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Contents

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INVITED REVIEW

Stereospecific