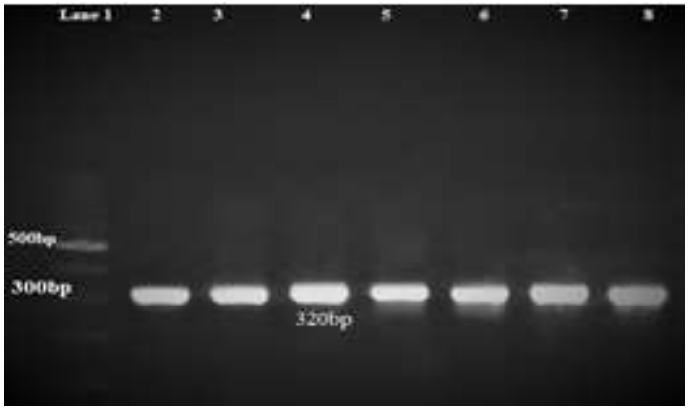


Fig. 1: Detection of *Enterococcus* spp. by PCR
Lane 1: 100bp DNA ladder; Lane 2-8: Positive samples

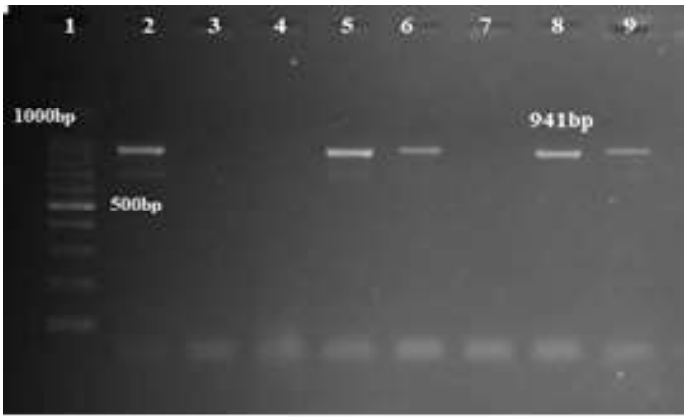


Fig. 2: Detection of *Enterococcus faecalis* by PCR
Lane 1: 100bp DNA ladder; Lane 2, 5, 6, 8 & 9 Positive; Lane 3-4 & 7 Negative

(83.33%). Similarly, Nam et al. (2010) observed that *Enterococcus* spp. isolates were sensitive to ampicillin, gentamicin and vancomycin, and resistant to ampicillin, tetracycline (69.5%), penicillin (64.7%), erythromycin (57.1%) and cephalothin (44.7%). Hamzah and Kadim (2018) results indicated resistance to vancomycin, penicillin, ofloxacin, ciprofloxacin, nitrofurantoin, tetracycline and amikacin whereas Gao et al. (2019) observed high resistance against penicillin, ceftiofur, tylosin, lincomycin, and oxytetracycline antibiotics in *Enterococcus* spp. which was similar to the findings of this study (Table 3).

P. aeruginosa isolates showed sensitivity towards cefuroxime, gentamicin, oxacillin (100%), amikacin, cefaperazone, erythromycin (25%) and resistance against ampicillin, ciprofloxacin, cephalothin, co-trimoxazole, ceftriaxone, nalidixic acid, penicillin G, streptomycin (100%), ampicillin/salbactam, amikacin, vancomycin, sparfloxacin, tetracycline, teicoplanin (75%) and amoxicillin, erythromycin (50%). *Enterococcus faecalis* isolates showed sensitivity towards nalidixic acid (100%), streptomycin (83.33%), cepalothin, ciprofloxacin, co-trimoxazole, ceftriaxone, cefuroxime, gentamicin, oxacillin and vancomycin (66.66%) and resistance against penicillin G (100%), erythromycin, tetracycline (83.33%) and ampicillin/salbactam, ampicillin (66.66%). The above results were similar to a study by Swetha et al. (2017) where they observed that isolates of *P. aeruginosa* from milk were resistant to ampicillin, penicillin, and oxacillin (100%) but sensitive to vancomycin (5.3%) and tetracycline (10.5%) indicating an alarming situation. Similarly Sekhri et al. (2021) studied antibiotic resistance of *P. aeruginosa* isolated from milk and observed that resistance to chloramphenicol, tetracycline, amoxicillin, erythromycin, cephalexin, teicoplanin (100%), azithromycin, doxycycline, ofloxacin, co-trimoxazole, vancomycin (80%), gatifloxacin, sparfloxacin (60%) and ciprofloxacin (50%), which was similar as findings of the present study (Table 3).

Polymerase chain reaction

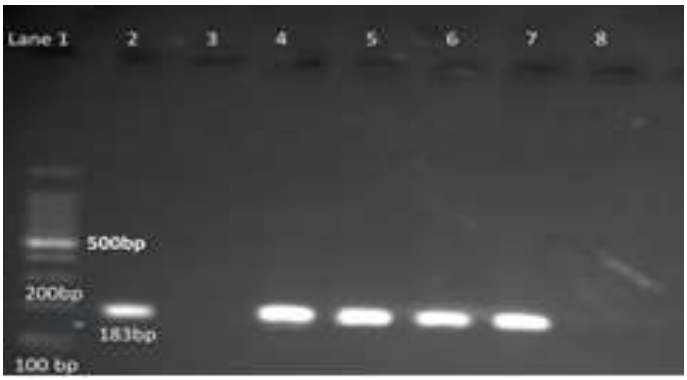


Fig. 3: Detection of *Pseudomonas aeruginosa* by PCR
Lane 1: Ladder; Lane 2, 4-7 Positive; Lane 3: Negative

On the basis of polymerase chain reaction using genus specific primers all the six isolates were identified as *Enterococcus* spp. producing a product size of 320bp (Fig.1). On the basis of species specific primer, all the isolates were *E. faecalis* producing a product size of 941bp (Fig. 2). Using genus specific primers all the six isolates were identified as *Enterococcus* spp. which was similar to El-Tawab et al. (2019), Devriese et al. (1996) and Jahan et al. (2013) where they used genus specific primers to confirm enterococci. Upon further analysis using species specific primers all the isolates were identified as *E. faecalis*. In various studies viz., Kariyama et al. (2000), Foka and Ateba (2019), Dutka-Malen (1995) and Mannu et al. (2003) used species specific primers for the confirmation of *E. faecalis* from mastitis milk. Similarly, Jahan et al. (2013) *E. faecalis* and *E. faecium* from meat and fermented meat products using species specific primer.

On the basis of polymerase chain reaction all the four isolates were *P. aeruginosa* producing a product size of 183bp (Fig. 3). In an earlier study Sekhri et al. (2021) used same species-specific primers for the confirmation of *P. aeruginosa* isolates. Similar to our study confirmation of *P.aeruginosa* using PCR has been

achieved by various earlier workers too (Iwasaki et al. 2019; Jangir et al. 2021; Schauer et al. 2021).

Detection of antibiotic resistance genes in *E. faecalis*

All the six *E. faecalis* isolates were tested for the presence of *vanA, vanB, vanC1, tetL, tetk* and *msrA/B* antibiotic resistant genes. It was revealed that one (16.66%) isolate was positive for *vanA* gene, three (50%) isolates were positive for *vanB* gene,

Table 1 Results of morphological, cultural and biochemical tests for *Enterococcus faecalis* identification

S. No.	Morphological & cultural characteristics	Interpretation
1	Growth on Bile Azide Esculin Agar	Small, round smooth dark brown colonies with black discoloration
2	Gram staining	Gram positive cocci (pairs or in chains)
3	Growth on Enterococcus Agar Base (1%TCC)	Maroon pin pointed coloured colonies
4	Growth on Blood Agar	Alpha or beta haemolytic
5	Sulphide Indole Medium Test	Non-Motile
	Biochemical Tests	Interpretation
1	Catalase	Negative
2	Oxidase	Negative
3	Indole	Negative
4	Methyl Red	Negative
5	Voges –Proskauer	Positive
6	Citrate Utilization	Negative
7	Urease	Negative
8	Nitrate reduction test	Positive
9	H_2S production	Negative
10	Esculin hydrolysis	Positive
11	Gas Production	Positive
12	6.5% NaCl (Salt tolerance)	Positive
13	Fermentation of sugar	
	a. Glucose	Positive
	b. Lactose	Positive
	c. Arabinose	Negative
	d. Sucrose	Positive
	e. Sorbitol	Positive
	f. Maltose	Positive

Table 2 Results of morphological, cultural and biochemical tests performed for *Pseudomonas aeruginosa* identification

S. No.	Morphological & cultural characteristics	Interpretation
1	Growth on BHI	Large round, opaque colonies with green discoloration.
2.	Growth on MLA	Pale colourless round colonies
3.	Gram's Staining	Gram Negative rods
4.	Growth on Cetrimide Agar	Cream medium sized flat, irregular edged colonies giving green pigmentation
5.	Sulphide Indole Medium Test	Motile
	Biochemical Tests	Interpretation
1	Catalase	Positive
2	Oxidase	Positive
3	Indole	Negative
4	Methyl Red	Negative
5	Voges-Proskauer	Negative
6	Citrate Utilization	Positive
7	Urease	Negative
8	Nitrate reduction test	Positive
9	ONPG	Negative

10	H_2S production	Negative
11	Arginine utilization	Positive
12	Triple sugar iron	K/K
13	Fermentation of sugar	
	a. Glucose	Positive
	b. Lactose	Negative
	c. Arabinose	Negative
	d. Sucrose	Negative
	e. Sorbitol	Negative
	f. Maltose	Negative

Table 3 Zone of antibiotic Resistance (Mean ± Standard Deviation) in mm in *Enterococcus faecalis* and *Pseudomonas aeruginosa* isolates

S. No.	Antibiotics	Mean ± SD in *Enterococcus faecalis* isolates (6)	Mean ± SD in *Pseudomonas aeruginosa* isolates (4)
1	Co-trimoxazole	17.2 ±8.5	5.3±6.1
2	Nalidixic Acid	11.2±10.2	7.0±9.5
3	Erythromycin	13.7±7.8	20.8±7.6
4	Tetracycline	14.5±2.4	14.8±2.5
5	Cephalothin	27.3±4.7	6.8±7.9
6	Streptomycin	10.2±5.1	19.3±7.0
7	Cefuroxime	24.0±4.7	4.3±8.5
8	Ceftriaxone	21.8±3.9	19.0±5.5
9	Teicoplanin	14.0±2.4	0.0±0.0
10	Enrofloxacin	23.8±4.7	30.0±3.6
11	Cefoperazone	20.7±6.6	17.5±4.2
12	Amikacin	16.3±1.2	18.0±8.1
13	Sparfloxacin	19.3±5.2	20.5±0.6
14	Ampicillin/sulbactam	28.2±3.5	0.0±0.0
15	Ciprofloxacin	19.8±4.0	30.8±1.5
16	Amoxicillin	0.0±0.0	0.0±0.0
17	Ampicillin	17.7±1.5	5.0±10.0
18	Oxacillin	14.8±7.5	0.0±0.0
19	Penicillin G	16.0±15.9	0.0±0.0
20	Vancomycin	15.2±1.2	0.0±0.0
21	Gentamicin	20.5±4.1	13.8±1.9

none of the isolates were positive for *vanCI* and *tetK*, five (83.3%) isolates were positive for *tetL* and all six (100%) isolates were positive for *msr A/B* gene. In an earlier study Erbas et al. (2016) observed one enterococci having *vanA* gene which was similar to the findings of the present study. Five (83.3%) isolates were positive for *tetL* was similar to the findings of a study by Jahan et al. (2013) where they stated that tetracycline efflux pumps *tetk* and *tetL* were positively amplified in 11 isolates. However, Huys et al. (2004) detected no *tetK* gene in their isolates, but Hummel et al. (2007) observed *tetL* in 94% and *tetK* in 56% isolates. In another study Stovcik et al. (2008) observed 63% isolates having *tetM* and 21% isolates having *tetL* genes whereas Frazzon (2010) observed 38% isolates having *tetM* and 9% having *tetL* and both *tetM* and *tetL* in 13% isolates, respectively. All the six (100%) isolates were positive for *msr A/B* gene which too is in tandem with the findings of Valenzuela, (2013) where they observed its presence in 66.66% isolates.

Upon comparison between phenotypic and genotypic resistance it was observed that for vancomycin phenotypically 66.66% isolates exhibited resistance while 50% isolates had *vanA*, *vanB* gene but none of the isolates had *vanC1* gene. For tetracycline, 83.33% isolates exhibited both phenotypic as well as genotypic resistance. For macrolide 83.33% isolates had phenotypic resistance while 100% isolates exhibited resistance genotypically.

Detection of antibiotic resistance genes in *P. aeruginosa*

All the four *P. aeruginosa* isolates were tested for the presence of *blaTEM, blaSHV, sulI, sulII, aadA, DHAM, MOXM, tetA, tetB, tetC, oxa-1, blaCTX-M, gyrA* and *gyrB* antibiotic resistant genes. It was revealed that three (75%) isolates were positive for *sulI*

Table 4 Primers used for the amplification of different organisms

S. No.	Organism	5' to 3'	Amplicon Size (bp)	Annealing Temp (°C)	Reference
1	*Enterococcus* rrs (16S rRNA)	F: GGATTAGATACCCTGGTAGTCC R: CGTTGCGGGACTTAACCCAAC	320	54	Devriese et al. (1996)
2	*E. faecalis*	F: ACGATTCAAAGCTAACTG R: ATCAAGTACAGTTAGTCT	941	54	Dutka-Malen et al. (1994)
3	*E. faecium*	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	54	Cheng et al. (1997)
4	*P. aeruginosa*	F: CTGGCCTTGACATGCTGAGA R:TCACCGGCAGTCTCCTTAGA	183	60	Sekhri et al. (2020)

Table 5 Sequence of primers used for the detection of antibiotic resistance genes in *Enterococcus faecalis*

S. No.	Antibiotics	Genes	Primers 5′-3′	Product (bp)	References
1.	Vancomycin	*vanA*	F:GCGAAAACGACAATTGC R:GTACAATGCGGCCGTTA	732	Dutka-Malen, (1995)
		vanB	F:ACGGAATGGGAAGCCGA R:TGCACCCGATTTCGTTC	647	Depardieu, (2004)
		vanC1	F:GGTATCAAGGAAACCTC R:CTTCCGCCATCATAGCT	822	Dutka-Malen, (1995)
2.	Tetracycline	*TetK*	F:TATTTTGGCTTTGTATTCTTTCAT R:GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski et al. (2000)
		TetL	F:ATAAATTGTTTCGGTCGGTAAT R:AACCAGCCAACTAATGACAATGAT	1077	Trzcinski et al. (2000)
3	Macrolide	*MsrA/B*	F:R:GCAAATGCTGTAGGTAAGACAACT R:ATCATGTGATGTAAACAAAAT	400	Wondrack et al. (1996)

gene, one (25%) isolate was positive for *sulII* gene, two (50%) isolates were positive for *tetC* gene and all the 4 (100%) isolates were positive for *aadA*, *DHAM*, *gyrA* and *gyrB* gene. None of the isolates were positive for *blaTEM, blaSHV, blaCTX-M, oxa-1, tetA, MOXM and tetB* genes. All the four *Pseudomonas aeruginosa* isolates were tested for the presence of *blaTEM, blaSHV, sulI, sulII, aadA, DHAM, MOXM, tetA, tetB, tetC, oxa-1, blaCTX-M, gyrA* and *gyrB* antibiotic resistant genes.

It was revealed that three (75%) isolates were positive for *sulI* gene, one (25%) isolate was positive for *sulII* gene, two (50%) isolates were positive for *tetC* gene and all the 4 (100%) isolates were positive for *aadA*, *DHAM*, *gyrA* and *gyrB* gene. However, none of the isolates were positive for *blaTEM, blaSHV, blaCTX-M, oxa-1, tetA, MOXM and tetB* genes.

In a study Das et al. (2017) observed 6 (12%) isolates possessed *blaTEM* resistance genes in *P. aeruginosa* and but didn't find any isolate carrying *blaSHV* genes similar to present study. Similarly, Meng et al. (2020) reported four isolates having *sul1* gene among 44 isolates whereas in the present study three (75%) isolates were positive for *sulI* gene, one (25%) isolate was positive for *sulII* gene.

Upon comparison between phenotypic and genotypic resistance it was observed that for ampicillin/sulbactam, 75% isolates exhibited phenotypic resistance while 100% isolates exhibited genotypic resistance. For sulphonamides, aminoglycosides and fluroquinolones 100% isolates exhibited both phenotypic as well as genotypic resistance. For tetracycline, 75% isolates exhibited phenotypic resistance while 50% exhibited genotypic resistance.

Conclusions

From the present study, we can conclude that there is prevalence of environmental pathogens like *E. faecalis* 7.05% and *P. aeruginosa* 4.70% in mastitis. On the basis of antibiotic sensitivity test, these isolates showed multidrug resistance towards many commonly used antibiotics. Phenotypic and genotypic resistance when compared revealed that there is partial correlation between these.

Acknowledgement

The authors are also grateful to the ICAR Niche area of Excellence on Antibiotic Resistance: Animal Human Interface

Table 6 Sequence of primers used for the detection of antibiotic resistance genes in *Pseudomonas aeruginosa*

S No.	Antibiotics	Genes	Primers 5′-3′	References
1.	Beta lactam	*BlaTEM*	F: GAGTATTCAACATTTTCGT R: ACCAATGCTTAATCAGTGA	Maynard et al. (2004)
		blaSHV	F:TCGCCTGTGTATTATCTCCC R:CGCAGATAAATCACCACAATG	
		blaCTX-M	F:TTTGCGATGTGCAGTACCAGTAA R:CGATATCGTTGGTGGTGCCATA	Edelstein (2003)
		OXA-1	F:ACACAATACATATCAACTTCGC R:AGTGTGTGTTTAGAATGGTGATC	Oliver et al. (2002)
2.	Tetracycline	*TetA*	F:CGATCTTCCAAGCGTTTGTT R:CCAGAAGAA CGAAGCCAGTC	Faldynova et al. (2013)
		TetB	F:TACAGGGATTATTGGTGAGC R:ACATGAAGGTCATCGATAGC	
		TetC	F:ACTTGGAGCCACTATCGAC R:CTACAATCCATGCCAACCC	Maynard et al. (2004)
3.	Sulphonamide	*SulI*	F:TTCGGCATTCTGAATCTCAC R:ATGATCTAACCCTCGGTCTC	Shehata et al. (2016)
		SulII	F:CGGCATCGTCAAACATAACC R:GTGTGCGGATGAAGTCAG	Maynard et al. (2004)
4.	Ampicillin	*MOXM*	F:GCTGCTCAAGGAGCACAGGAT R:CACATTGACATAGGTGTGGTGC	Van et al. (2008)
		DHAM	F:AACTTTCACAGGTGTGCTGGGT R:CCGTACGCATACTGGCTTTGC	
5.	Streptomycin	*aadA*	F:TGATTTGCTGGTTACGGTGAC R:CGCTATGTTCTCTTGCTTTTG	
6.	Fluoroquinolone	*gyrA*	F:GGATAGCGGTTAGATGAGC R:CGTTAATCACTTCCGTCAG	Yue et al. (2008)
		gyrB	F:CAGCAGATGAACGAACTGCT R: AACCAAGTGCGGTGATAAGC	

for providing necessary scientific reagents and chemicals required during research work. The authors are also thankful to the Director Research, GADVASU for providing the necessary laboratory facilities.

References

Ali MA, Ahmad MD, Muhammad K, Anjum AA (2011) Prevalence of sub clinical mastitis in dairy buffaloes of Punjab, Pakistan J Anim Plant Sci 21(3): 477-480.

Awandkar SP, Kulkarni MB, Khode NV (2022) Bacteria from bovine clinical mastitis showed multiple drug resistance, Vet Res Commun 46(1): 147-158.

Bachaya HA, Raza MA, Murtaza S, Akbar IUR (2011) Subclinical bovine mastitis in Muzaffargarh district of Punjab (Pakistan), J Anim Plant Sci 21(1): 16-19.

Banerjee S, Batabyal K, Joardar SN, Isore DP, Dey S, Samanta I, Samanta TK, Murmu S (2017) Detection and characterization of pathogenic *Pseudomonas aeruginosa* from bovine subclinical mastitis in West Bengal, India, Vet World 10(7): 738-42.

Bauer AW, Kirby WM, Sherirs JC, Turck M (1966) Antibiotic susceptibility testing by standardized single disk method, Am J Clinic Path 45: 433-96.

CDC (2002) *Staphylococcus aureus* resistant to vancomycin United States, MMWR https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5126a1.htm.

Cheng S, Mccleskey FK, Gress MJ, Petroziello JM, Liu R, Namdari H, Beninga K, Salmen A, DelVecchio VG (1997) A PCR assay for identification of *Enterococcus faecium*, J Clin Microbiol 35(5): 1248-1250.

CLSI (2018) Performance Standards for Antimicrobial Susceptibility testing, M100, 28th Edition.

Constable PD, Hinchcliff KW, Done SH, Grunberg W (2017) Diseases of mammary gland, in: Veterinary Medicine, 11th Edn. Elseveir Ltd. St. Louis, Missouri, pp 1904- 2001

Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) (2003) Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Foods and Humans in Denmark, DANMAP, Soborg, Denmark.

Das A, Guha C, Biswas U, Jana PS, Chatterjee A, Samanta I (2017) Detection of emerging antibiotic resistance in bacteria isolated from subclinical mastitis in cattle in West Bengal, Vet World 10(5): 517.

Devriese LA, Ieven M, Goossens H, Vandamme P, Pot B, Hommez J, Haesebrouck F (1996) Presence of vancomycin resistant enterococci in farm and pet animals, Antimicrob Agents Chemo 40(10): 2285-2287.

Depardieu F, Perichon B, Courvalin P (2004) Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR, J Clin Microbiol 42(12): 5857-60.

Dutka Malen S, Evers S, Courvalin P (1995) Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR, J Clin Microbiol 33(1): 24-27.

Elhadidy M, Elsayyad A (2013) Uncommitted role of enterococcal surface protein, Esp, and origin of isolates on biofilm production by *Enterococcus faecalis* isolated from bovine mastitis, L Microbiol Immunol Infect 46(2): 80-84.

El-Tawab A, Awad A, Elhofy FI, Mahmoud MA, Amin EK (2019) Genotyping and resistance genes of *Enterococcus faecalis* isolated from different food sources in Egypt, Benha Vet Med J 37(1): 149-153.

Erbas G, Parin U, Turkyilmaz S, Ucan N, Ozturk M, Kaya O (2016) Distribution of antibiotic resistance genes in *Enterococcus* spp. isolated from mastitis bovine milk, Acta Vet Beograd 66: 336-346.

Faldynova M, Videnska P, Havlickova H, Sisak F, Juricova H, Babak V, Steinhauser L, Rychlik I (2013) Prevalence of antibiotic resistance genes in faecal samples from cattle, pigs and poultry, Vet Med 58(6): 298-304.

Foka FET, Ateba CN (2019) Detection of virulence genes in multidrug resistant enterococci isolated from feedlots dairy and beef cattle: Implications for human health and food safety, BioMed Res Int, https://doi.org/10.1155/2019/5921840.

Frazzon AG, Gama BA, Hermes V, Bierhals CG, Pereira RI, Guedes AG, Azevedo PA, Frazzon J (2010) Prevalence of antimicrobial resistance and molecular characterization of tetracycline resistance mediated by tet(M) and tet(L) genes in Enterococcus spp. isolated from food in Southern Brazil, World J Microbiol Biotechnol 26: 365-70.

Gao X, Fan C, Zhang Z, Li S, Xu C, Zhao Y, Han L, Zhang D, Liu M (2019) Enterococcal isolates from bovine subclinical and clinical mastitis: Antimicrobial resistance and integron-gene cassette distribution, Microb Pathog 129(4): 82-87.

Hamzah AM, Kadim HK (2018) Isolation and identification of *Enterococcus faecalis* from cow milk samples and vaginal swab from human, J Entomol Zool Stud 6: 218-222.

Huys G, D'Haene K, Swings J (2002) Inûuence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method, Lett Appl Microbiol 34: 402-406.

Hummel A, Holzapfel WH, Franz CMAP (2007) Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food, Syst Appl Microbiol 30(1): 1-7.

Iwasaki M, Qi G, Endo Y, Pan Z, Yamashiro T, Andriamanohiarisoamanana FJ, Umetsu K (2019) Quantity changes in *Pseudomonas* species in dairy manure during anaerobic digestion at mesophilic and thermophilic temperatures, J Mater Cycles Waste Manag 21(3): 423-432.

Jahan M, Krause DO, Holley RA (2013) Antimicrobial resistance of *Enterococcus* species from meat and fermented meat products isolated by a PCR-based rapid screening method, Int J Food Microbiol 163(2-3): 89-95.

Jangir K, Mir IA, Saleem T (2021) Virulence characterization of exo-S, exo-U and algD genes and antibiogram atudy of *Pseudomonas aeruginosa* isolated from goat mastitis milk in India, Isr J Vet Med 76(3): 108-115.

Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H (2000) Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci, J Clin Microbiol 38(8): 3092-3095.

Klare I, Konstabel C, Badstübner D, Werner G, Witte W (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*, Int J Food Microbiol 88(2-3): 269-290.

Lambert PA (2002) Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*, J R Soc Med 95(41): 22-26.

Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Duprè I, Sechi LA (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin, Int J Food Microbiol 88(2-3): 291-304.

Maynard C, Bekal S, Sanschagrin F, Levesque CR, Brousseau R, Masson L, Larivie're S, Harel J (2004) Heterogeneity among virulence and antimicrobial resistance gene profiles of extraintestinal *Escherichia coli* isolates of animal and human origin, J Clin Microbiol 42(12): 5444-52.

Melchior MB, Vaarkamp H, Fink-Gremmels J (2006) Biofilms: a role in recurrent mastitis infections, Vet J 171(3): 398-407.

Meng H Liu L, Lan T, Dong L, Hu H, Zhao S, Zhang Y, Zheng N, Wang J (2020) Antibiotic resistance patterns of *Pseudomonas* spp. isolated from raw milk revealed by whole genome sequencing, Front Microbiol 11: 1005.

Nam HM, Lim SM, Moon JS, Kang HM, Kim JM, Jang KC, Kang MI, Joo YS, Jung SC (2010) Antimicrobial resistance of enterococci isolated from mastitic bovine milk samples in Korea, Zoonoses Public Health 5(7-8): e59-e64.

Różańska H, Lewtak-Piłat A, Kubajka M, Weiner M (2019) Occurrence of enterococci in mastitic cow's milk and their antimicrobial resistance, J Vet Res 63(1): 93.

Schauer B, Wald R, Urbantke V, Loncaric I, Baumgartner M (2021) Tracing mastitis pathogens epidemiological investigations of a *Pseudomonas aeruginosa* mastitis outbreak in an Austrian dairy herd, Anim 11(2): 279.

Sekhri I, Chandra M, Kaur G, Narang D, Gupta DK, Arora AK (2021) Prevalence of *Pseudomonas aeruginosa* and other microorganisms from mastitis milk and their antimicrobial resistance pattern, Ind J Anim Res 55(6): 716-721.

Sharma A, Sindhu N (2007) Occurrence of clinical and subclinical mastitis in buffaloes in the state of Haryana (India), Ital J Anim Sci 6(2): 965-967.

Singh K, Chandra M, Kaur G, Narang D, Gupta DK (2018) Prevalence and antibiotic resistance pattern among the mastitis causing microorganisms, Open J Vet Med 8(04): 54.

Stovcik V, Javorsky P, Pristas P (2008) Antibiotic resistance patterns and resistance genes in enterococci isolated from sheep gastrointestinal tract in Slovakia, Bull Vet Inst Puławy 52 (1): 53-57.

Swetha CS, Babu AJ, Rao KV, Bharathy S, Supriya RA, Rao TM (2017) A study on the antimicrobial resistant patterns of *Pseudomonas aeruginosa* isolated from raw milk samples in and around Tirupati, Andhra Pradesh, Asian J Dairy Food Res 36(2): 100-105.

Trzcinski K, Cooper BS, Hryniewicz W, Dowson CG (2000) Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*, *J Antimicrob Chemother* 45(6): 763-770.

Wondrack L, Massa M, Yang BV, Sutcliffe J (1996) Clinical strain of *Staphylococcus aureus* inactivates and causes efflux of macrolides, Antimicrob Agents Chemother 40(4): 992-998.

Yadav R, Chhabra R, Shrinet G, Singh M (2020) Isolation of *Pseudomonas aeruginosa* from bovine mastitic milk sample along with antibiogram study, J Anim Res 10(2): 269-273.

Yang F, Zhang S, Shang X, Wang X, Yan Z, Li H, Li J (2019) Antimicrobial resistance and virulence genes of *Enterococcus faecalis* isolated from subclinical bovine mastitis cases in China, J Dairy Sci 102(1): 140-144.

Indian J Dairy Sci 78(2): 182-186
https://doi.org/10.33785/IJDS.2025.v78i02.011

RESEARCH ARTICLE

Seasonal variation in composition, physicochemical properties and microbial load of raw milk: A comparative study between organized and unorganized dairy farms

Subarna Sarkar[a] (✉), Jitendra Saharia[a], Raj Jyoti Deka[a], Masuk Raquib[b], Purabi Kaushik[c], Papori Talukdar[d], Ajoy Das[e], Ranajoy Choudhury[f]

Received: 20 November 2024 / Accepted: 17 January 2025 / Published online: 23 April 2025

Abstract: Raw milk is a highly perishable agricultural product that plays an important role in the dairy industry and human nutrition. The present study investigated the seasonal variation in the composition, physicochemical properties, and microbial load of cow raw milk in Guwahati, Assam. A total of 144 raw milk samples were collected from organized and unorganized dairy farms in different seasons. In comparison to unorganized farms, organized farms had significantly higher (P<0.01) levels of fat, SNF, protein, lactose, and ash. In comparison to summer milk, winter milk had higher (P<0.05) levels of fat, SNF, protein, and ash except for the lactose content which was lower (P<0.05) in winter. The pH of raw milk was also higher (P<0.01) in the winter. Furthermore, organized farms had a higher (P<0.01) raw milk specific gravity than unorganized farms. Both room and refrigeration storage temperatures caused significantly higher (P<0.01) total viable count (TVC) and coliform count in milk during the summer. In conclusion, the findings of this study illustrated the dynamic nature of raw milk quality in various seasons and farm types. These variations have implications for raw milk quality and safety, emphasizing the need to implement appropriate management practices in dairy farms to maintain high-quality and safe milk throughout the year.

Keywords: Coliform, Milk protein, Seasons, Specific gravity, Subtropical region

Introduction

Seasonal variation in the composition, physicochemical properties and microbial load of cow raw milk is an essential consideration for ensuring food safety and quality, particularly in the context of dairy farms operating in tropical regions. Temperature, humidity, and rainfall can affect the health of dairy cattle, the quality of forage, and the incidence of infections, all of which influence milk composition and microbial load (Oliver and Page 2016; Sahaet al. 2024). The microbial load of raw milk not only shortens its shelf life but also is potentially hazardous to consumers' health if ingested without proper processing and treatment (Terefe and Walelegne, 2024). Additionally, milk's physicochemical attributes such as its acidity, fat content, and total solids, are very important in deciding whether it is suitable to make other dairy products (Coulon et al. 1998; Yasmin et al. 2012; Mohsinet al. 2024). The nutrient content (Fat, protein, lactose, vitamins, and minerals) of milk is frequently highly affected by seasonal changes in environmental factors such as temperature, humidity, and forage availability (Vélez-Terranova et al. 2023). Additionally, factors such as environmental factors, animal health, and milking hygiene practices influence the microbial load of raw milk (Lakew et al. 2019; Terefe and Walelegne, 2024). Therefore, to address the challenges posed by climatic conditions in subtropical areas, understanding seasonal variations in milk quality is crucial.

To our best knowledge, no studies have been conducted on seasonal variation in composition, physicochemical properties and microbial load of raw milk in the subtropical eastern Himalayan region. This comparative study is aimed at developing targeted policies and interventions that can improve dairy farm practices, ensuring safer and higher-quality milk for consumers. Therefore, the main objective of this study was to find out how cow milk differed from organized and unorganized farm management in terms of nutritional composition and other physicochemical properties throughout different seasons.

[a]Department of Livestock Production and Management, College of Veterinary Science, AAU, Khanapara, Guwahati-781 022, India.
[b]Department of Livestock Products Technology, College of Veterinary Science, AAU, Khanapara, Guwahati-781 022, India.
[c]Department of Animal Genetics and Breeding, College of Veterinary Science, AAU, Khanapara, Guwahati-781 022, India.
[d]Department of Animal Nutrition, College of Veterinary Science, AAU, Khanapara, Guwahati-781 022, India.
[e]Livestock Production and Management Section, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, UP- 243 122, India
[f]Division of Bacteriology and Mycology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, UP- 243 122, India

Subarna Sarkar(✉)
E-mail: subarnasarkar856@gmail.com

Materials and Methods

Location and collection of milk samples

The experiment was carried out in the College of Veterinary Science, Assam Agriculture University (AAU), Khanapara, Guwahati, India. A total of 144 samples were collected, with 36 samples each from organized and unorganized farms during both winter and summer seasons, respectively. Raw milk samples were collected from the region of Khanapara and nearby regions. Organized farms had Sahiwal, crossbred of Holstein Friesian and Jersey cows, while unorganized farms predominantly had local indigenous breeds and crossbred of Jersey cows. Milk samples were collected from 3 to 4 cows, mixed with a sterile plunger, and stored in sterile containers. Samples were collected during summer (May to July) and winter (December to February) and transported in iceboxes at 2–8°C to preserve quality. Upon arrival at the laboratory, they were promptly processed and stored under appropriate conditions until analysis.

Analysis of milk sample for composition

Immediately after collection, the milk samples were brought to the laboratory and Quick judging of raw cow's milk, immediately on receipt, was done in an Ultrasonic Milk Analyser (Master Classic, Bengaluru, India). Fat, solids not fat (SNF), Protein, Lactose, and Ash of the raw milk were estimated.

Physicochemical assessment of collected milk samples

The following physicochemical assessment of collected raw milk samples was done during the experimental periods. The pH of milk was determined by using a digital pH meter Model 780 (Metrohm, Switzerland). Tritrable acidity was determined by following the standard method described by Artherton and Newlander (1977). The specific gravity and freezing point raw of milk samples were determined using an Ultrasonic Milk Analyser (Master Classic, Bengaluru, India).

Microbiological assessment

The total viable count (TVC) and the coliform count was done as per the method described by Harrigan and McCance (1976).

Statistical analysis

Statistical analysis was performed using SPSS for the collected data. An analysis of variance was conducted using the general univariate linear model (GLM) and the Scheffe test was used to compare least-square means of significant effects. Significant levels were set at $P<0.05$. To address the wide distribution variation of microbial data, a logarithmic conversion was applied to the TVC and coliform count values (Log_{10}) to achieve normalization. The following linear model was utilized in the statistical analysis:

$$Y_{ijk} = \mu + a_i + b_i + (ab)_{ij} + e_{ijk}$$

Where,

μ = overall mean

a_i = effect of i^{th} farms (i=1,2; organized or unorganized)

b_i = effect of j^{th} seasons (j=1,2; summer or winter)

$(ab)_{ij}$ = interaction between i^{th} farms and j^{th} seasons

e_{ijk} = Random error of observation or residual effect 4 NID $(0, \sigma^2_e)$

Results and Discussion

Milk composition

The interaction between season × farm did not have a significant impact on the average fat percentage of raw milk. However, both season ($P<0.01$) and farm ($P<0.05$) individually affected the fat percentage of raw milk (Table 1). Specifically, within the farms, the organized farm had a higher fat percentage in raw milk compared to the unorganized farm. Additionally, during the winter season, the fat percentage was greater than during the summer season. The SNF percentage was affected by the farm ($P<0.01$). Among the different farms, the organized farm had a higher SNF percentage in raw milk than the unorganized farm (Table 1). The percentage of protein in raw milk was influenced by multiple factors, including the farm ($P<0.01$), the season ($P<0.05$), and the season x farm interaction ($P<0.05$). A higher protein percentage was observed in raw milk from the organized farm compared to the unorganized farm. Further, when considering the influence of the season, the percentage of protein was higher during the winter than during the summer. The fat percentage of raw milk was also influenced by season ($P<0.05$) and farm ($P<0.01$). Among the different farms, the organized farm displayed a higher lactose percentage in raw milk compared to the unorganized farm. Furthermore, during the winter season, the lactose percentage was observed to be lower than during the summer season. The proportion of ash percentage in raw milk remained unaltered by both the season and the interaction between season × farm. However, the ash percentage was significantly impacted by the farm ($P<0.01$). When comparing the various farms, the organized farm demonstrated a greater ash percentage in raw milk in contrast to the unorganized farm.

Studies have demonstrated that milk contains more fat and SNF in the winter than in the summer (Arora and Bhojak 2013; Lakew et al. 2019; Ramadaniet al. 2024). A similar result was observed in the present study. Variations in factors such as temperature, humidity, hygiene, and stress levels in different seasons as well as different types of organized and unorganized farms can affect the cow's overall health and milk production, subsequently

influencing the fat and SNF content (Vélez-Terranova et al. 2023). Protein composition in milk also exhibits seasonal changes (Arora and Bhojak 2013; Bokharaeianet al. 2023). Multiple factors contribute to the variation in protein contents of milk in different farms, including, the cleanliness of cows, the stage of lactation, milk somatic cell count, nutritional factors, and genetic variants of casein (Coulon et al. 1998). Furthermore, seasonal variations impact the lactose content of milk (Yasmin et al. 2012). Studies have revealed that lactose concentrations tend to be higher in milk during the summer months (Arora and Bhojak 2013; Richardset al. 2023). Genetic differences in breeds, different feeding strategies, general management, and environmental conditions might account for this variation in lactose content. Minerals in milk, which contribute to its ash content, are directly related to the diet of the cow (Sirinayakeet al. 2023). In organized farms, high-quality forage is usually available and mineral-rich feed additives and supplements may be administered to the cows, which may result in the intake of more minerals, which can increase milk ash content.

Physicochemical properties

The density of raw milk, as indicated by its specific gravity, did not demonstrate any significant variation due to the season and the interaction between season × farm (Table 1). However, the specific gravity was found to be influenced by the farm ($P<0.01$). Notably, among the farms examined, the organized farm exhibited a higher specific gravity of raw milk compared to the unorganized

Table 1: Seasonal and farm-specific variations in the nutritional composition and physicochemical properties (mean ± SE) of raw milk (n=144)

Parameters	Season (S)	Farm (F)		Season mean	F value		
		Organized	Unorganized		Season	Farm	S × F
Fat (%)	Winter	4.40 ± 0.12^{Aa}	3.92 ± 0.28^{Ab}	4.16 ± 0.32^{A}	$F=18.526^{**}$	$F=7.5023^{*}$	$F=0.0096^{NS}$
	Summer	3.65 ± 0.08^{B}	3.20 ± 0.13^{B}	3.43 ± 0.10^{B}			
	Farm mean	4.03 ± 0.13^{a}	3.56 ± 0.18^{b}	-			
SNF (%)	Winter	9.40 ± 0.10^{a}	8.97 ± 0.26^{b}	9.18±0.15	$F=4.273^{NS}$	$F=17.917^{**}$	$F=2.8003^{NS}$
	Summer	9.33 ± 0.04^{a}	8.33 ± 0.18^{b}	8.83±0.18			
	Farm mean	9.37 ± 0.05^{a}	8.65 ± 0.18^{b}				
Protein (%)	Winter	3.58±0.03	3.55 ± 0.12^{A}	3.57 ± 0.06^{A}	$F=5.3476^{*}$	$F=9.037^{**}$	$F=6.471^{*}$
	Summer	3.60 ± 0.03^{a}	3.20 ± 0.07^{Bb}	3.4 ± 0.07^{B}			
	Farm mean	3.60 ± 0.02^{a}	3.38 ± 0.08^{b}				
Lactose (%)	Winter	5.33 ± 0.03^{Aa}	4.81 ± 0.10^{Ab}	5.08 ± 0.09^{A}	$F=5.615^{*}$	$F=17.575^{**}$	$F=2.126^{NS}$
	Summer	5.42 ± 0.06^{B}	5.17 ± 0.14^{B}	5.30 ± 0.08^{B}			
	Farm mean	5.38 ± 0.04^{a}	4.99 ± 0.10^{b}				
Ash (%)	Winter	0.77±0.02	0.70±0.03	0.73±0.02	$F=0.152^{NS}$	$F=12.273^{**}$	$F=0.152^{NS}$
	Summer	0.77 ± 0.02^{a}	0.68 ± 0.02^{b}	0.73±0.02			
	Farm mean	0.77 ± 0.01^{a}	0.69 ± 0.01^{b}				
Specific gravity	Winter	1.0333±0.0041	1.0321±0.0094	1.0327±0.0052	$F=3.982^{NS}$	$F=10.418^{**}$	$F=1.619^{NS}$
	Summer	1.0329±0.0033	1.0301±0.0062	1.0315±0.0054			
	Farm mean	1.0331 ± 0.0026^{a}	1.0311 ± 0.0062^{b}	-			
Freezing point depression (°C)	Winter	0.64±0.01	0.60±0.03	0.62±0.01	$F=1.844^{NS}$	$F=1.306^{NS}$	$F=2.443^{NS}$
	Summer	0.64±0.00	0.55±0.02	0.59±0.01			
	Farm mean	0.64±0.00	0.58±0.02	-			
pH	Winter	7.06 ± 0.05^{A}	6.88 ± 0.06^{A}	6.97 ± 0.04^{A}	$F=58.962^{**}$	$F=1.939^{NS}$	$F=0.0105^{*}$
	Summer	6.75 ± 0.02^{B}	6.57 ± 0.02^{B}	6.66 ± 0.03^{B}			
	Farm mean	6.90±0.05	6.73±0.06	-			
Titratable Acidity (%)	Winter	0.16 ± 0.01^{A}	0.15 ± 0.01^{A}	0.16 ± 0.01^{A}	$F=162.598^{**}$	$F=0.0063^{NS}$	$F=0.4489^{NS}$
	Summer	0.18 ± 0.02^{B}	0.17 ± 0.01^{B}	0.18 ± 0.01^{B}			
	Farm mean	0.17±0.01	0.16±0.00	-			

Row-wise (a, b) and column-wise (A, B) means with different superscripts differ significantly

**Significant= P<0.01, *Significant= P<0.05, NS= non-significant

farm. The cryoscopic test, measuring the freezing point depression (!) of raw milk, did not exhibit any significant alterations due to the season, farm, and the interaction between season × farm. Nonetheless, the pH was observed to be impacted by the season (P<0.01) and the interaction between season × farm (P<0.01). Within the different seasons, a higher raw milk pH was observed during the winter season compared to the summer season. The titratable acidity was found to be influenced by the season (P<0.05). When comparing different seasons, a lower titratable acidity percentage of raw milk was observed during the winter season in contrast to the summer season (Table 1). As a result of changes in diet and nutrient composition of fodder during different seasons, the fermentation process in the rumen may differ, subsequently impacting the pH of the milk (Ponnampalamet al. 2024). Furthermore, environmental factors like temperature and humidity also affect the pH of raw milk (Estremadoyroet al. 2024); which has also been similarly observed in our study. Microbial activity in milk increases during warmer seasons, potentially affecting pH levels. The titratable Acidity (%) of raw milk has been observed to be higher in the winter than in the summer. Different forage availability and composition throughout the year can impact the microbial activity in the cows' rumen, subsequently affecting the production of volatile fatty acids (Butler et al. 2008), which in turn influence the titratable acidity of milk.

Microbial load

The total viable count (TVC) of raw milk on the day of collection (day 0 at room temperature) remained unaffected by the interaction between season × farm. However, it was influenced by both the season (P<0.01) and the farm (P<0.01). On the other hand, the TVC of raw milk on day 1 (at refrigeration temperature) was only influenced by the season (P<0.01). During both day 0 and day 1, a lower TVC of raw milk was observed during the winter season in comparison to the summer season (Table 2). Furthermore, among the different farms, the organized farm displayed a lower TVC of raw milk on the day of collection (day 0) when compared to the unorganized farm (Table 2). The coliform count of raw milk on day 0 (at room temperature) and day 1 (under refrigeration) exhibited no significant influence from the farm and the interaction between season × farm. However, the season had a significant (P<0.01) impact on the coliform count. It was observed that coliform counts in raw milk were lower during the winter season compared to the summer season, both on day 0 and day 1 (Table 2). The microbial load of milk is influenced by a variety of factors, such as healths, controlled feeding and housing conditions, and hygiene practices used during the milking process, storage conditions, and the overall microbial environment (Terefe and Walelegne, 2024). The warmer temperatures create an ideal condition for the rapid growth of bacteria, coliforms and other microorganisms present in the milk (Oliver and Page 2016; Sahaet al. 2024), leading to an increase in the total viable count as well as coliform count.

Table 2: Seasonal and farm-specific variations in microbial load (mean ± SE) of milk at different storage temperatures (n=144)

Parameters	Days	Seasons (S)	Farm (F)	Season mean	F value			
			Organized	Unorganized		Season	Farm	S × F
TVC (log10) cfu/ml	Day 0 (Room temp.)	Winter	5.03 ± 0.07^{Aa}	4.66 ± 0.10^{b}	4.84 ± 0.08^{A}	F=8.9096**	F=36.247**	$F=3.5329^{NS}$
		Summer	5.46 ± 0.10^{Ba}	4.75 ± 0.09^{b}	5.11 ± 0.12^{B}			
		Farm mean	5.25 ± 0.09^{a}	4.70 ± 0.05^{b}	-			
	Day 1 (Refg. Temp.)	Winter	4.10±0.31	3.91 ± 0.22^{A}	4.00 ± 0.18^{A}	F=9.7826**	$F=0.7137^{NS}$	$F=0.0171^{NS}$
		Summer	4.67±0.05	4.53 ± 0.03^{B}	4.60 ± 0.03^{B}			
		Farm mean	4.38±0.17	4.22±0.14	-			
Coliform (log10) cfu/ml	Day 0 (Room temp.)	Winter	3.07 ± 0.23^{A}	2.53 ± 0.26^{A}	2.80 ± 0.18^{A}	F=48.4895**	$F=2.2046^{NS}$	$F=0.2392^{NS}$
		Summer	4.85 ± 0.36^{B}	4.58 ± 0.23^{B}	4.71 ± 0.21^{B}			
		Farm mean	3.96±0.33	3.55±0.35	-			
	Day 1 (Refg. Temp)	Winter	2.20 ± 0.28^{A}	2.06 ± 0.22^{A}	2.13 ± 0.17^{A}	F=97.9951**	$F=3.6907^{NS}$	$F=0.3138^{NS}$
		Summer	4.67 ± 0.39^{B}	4.53 ± 0.31^{B}	4.60 ± 0.16^{B}			
		Farm mean	3.43±0.43	3.36±0.38	-			

Row-wise (a, b) and column-wise (A, B) means with different superscripts differ significantly
**Significant= P<0.01, *Significant= P<0.05, NS= non-significant

Conclusion

The findings of this study indicate significant differences in microbial load, physicochemical properties, and nutritional composition of raw milk between seasons and farm types. These variations have implications for raw milk quality and safety, emphasizing the need to implement appropriate management practices in dairy farms to maintain high-quality and safe milk throughout the year. Policymakers can use this information to promote the adoption of better farm management techniques, provide training for dairy farmers, and implement regulations that standardize milk production processes across both organized and unorganized farms. Further research and intervention are needed to enhance quality control in dairy farms in the Eastern Himalayan regions.

Acknowledgment

The authors thankfully acknowledge Assam Agricultural University (AAU), Khanpara, Assam, India, for providing the necessary facilities to conduct the research.

References

Arora R and Bhojak N (2013) Physiochemical and environmental factors responsible for change in milk composition of milking animal. Int J Eng Sci Techno 2: 275-277

Atherton HV and Newlander JA (1977) In Chemistry and testing of dairy products. 4th ed., AVI Publishing Company, Inc. west Port, Connecticut

Bokharaeian M, Toghdory A, Ghoorchi T, Ghassemi Nejad J and Esfahani IJ (2023) Quantitative associations between season, month, and temperature-humidity index with milk yield, composition, somatic cell counts, and microbial load: a comprehensive study across ten dairy farms over an annual cycle. Anim 13(20): 3205

Butler G, Nielsen JH, Slots T, Seal C, Eyre MD, Sanderson R and Leifert C (2008) Fatty acid and fat soluble antioxidant concentrations in milk from high and low input conventional and organic systems: seasonal variation. J Sci Food Agric 88: 1431-1441

Coulon JB, Hurtaud C, Rémond B and Verite R (1998) Factors contributing to variation in the proportion of casein in cows' milk true protein: a review of recent INRA experiments. J Dairy Res 65: 375-387

Estremadoyro LJG, Salome PH, Carhuas JN, Guzman SO, Tacza AA, Guillen MAF and Garcia-Olarte E (2024) Effects of different seasons on milk quality: A study on two cattle breeds in rainy and drought contexts. World's Vet J 14(2): 213-219.

Harrigan WF and McCance ME (1976) Laboratory methods in Food and Dairy Microbiology. Academic press Inc., London

Lakew A, Goshu G, Mengistu A, Mamo G and Demissie T (2019) The Effect of Different Seasons on the Milk Quality in Central Highlands of Ethiopia. Glob Vet 21: 77-81

Mohsin AZ, Hui Ci N, Ismail AR, Marzlan AA, Abd Rahim MH and Meor Hussin AS (2024) Gouda cheese with different coagulants and types of milk: physicochemical, biochemical, microbiological, and sensory properties. J Food Meas Charact 18(2): 1065-1074.

Oliver DM and Page T (2016) Effects of seasonal meteorological variables on E. coli persistence in livestock faeces and implications for environmental and human health. Sci Rep 6: e37101

Ponnampalam EN, Priyashantha H, Vidanarachchi JK, Kiani A and Holman BW (2024) Effects of nutritional factors on fat content, fatty acid composition, and sensorial properties of meat and milk from domesticated ruminants: an overview. Anim 14(6): 840

Ramadani X, Kryeziu A, Kamberi M and Zogaj M (2024) Influence of the farm location and seasonal fluctuations on the composition and properties of the milk. Agron Res 22(1): 238-252

Richards AT, Knapp JR, Summer P, Ohta Y and Boerman JP (2023) Bioavailability of a novel rumen protected methionine supplement and its effect on milk production and body composition in dairy cows. Anim Feed Sci Technol 304: 115750.

Saha S, Majumder R, Rout P and Hossain S (2024) Unveiling the significance of psychrotrophic bacteria in milk and milk product spoilage-A review. The Microbe e100034.

Sirinayake Lokuge GM, Johansen M, Lund P, Larsen LB and Poulsen NA (2023) Effects of proportion and digestibility of grass-clover silage on milk protein, mineral and fatty acid compositions in Danish Holstein cows. Acta Agric Scand Sect A Anim Sci 72(3-4): 123-134

Terefe G and Walelegne M (2024) Effect of feeds and hygienic practices on milk production and its nutritional and microbiological quality. CABI Rev (2024)

Vélez-Terranova M, Campos Gaona R, Salamanca-Carreño A, Velasco Daza RA, Arenas Rodríguez BA and Chaparro-Ortegón JS (2023) Environmental Factors That Affect the Sanitary and Nutritional Variability of Raw Milk in Dual Purpose Livestock Systems of Colombian Orinoquia. Animals. 13: 1385-1389

Yasmin A, Huma N, Butt MS, Zahoor T and Yasin M (2012) Seasonal variation in milk vitamin contents available for processing in Punjab, Pakistan. Saudi So. Agric Sci 11: 99-105

Indian J Dairy Sci 78(2): 187-191
https://doi.org/10.33785/IJDS.2025.v78i02.012

RESEARCH ARTICLE

Epidemiological diversity and diagnostic accuracy of cow-side test for subclinical mastitis in cows of Braj region of India

Shubhangi Choudhary[1], Alok Kumar Chaudhary[1]*, Sanjay Kumar Bharti[2], Ruchi Tiwari[3] and Nisha Chaudhary[1]

Received: 05 January 2024 / Accepted: 18 May 2024 / Published online: 23 April 2025

Abstract: The aim of this study was to determine the prevalence of subclinical mastitis in native breeds of cows in the Brij region of Mathura and to assess the accuracy of the cow-side test. Milk samples were taken from selected population and screened through California mastitis test. Positive and ambiguous findings were further tested for Somatic cell count. The overall clinical mastitis prevalence was 55.83%. Organized farms had a lower prevalence of 44.12%, while unorganized farms exhibited a significantly ($p<0.05$) higher prevalence of 71.15%, indicating epidemiological diversity. In the context of two commonly reared breeds, Sahiwal cows showed a higher prevalence at 61%, in contrast to Haryana cows with a prevalence of 49%. Group with a paroty of 3-5 demonstrated the highest prevalence at 63.7%. Diagnostic tests, including the California mastitis test (CMT) and Somatic cell count (SCC), unveiled variations in prevalence rates.

Keywords: SCC, Udder health, milk and fat, sensitivity, accuracy

Introduction

Subclinical mastitis is the most frequently occurring form of mastitis in dairy cattle where there are no visible signs of abnormalities in the milk or udder (Radostits et al. 2007). It is detected through silent evidences of some inflammatory indicators such as a high somatic cell count level above to 200,000 cells/ ml (Satu ,2003) , increased serum proteins due to cellular damage in the udder tissue (Hussein et al. 201) and alterations in milk pH due to efflux of bicarbonates of serum (Qayyum et al. 2016 and Sani , 2021) . Diagnosing and managing of subclinical mastitis in dairy cattle still remains a significant challenge, because of its elusive nature also posing a potential source of infection for healthier animals (Abebe et al. 2016 and Ruegg 2017). Apart from these, the milk from antibiotic-treated animals with subclinical mastitis poses a dual risk, carrying both a potential zoonotic threat and the presence of antibiotic residue. In the One Health concept, it is necessary to map subclinical mastitis with a holistic approach because it directly impacts animal health, human health, and the economy of farmers as well as the country. Several contributing factors involves such as animal-related aspects like host age, breed, lactation level and parity, along with environmental factors such as temperature, humidity, human interactions and hygiene (Almaw et al. 2008; Doherr et al. 2007; Karimuribo et al. 2008; Madut et al. 2009). The most commonly reported contagious pathogen is *Staphylococcus aureus* and the environmental pathogen *Escherichia coli* (Cheng et al. 2020) Considering these factors, this study aims to assess the holistic diversity and accuracy of commonly used diagnostic tests for subclinical mastitis in lactating cows in the Brij region of India. By investigating epidemiological aspects, we strive to enhance understanding diversity of subclinical mastitis in dairy animas and insights on its diagnosis tools. These findings might be helpful to develop suitable preventive measure in this specific geographical area.

Materials and Methods

Location and geography of study area

Mathura, is an ancient district in the north-central part of Uttar Pradesh state, India, situated between Lat. 27°14' and 27°58' N and Long. 77°17' and 78°12' E, hold a renowned legacy for cow rearing associated with Lord Krishna. The region is currently overpopulated with indigenous cattle. The climatic conditions in the area exhibit variable temperature and humidity, averaging 30.6°C and 86.7%, respectively, throughout the year.

[1] Department of Veterinary Medicine, College of Veterinary & Animal Sciences, DUVASU Mathura, U.P., INDIA.
[2] Department of Livestock Production and Technology, College of Veterinary & Animal Sciences, DUVASU Mathura, U.P., INDIA
[3] Department of Veterinary Microbiology, College of Veterinary & Animal Sciences, DUVASU Mathura, U.P., INDIA

(✉)Alok Kumar Chaudhary
Email: drvetalok@gmail.com

Epidemiological study:

Population and sample collection

The selected population comprised 57 cows of Haryana and 63 cows of Sahiwal breeds from various age groups, all with multiple parities and over 30 days of lactation. Sixty-eight cows were sourced from the organized farm LFC, and 52 were sourced from Gaushalas in Mathura District. A total of 480-quarter milk samples were aseptically collected from 120 lactating cows and tested for the presence of subclinical mastitis using California Mastitis Test (CMT and somatic cells count. After discarding the first few milk squirts, 15 ml milk sample was collected in a sterile plastic bottle in a clean environment from each individual cow's quarters and leveled properly from randomly selected population.

Assessment of subclinical mastitis

Cow-side assessment

All the 120 samples examined at cow-side by used of California Mastitis Test (CMT) with modified Scandinavian scoring system 0 to 4. In the scoring system, 0 is negative (no gel formation), 1 is trace (possible infection), and 2 or 3 indicates a positive result and 4 has the thickest gel formation. A sample was considered as positive to subclinical mastitis when one or more quarters with more than 2 score were detected a Schukken, et al. 2003.

Laboratory assessment

The somatic cell count of milk samples were done using an automatic somatic cell counter (Lacto scan SSC, Milkotronic Ltd.; Nova Zagora, Bulgaria) in accordance with the manufacturer's manual and method in the literature (Salvador et al. 2014). The SCC is quantified as the number of cells per ml of milk. All the collected samples having CMT score 0 and 1,2, 3 and 4 were tested of individual cows were categorized having SCC of 100,000 or less indicates an 'uninfected' cow and cattle having SCC of 200,000 would determine whether a cow is infected with mastitis. Cows with a result of greater than 200,000 are highly likely to be infected on at least one quarter (Salvador et al. 2014)

Assessment of diagnostic accuracy

Data of both the test statistically tabulated as true positive, false positive, false negative (Table-4). The values of sensitivity, specificity, positive prediction, negative prediction and accuracy were calculated by using following formula:

a) Sensitivity = True positive/True positive + False negative

b) Specificity = True negative/True negative + False positive.

c) Positive predictive value = True positive/True positive + False positive.

d) Negative predictive value = True negative/True negative + False negative.

e) Accuracy = True negative/Total value

Results and Discussion

The overall prevalence of subclinical mastitis in indigenous cows of Brij region, Mathura district, was 45% based on cow-side California mastitis test, while laboratory analysis showed 55.83% positivity in somatic cell count (Fig. 1).Similarly, prevalence of subclinical mastitis also have been reported by Birhanu et al. (2017), Reddy et al. (2015), and Badiuzzaman et al. (2015) .

Grading of bovine sub-clinical mastitis

Based on cow side test, out of 120 lactating cows 66 cows were found CMT negative, 10 were identified as weakly positive (+),14 were distinctly positive (++) and 30 were diagnosed as strongly positive (+++) for sub-clinical mastitis (Table). However, laboratory analysis of somatic cell counts revealed that 67 milk samples found positive and having more than 2 lakhs SCC per ml of milk. Among these, 26 samples showed more than 10 lakhs/ml, 30 samples were ranges in 5-10 lakhs/ml and 11 samples were range lower range of somatic cell count 2-5 lakhs/ml (Table 2)

Epidemiological diversity

Based on somatic cell count epidemiological diversity of SCM in unorganized farm was significantly ($p<0.05$) higher as compared to organized farm as 71.15% and 44.12% respectively (Table -1). The higher infection rate in unorganized farms potentially influenced by various detrimental factor such as nutritional imbalance, poor hygiene, lack of awareness and improper ways to mange others farm practices. Cheng et al. (2020),Bangar et al. (2015), Krishnamoorthy et al. (2021), Khan et al. (2021) and Mbindyo et al. (2020).

Among, two most commonly indigenous breed of cows in brij region, subclinical mastitis was detected higher in Sahiwal breed compared to the Haryana breed as recorded with prevalence of 61% and 49% respectively but it was non-significant (Table-2). It

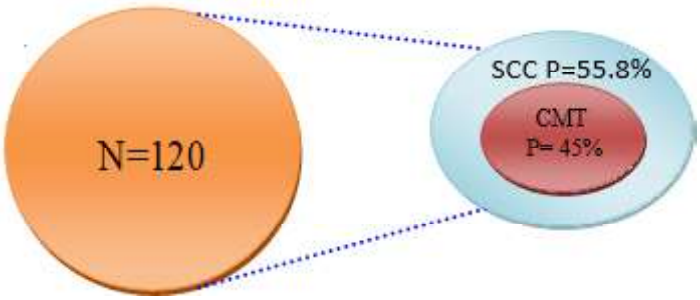


Fig1 -: Overall Status of sub-clinical mastitis in lactating cow in brij region of Mathura

might be due to the phenotypic characteristic of udder that mostly having pendulous and large udder and synthesis more milk as

Table: 1 Overall Status of sub-clinical mastitis in indigenous cattle

S.N.	Overall Status of Subclinical mastitis in indigenous dairy cattle	
1	Total Number of cows (N)	120
2	Total Positive of cows (P/%)	67/ 55.83%

Table-2 Status of subclinical mastitis based on Farming

S.N	Type of Breed	Total cow	Positive cow (N)	Positive cow (%)	(p>0.05)
1	Sahiwal	63	39	61.90	$X^{2=}$1.738
2	Haryana	57	28	49.12	*P* =0.18
	Total	120	67	55.83	

Table3: Status of subclinical mastitis based on breed of population

S.N	Parity	Total cow	Positive cow (N)	Positive cow (%)	(p<0.05)
1	3-5	80	51	63.75	$X^{2=}$6.099*
2	More than 5	40	16	40	*P*=.013
	Total	120	67	55.83	

Table: 4 Status of subclinical mastitis based on Parity

S.N	Parity	Total cow	Positive cow (N)	Positive cow (%)	(p<0.05)
1	3-5	80	51	63.75	$X^{2=}$6.099*
2	More than 5	40	16	40	*P*=.013
	Total	120	67	55.83	

Table-5: Comparative analysis CMT and SCC against subclinical mastitis

Comparative study of CMT and SCC test				
Test	Scoring system	Positive cow (N)	Positive cow (%)	Total (N/%)
CMT	+	10	18.52	54/ 45%
	++	14	25.93	
	+++	30	55.56	
SCC	* (2- 5 lakhs)	11	16.42	67/ 55.83%
	**(5- 10 lakhs)	30	44.78	
	***(< 10 lakhs)	26	38.81	

Accuracy of diagnostic test			
True positive	False positive	False negative	True negative
48	6	13	53

Comparative Diagnostic accuracy of CMT		(95% CI)
Sensitivity	78.69%	66.32% to 88.14%
Specificity	89.83%	79.17% to 96.18%
Positive Likelihood Ratio	7.74	3.58 to 16.70
Negative Likelihood Ratio	0.24	0.15 to 0.39
Disease prevalence (*)	55.83%	
Positive Predictive Value (*)	90.72%	81.92% to 95.48%
Negative Predictive Value (*)	76.93%	67.14% to 84.48%
Accuracy (*)	83.61%	75.75% to 89.74%

Table6: Status of subclinical mastitis based on affected udder and teats of cow

Udder Morphology		Status of SCM			
Shape of udder	Total	Cow negative	Cow positive (N)	Cow positive (%)	(p>0.05)
Pendulous	50	16	34	68.00	
Bowl	33	18	15	45.45	
Round	37	19	18	48.65	
Total	120	53	67	55.83	$X^{2=}5.217$ p=.073
Teat Morphology			Status of SCM		
Teat Shape	Total	Cow negative	Cow positive (N)	Cow positive (%)	(p>0.05)
Bottle	90	40	50	55.5	$X^{2=}85.27$ P=.001
Cylindrical	250	80	170	68	
Funnel	140	65	75	53.5	
Total	480	185	295	61.4	

well also lead physiological stress, it allowed to easily contacting to environmental opportunistic.

Among all the three parity groups, 3-5 was one of significantly ($p<0.05$) affected parity group with the highest 63.7% prevalence rate (Table-3). Lactating animals under this range, having fully grown udder that optimized our peak milk production. However, within the same age group, animals experienced more udder stress and possibility to breakdown the teats canal barriers and udder parenchyma tissue with progressive ageing the body's immune system undergoes a transition, adapting to shifts in various patho-physiological pathways from adulthood to old age. Similarly, the results and facts behind it agreed with (Cheng et al. (2020);Lakew et al. (2009) and Badiuzzaman et al.(2015).

Statistical validation of diagnostic test against subclinical mastitis

Statistical validation of the tests showed that the sensitivity, specificity, and diagnostic accuracy of the CMT test against SCC were 78.69%, 89.83%, and 83.61%, respectively. (Table - 4).These values are consistent with the findings of Kandiwa et al. (2017); Badiuzzaman et al. (2015) and Sumon et al. (2020), who reported sensitivity of CMT as 80.08% and specificity as 69.40%, with diagnostic accuracy ranging from 87.1% to 97.4%.

Conclusion

In the present study, overall prevalence rate of subclinical mastitis was found slightly higher, in indigenous cows of the Brij region in Mathura district. Unorganized farms exhibited significantly higher SCM prevalence compared to organized farms likely influenced by factors such as nutritional imbalance and poor hygiene. Sahiwal breed showed a higher subclinical mastitis rate compared to Haryana breed possibly due to phenotypic characteristics. Animals in the 3-5 parity group had the highest prevalence reflecting the challenges of udder stress and tissue breakdown with aging .This study also concluded that CMT test, demonstrated low sensitivity of 78.69%, specificity of 89.83%, and diagnostic accuracy of 83.61% against SCC. It indicates that screening criteria should be based on laboratory analysis rather than cow side and furthermore develop advance cow side test based on modern technology such as dye, markers or sensor with higher accuracy.

Acknowledgements

We would like to express our sincere gratitude to all the competent authorities of LFC/TVCC, and other competent authorities of DUVASU, Mathura for their support and cooperation and guidance throughout this study.

References

Abebe R, Hatiya H, Abera M, Megersa B, Asmare K (2016) Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed South Ethiopia. BMC Vet Res 121: 1-11

Almaw G, Zerihun A, Asfaw Y (2008) Bovine mastitis and its association with selected risk factors in small holder dairy farms in and around Bahir Dar, Ethiopia. Anim Health Prod 40: 427-432

Badiuzzaman M, Samad, M A, Siddiki S, Islam MT, Saha S (2015) Subclinical mastitis in lactating cows: Comparison of four screening tests and effect of animal factors on its occurrence. Bangladesh J Vet Med 13(2): 41-50.

Birhanu M, Leta S, Mamo G, Tesfaye S(2017) Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. BMC Resh Notes 10(1): 1-6

Cheng WN, Han SG (2020) Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments-A review. Asian-Australas J Anim Sci 33(11): 1699-1713

Doherr MG, Roesch M, Schaeren W, Schallibaum M, Blum JW (2007)Risk factors associated with subclinical mastitis in dairy cows on Swiss organic and conventional production system farms. Veterinarni Medicina-praha 52(11): 487

Kandiwa E, Iraguha B, Mushonga B, Hamudikuwanda H, Mpatswenumugabo J P (2017) Comparison of cow-side diagnostic tests for subclinical mastitis of dairy cows in Musanze district, Rwanda. J South African Vet Association 88(1): 1-6

Kathiriya J, Kabaria B, Saradava A, Sanepara Y (2014) Pervalence of subclinical mastitis in dairy cows in Rajkot district of Gujarat. Int J Sci Nature 5: 35-43

Khan I, Javaid A (2021) Identification of biologically important compounds in neem leaves through GC-MS analysis. Jordan J Pharmaceutical Sci 14(3)

Krishnamoorthy P, Goudar AL, Suresh KP, Roy P (2021) Global and countrywide prevalence of subclinical and clinical mastitis in dairy cattle and buffaloes by systematic review and meta-analysis. Res Vet Sci 136: 561-586

Lakew M, Tolasa T, Tigre W (2009) Prevalence and major bacterial causes of bovine mastitis in Asella,South Eastern Ethiopia. Tropical Anim Health Prod 41: 1525-1530

Madut NA, Godir AEA,El-Jalil I M (2009) Host determinants of bovine mastitis in semi-intensive production system of Kharfoum State, Sudan. J Cell Animal Biol 3: 71-77

Mbindyo CM, Gitao GC, Mulei CM (2020) Prevalence, etiology, and risk factors of mastitis in dairy cattle in Embu and Kajiado Counties, Kenya. Vet Med Int (1)

Pyorala S(2003)Indicators of inflammation in the diagnosis of mastitis. Vet Res 34 (5): 565-578

Radostitis O, Gay C, Hinchcliff K and Constable P (2007) Veterinary medicine. A text book of the diseases of cattle, horses, sheep, pigs and goats. 10th Edn. Saunders Elsevier

Reddy BS, Shobhamani B, Sreedevi B, Kumari KN, Reddy YR (2015)Diagnosis of subclinical mastitis in cross bred cattle. Res Rev J Vet Sci Technol 4: 39-43

Salvador RT, Soliven RL, Balagan EJY, Abes NS, Gutierrez CA, Mingala CN(2014) Evaluation of a portable somatic cell counter in the diagnosis of bubaline subclinical mastitis. Thai J Agric Sci 47(4): 205-209

Sumon SM, Parvin MS, Ehsan MA, Islam MT(2020) Relationship between somatic cell counts and subclinical mastitis in lactating dairy cows. Vet World 13(8):170

Sani N, (2021) Alteration of milk ph, somatic cell count (scc), lactate dehydrogenase (ldh) and alkaline phosphatase (alp) activities in buffalo milk related to udder health status Buffalo Bull 40 (3): 443-450

Hussein HA, Abd El-Razik KA, Gomaa AM, Elbayoumy MK, Abdelrahman KA, Hosein HI (2018) Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle, Vet World 11(1): 34-41

Indian J Dairy Sci 78(2): 192-199
https://doi.org/10.33785/IJDS.2025.v78i02.013

RESEARCH ARTICLE

Impact of Dairying on Livelihood Security of Farmers in Aspirational Districts of Karnataka

Abhishek, K.M[1] Somasekaran Subash[2] (✉), Devi, M.C.A[3] and Muniandy Sivaram[4]

Received: 30 September 2024 / Accepted: 25 October 2024 / Published online: 23 April 2025

Abstract: This study examines the socio-economic impact of dairying on the livelihood security of farmers in the Aspirational districts of Karnataka, viz., Raichur and Yadgir districts. Dairying is a vital component of rural economies, contributing significantly to food security and poverty alleviation. Livelihood Security Index (LSI) was constructed to assess the differences between dairy and non-dairy farmers across seven dimensions; food and nutritional security, economic security, health security, educational security, social security, institutional security, and infrastructural security. Findings indicate that dairy farmers enjoy higher LSI compared to their non-dairy counterparts, attributed to consistent income generation and better integration into support systems. The study also highlights essential management practices adopted by dairy farmers, including housing, feeding, health care, breeding, and milking techniques. These practices were crucial for enhancing productivity and profitability in the dairy sector. The research underscores the importance of promoting dairying as a sustainable livelihood strategy to improve rural livelihoods of the farmers especially in Aspirational districts of our country.

Keywords: Socio-economic impact, Livelihood Security, Dairying, Aspirational District

[1,2, 3,4] Southern Regional Station
ICAR-National Dairy Research Institute
Bengaluru, Karnataka 560 030, India
(✉)Somasekaran Subash
Email: *subashagri@gmail.com

Introduction

As an agrarian nation, India relies heavily on agriculture and allied sectors, with the livestock sector contributing significantly to the national economy (Singh et al. 2020). The dairy industry alone accounts for 4.11% of the national GDP and 25.60% of the agricultural GDP (Chadda et al. 2022). Karnataka ranks 8th in milk production, contributing 5.56% to the nation's total milk output (DAH&D, 2023). With a bovine population of 114.4 lakh, the state's dairy sector is primarily supported by small-scale farmers who manage less than two hectares of land and own one to four dairy animals (DAH&D, 2019). This sector not only ensures food security for millions of rural households but also acts as a buffer against poverty and economic instability (Rodríguez et al. 2016).

The concept of livelihood encompasses a range of activities and resources that individuals or households utilize to secure their basic needs (Frankenberger, 1996). Livelihood security is defined as having adequate and sustainable access to income and resources to meet these needs while managing risks associated with various uncertainties (Chambers and Conway, 1992). In rural areas, particularly in aspirational districts where the agricultural landscape is fraught with challenges such as fluctuating market prices, depleting natural resources, and climate variability, dairying emerges as a viable alternative for enhancing livelihood security (Lazard and Youngs, 2021). The Government of India's Aspirational Districts Programme (ADP), launched in 2018, aims to uplift underdeveloped regions by improving living standards through integrated development initiatives across various sectors, including agriculture.

The present study investigates the impact of dairying on the socio-economic profile of dairy farmers in Karnataka's aspirational districts. It aims to document the current status of dairy farming, to analyse its role in enhancing livelihood security, and to identify the challenges faced by dairy farmers. By focusing on both dairy and non-dairy farmers in Raichur and Yadgir, the research seeks to provide insights into their socio-economic conditions and how dairying contributes to their livelihoods. The findings will be instrumental for policymakers and stakeholders in formulating effective strategies that promote sustainable dairy farming

practices and improve the overall economic stability of rural households.

This study delves into seven key indicators of livelihood security namely income stability, food security, health access, education opportunities, asset ownership, social capital, and risk management capabilities. By exploring these dimensions, the research aims to highlight how dairying not only serves as a source of income but also enhances overall quality of life for farmers and their families. Overall, this paper underscores the importance of dairying in securing livelihoods for farmers in Karnataka's aspirational districts. It emphasizes that enhancing support for this sector can lead to significant improvements in socio-economic conditions for rural households. Through targeted interventions and policy support, dairying can play a pivotal role in alleviating poverty and fostering sustainable development in these aspirational districts regions.

Materials and methods

Sampling plan: The present study was conducted during the year 2022 in the Aspirational districts of Karnataka state viz., Raichur and Yadgir Districts. The districts were chosen purposively based on the Government of India's Aspirational Districts Programme (ADP) by NITI Aayog. Two blocks from each district were selected randomly, i.e. Raichur and Sindhanur blocks of Raichur; Surpur and Shahapur blocks of Yadgir. A Cluster of five villages from each block were randomly selected. Thus, a total of 20 villages were selected for the study. Respondents for this study are dairy farmers who had at least one dairy animal at the time of investigation and non-dairy farmers who are not active in dairy farming. A total of, 50 respondents from each block were selected randomly, among those 25 respondents are dairy farmers and remaining 25 are non-dairy farmers. Thus, a total of 200 respondents from two aspirational districts were selected for the study.

***Measuring dairy management practices*:** For measuring the management practices of dairy farming, adopted, by the respondents, a list of practices regarding housing, feeding, health care, breeding, milking and general management practices was prepared by referring the published literatures and by consulting the experts in the field. The respondents were categorized based on the frequency and percentage.

Construction of LSI: The Livelihood Security Index (LSI) was developed in the present study to evaluate the impact of dairy farming on the livelihoods of respondents. By comparing dairy farmers with non-dairy farmers, the study aimed to assess the significance of dairy farming in securing livelihoods within the research area. Through a review of various studies on the Livelihood Security Index (LSI), a comprehensive framework was developed consisting of seven key components: food and nutritional security, economic security, health security, educational security, social security, institutional security, and infrastructural security.

The construction of a Livelihood Security Index (LSI) hinges on assigning weights (scale values) to the seven key components of LSI. These weights reflect the perceived importance of each component in determining a Livelihood Security of respondents. The Normalized Rank Order Method, developed by Guilford (1954), provides a structured approach for this weighting process. The 90 judges were asked to rank the seven components based on their perceived importance in assessing the livelihood security of respondents. Out of the initial 90 judges selected for the study , 38 responded. After a thorough evaluation, 6 responses were excluded due to inconsistencies or incomplete data. The remaining 32 responses were used for further analysis. Below formula was used to calculate proportions (p-values) for each rank assigned by the judges. This formula considers the assigned rank and the total number of components being ranked (7). Finally, for each dimension, a 'scale value' was obtained by multiplying the frequency of each rank by its corresponding C-value and then summing these products. The sum was then divided by the total number of judges (32). This process resulted in a unique scale value for each livelihood security component, reflecting its relative importance in the overall LSI, which is presented in Table 1.

$$p = [(Ri-0.5)*100]n$$

Where, Ri = stands for the rank value of the dimension i in the reverse order as 7 to 1, n indicates the number of dimensions ranked by the judges.

Ensuring the validity and reliability of the vulnerability index is crucial. To achieve this, a critical step called item analysis and relevance test was conducted. The judges were asked to evaluate the relevance of each indicator using a three-point scale: 'Most Relevant' (3 points), Relevant' (2 points), and 'Least Relevant' (1 point). This process helped to assess the importance of each indicator in the context of the index. Two key metrics were calculated for each indicator: Relevancy Weightage (RW) and Mean Relevancy Score (MRS). These metrics helped to determine which indicators should be included in the final index.

Each dimension of LSI consists of various number of indicators and therefore, their range of total scores were different. Hence, total score of each dimension was converted into unit score by using simple range and variance as given below.

$$Uij = (Yij - Min Y\_ij) / (Max Y\_j - Min Y\_j)$$

Where, Uij = Unit score of the ith respondents on jth dimension, Yij = Value of the ith respondent on the jth dimension, Max Yj = Maximum score on the jth dimension, Min Yj = Minimum score on the jth dimension

Thus, the score of each dimension will be ranging from 0 to 1 i.e. when Yij is minimum, the score is 0 and when Yij is maximum the score is 1. Then, the unit scores of every respondent will be multiplied by respective scale value of each dimension and summed up. Thus, the score obtained was divided by the sum of scale values in order to get the LSI for each respondent.

LSIi= Σ Uij*Sj/ Sum of scale values

Where, LSIi =Livelihood Security Index of ith respondent, Uij = Unit score of the ith respondent on jth component, Sj = Scale value of the jth component

***Propensity Score Matching (PSM) technique*:** Propensity score matching technique (Rosenbaum and Rubin 1983) was employed for comparing dairy farmers and non-dairy farmers with respect to overall livelihood security. This technique and its application to the present study are explained below.

Estimation of Propensity Score (PS) value: The PS for each dairy farmer was calculated using a logistic regression model. This model predicts the probability that a farmer is a dairy farmer, given their specific characteristics (Xi). The PS is calculated as P(Xi) = Pr (Di = 1 | Xi), where Di indicates whether the individual is a dairy farmer (Di=1) or not (Di=0). To calculate the PS, both dairy farmers and non-dairy farmers were included in the analysis. The factors (Xi) that influence the livelihood security of both groups were used as covariates in the logistic regression model.

Matching of PS: To ensure that the dairy farmers and non-dairy farmers have similar characteristics, they were matched based on their PS. This means that dairy farmers were paired with non-dairy farmers who had similar probabilities of being dairy farmers. The kernel-based matching method was used to find the best matches between the two groups.

Assessment of matching quality: The balance requirement will be evaluated to determine if there are any statistically significant differences between the two groups after resampling the data. This is done to ensure that the matching procedures successfully balanced the data and created a randomized experimental design like effect.

Calculation of average treatment effect (ATT): The ATT and implication of dairy farming on the control and treated groups after matching will be compared.

$$ATT = E[Y1i - Y0i \mid Di = 1] = E\{E[Y1i - Y0i \mid Di = 1, p(Xi)]\} = E\{E[Y1i \mid Di = 1, p(Xi)] - E[Y0i \mid Di = 0, p(Xi)] \mid Di = 1\}$$

Here, Y1i and Y0i represent overall livelihood security of the sample dairy farmers in the treated group and non-dairy farmers in the control group, respectively.

Index gap analysis with respect to different groups: Index gap analysis (% ΔIg) was made for comparing the overall livelihood security index value of dairy and non-dairy farmers of the study area.

Index gap (%ΔIg) = (Index value of dairy farmers- Index value of non-dairy farmers)/(Index value of dairy farmers) x 100

Results and Discussion

***Socio-economic profile*:** The study from Table 2 found that 72 per cent of dairy farmers belonging to middle to old age compared to 79 per cent of non-dairy farmers. While most dairy farmers were male (73.00%), non-dairy farmers had a slightly higher proportion of males (79.00%). Both groups had similar average family sizes, with dairy farmers reporting 6.31 members per household and non-dairy farmers reporting 6.60. The majority of farmers in both categories had completed secondary education,

Table 1: Seven Dimensions of Livelihood Security Index (LSI)

r_i	R_i								Σ f	p	C
1	7	12	6	4	2	1	3	4	32	92.85	8
2	6	9	6	3	5	2	2	5	32	78.57	7
3	5	2	10	7	2	2	1	8	32	64.28	6
4	4	5	3	8	10	0	3	3	32	50.00	6
5	3	3	1	4	8	9	1	6	32	35.71	5
6	2	1	2	4	4	2	14	5	32	21.42	5
7	1	0	4	2	1	16	8	1	32	7.14	4
Σ f		32	32	32	32	32	32	32		350	41
Σ fc		221	199	191	187	153	169	192	1280		
Sc		6.90	6.21	5.96	5.84	4.78	5.28	6.00	M=4.57 σ=0.67 SE=0.11		

ri = Correct rank order, Ri = Reverse rank order, Σ = Sum, *p*= Proportion, C = C values of respective ranks, Sc = Scale value, M= mean value, σ = Standard Deviation, Standard Error = σ/vN

and dairy farmers reported a higher average annual income (0.91) compared to non-dairy farmers (0.78). Both groups demonstrated moderate levels of social participation, but dairy farmers had slightly higher levels of mass media exposure and extension contact.

***Status of the dairying in the Aspirational Districts**:* The data from Table 3 suggests that dairy farming experience, livestock possession, and milk production are closely related factors that influence the success of dairy farmers. Experienced farmers with a medium number of livestock tend to have higher milk production, indicating the importance of knowledge and resources in optimizing dairy operations. However, the data also revealed that most farmers consume a significant portion of their milk production, potentially limiting their income from sales. To enhance profitability, dairy farmers may benefit from exploring ways to increase milk sales while maintaining sustainable production levels.

Dairy Management practices adopted: The data from Table 4 highlights the various management practices adopted by dairy farmers, including housing, feeding, health, breeding, milking, and general management. It suggests that most farmers use semi-kutcha sheds attached to their houses, with a focus on ventilation. Feeding practices involve a combination of grazing and stall feeding, with a majority providing roughages and concentrates. Health management emphasizes deworming and vaccination, while breeding was predominantly through artificial insemination.

Table 2: Socio-economic profile of the dairy farm households

Variables	% of Dairy farmer	% of Non-dairy farmers
Age		
Young (Up to 35)	28.00	21.00
Middle age (36-50)	38.00	46.00
Old (More than 50)	34.00	33.00
Gender		
Male	73.00	79.00
Female	27.00	21.00
Family size		
Small (Up to 5)	47.00	43.00
Medium (6-8)	37.00	33.00
Large (More than 8)	16.00	24.00
Family type		
Nuclear family	54.00	57.00
Joint family	46.00	43.00
Education		
Illiterate	12.00	15.00
Read and write	16.00	19.00
Primary level	12.00	19.00
Middle school	16.00	13.00
Secondary education	33.00	25.00
Graduate and above	11.00	09.00
Annual income		
Low (Up to 0.79 lakh)	28.00	50.00
Medium (0.80-1.20 lakh)	47.00	44.00
High (More than 1.21 lakh)	25.00	6.00
Social participation		
Low (Up to16.50)	37.00	46.00
Medium (16.51-20.91)	40.00	38.00
High (More than 20.92)	23.00	16.00
Mass media exposure		
Low (Up to12.35)	22.00	26.00
Medium (12.36-15.46)	43.00	55.00
High (More than 15.47)	35.00	19.00
Extension contacts		
Low (Up to16.59)	29.00	49.00
Medium (16.60-20.15)	50.00	42.00
High (More than 20.16)	21.00	9.00

Milking practices prioritize udder washing, and most farmers rely on tap water and have their animals insured. However, record-keeping remains an area for improvement. Adopting best practices in these areas can enhance dairy productivity and profitability.

Livelihood security of dairy and non-dairy farmers: The analysis of the seven dimensions of livelihood security from Table 5 reveals significant differences between dairy and non-dairy farmers. Dairy farmers generally exhibit higher levels of food, economic, health, educational, social, institutional, and infrastructural security compared to their non-dairy counterparts. This advantage could be attributed to the consistent income generated from dairy farming, which enhances food and nutritional security throughout the year. In contrast, non-dairy farmers face greater vulnerabilities due to reliance on unpredictable agricultural conditions, leading to lower economic stability.

Social and institutional security levels also indicate that dairy farmers are better integrated into support systems, although both groups showed a need for improved participation in cooperative organizations and access to training programs. Furthermore, infrastructural security is notably higher among dairy farmers, likely due to better access to essential services and facilities. Overall, the livelihood security index reflects that dairy farming provides a more robust foundation for economic and social well-being. This underscores the importance of promoting dairy

Table 3: Status of the dairying in the Aspirational Districts

Variables	% of Dairy farmer
Experience in dairy farming	
Low (Up to 11 years)	26.00
Medium (12-19 years)	41.00
High (More than 20 years)	33.00
Livestock Possession	
Low (<3)	26.00
Medium (4-6)	57.00
High (>7)	17.00
Milk Production	
Low (Up to 7 litres per day)	37.00
Medium (8-12 litres per day)	46.00
High (More than 13 litres per day)	17.00
Milk Consumption (litres/day)	
Low (Up to 1.00 litres per day)	10.00
Medium (1.01-2.00 litres per day)	80.00
High (More than 2.01 litres per day)	10.00
Milk sale (litres/day)	
Low (Up to 5 litres per day)	38.00
Medium (6-11 litres per day)	43.00
High (More than 12 litres per day)	19.00

Table 4: Dairy Management practices adopted by the respondents

Practices		Percentage
i. Housing management		
Type of shed	Pucca	37.00
	Semi kutcha	63.00
Sheds proximity to farmers house	Away	18.00
	Attached	82.00
Ventilation of the shed	Ventilated	72.00
	Non-ventilated	28.00
ii. Feeding management		
Feeding method	Grazing	23.00
	Stall feeding	12.00
	Both	65.00
Type of feed	Roughages only	21.00
	Roughages+concentrates	79.00
Feeding of pregnant animals with an extra ration during the advanced stage of pregnancy		65.00
iii. Health management		
Deworming practices	Yes	83
	No	17
Vaccination carried out	At Farm	40.00
	At Veterinary centre	60.00
Sick animals' management	Separately	26.00
	With others	74.00
iv. Breeding management		
Breeding method	Natural	41.00
	A. I	59.00
A.I available from	State department	100.00
	Private	0.00
A.I availed at	Door step	37.00
	Veterinary centre	63.00

v. Milking management		
Washing of udder prior to milking	Yes	100.00
	No	0.00
Concentrate feeding at milking time	Yes	39.00
	No	61.00
Frequency of milking	Twice	100.00
	Thrice	0.00
vi. General management		
Record keeping	Yes	14.00
	No	86.00
Source of water for animal	Village pond	19.00
	Bore well	26.00
	Tap water	55.00
Animal insurance	Yes	68.00
	No	32.00

Table 5. Distribution of respondents according to different livelihood security index dimensions

Dimensions	% of dairy farmers	% of non-dairy farmers
i. Food and nutritional security		
Low (Up to 0.67)	12.00	30.00
Medium (0.68-0.85)	28.00	33.00
High (More than 0.86)	60.00	37.00
ii. Economic security		
Low (Up to 0.41)	38.00	54.00
Medium (0.41-0.63)	43.00	36.00
High (More than 0.63)	19.00	10.00
iii. Health security		
Low (Up to 0.74)	22.00	30.00
Medium (0.74-0.85)	30.00	42.00
High (More than 0.85)	48.00	28.00
iv. Educational security		
Low (Up to 0.60)	16.00	40.00
Medium (0.60-0.75)	45.00	36.00
High (More than 0.75)	39.00	24.00
v. Social security		
Low (Up to 0.33)	40.00	45.00
Medium (0.33-0.73)	36.00	38.00
High (More than 0.73)	24.00	17.00
vi. Institutional security		
Low (Up to 0.52)	34.00	57.00
Medium (0.52-0.72)	37.00	23.00
High (More than 0.72)	29.00	20.00
vii. Infrastructural security		
Low (Up to 0.58)	11.00	46.00
Medium (0.58-0.79)	64.00	41.00
High (More than 0.79)	25.00	13.00
viii. Overall livelihood Security		
Low (Up to 0.59)	9.00	47.00
Medium (0.60-0.70)	52.00	41.00
High (More than 0.71)	39.00	12.00

farming as a viable livelihood option to enhance the overall quality of life in rural communities of aspirational districts.

Comparison of characteristics between dairy and non-dairy farmers using propensity score matching: "Propensity Score Matching (PSM) was employed to statistically compare the livelihoods of dairy and non-dairy farmers. Figure 1 illustrates that the background characteristics of both groups overlap significantly, suggesting that their livelihoods can be meaningfully compared based on these characteristics."

By calculating the index gap analysis i.e., the percentage difference between the overall livelihood security index value of dairy and non-dairy farmers. It can be observed from the Table 6

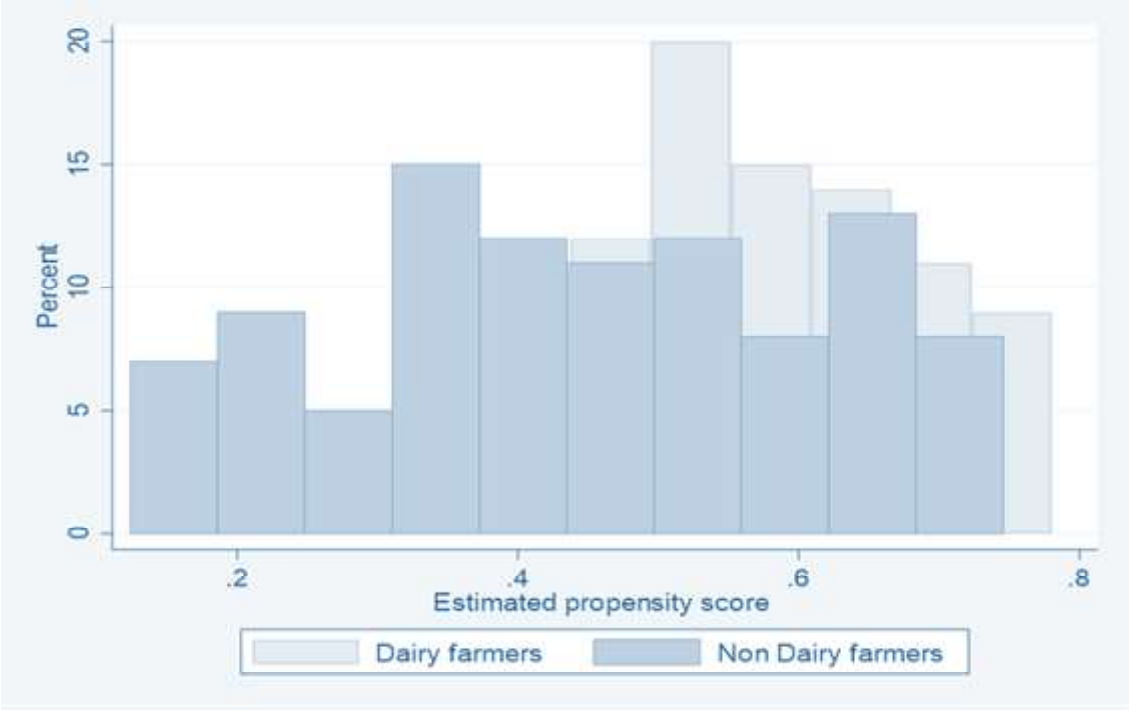


Fig. 1 Common support showing the frequency distribution of propensity score of dairy and non-dairy farmers

Table 6: Average difference and gap analysis after propensity score matching of dairy and non-dairy farmers

Number of matches	Dairy	%Gap
m=1	0.0958***	14.08
	(.01482)	
m=3	0.0959***	14.10
	(0.013)	
m=5	0.0952 ***	14
	(0.013)	
Observations	200	

that livelihood security of dairy farmers had significantly higher than that of non-dairy farmers by 14.10 per cent. Therefore, farmers who were practising dairying had more secured livelihood than the non-dairy farmers.

Conclusions

The findings of this study reveal that dairying significantly enhances livelihood security among farmers in Karnataka's aspirational districts. By employing a comprehensive Livelihood Security Index (LSI), it is evident that dairy farmers experience superior food, economic, health, educational, social, institutional, and infrastructural security compared to non-dairy farmers. The management practices identified, ranging from housing and feeding to health care and milking, play a critical role in ensuring the success of dairy operations. The 14.10% advantage in livelihood security underscores the need for targeted interventions to support dairy farming as a viable livelihood option in across the Aspirational Districts of our country. Hence, strengthening dairy based developmental programmes can be one of the important policy interventions for securing the livelihood of farmers in Aspirational districts of our country. Strengthening management practices and providing access to training programs can further enhance productivity and profitability in this sector. Ultimately, promoting dairying not only contributes to individual farmer resilience but also fosters sustainable development and poverty alleviation in rural communities across aspirational districts.

Acknowledgement

The first author sincerely acknowledges ICAR-National Dairy Research Institute for providing financial assistance for pursuing Postgraduate research work on this topic.

References

Chadda A, Jadoun YS, Singh J, Kansal SK (2022) Role of Self-help Groups in Promoting Adoption of Scientific Dairy Practices among Rural Women: A Comparative Analysis. J Community Mobilization and Sustainable Dev 709

Chambers R, Conway G (1992) Sustainable Livelihoods: Practical Concepts for the 21st Century. Discussion Paper 296, Institute of Development Studies, London

Department of Animal Husbandry & Dairying (2019) 20th Livestock Census 2019: All India Report. Ministry of Fisheries, Animal Husbandry & Dairying, Government of India.

Department of Animal Husbandry and Dairying (DAH&D) (2023) Basic Animal Husbandry Statistics-2023. Government of India. https://dahd.nic.in/sites/default/filess/BAHS2023.pdf

Frankenberger T (1996) Measuring Household Livelihood Security: An Approach for Reducing Absolute Poverty. J Global Food Forum 34(2): 1-5

Guilford JP (1954) Psychometric Methods. Tata McGraw Hill Publishing Company, Bombay.

Lazard O, Youngs R (2021) The EU and Climate Security: Toward Ecological Diplomacy. Carnegie Europe, 12

Rodríguez DI, Anríquez G, Riveros JL (2016) Food Security and Livestock: The Case of Latin America and the Caribbean. Ciencia e Investigación Agraria, 43(1): 5-15

Rosenbaum PR, Rubin DB (1983) The central role of the propensity score in observational studies for causal effects. Biometrika 70(1): 41–55

Singh AK, Upadhyaya A, Kumari S., Sundaram PK, Jeet P (2020) Role of Agriculture in making India $5 trillion Economy under Corona Pandemic Circumstance: Role of agriculture in Indian economy. J AgricSearch 7(2): 54-58

Impact Factor: 0.2; NAAS Score: 6.20 (January 2025)

Regd. No. 15665/68

Covered by Clarivate Analytics Services: Emerging Sources Citation Index

https://mjl.clarivate.com/search-results

INDIAN JOURNAL OF DAIRY SCIENCE **MAY-JUNE VOL.78, NO.3, 2025**

Contents

ISSN 0019-5146 (Print)
ISSN 2454-2172 (Online)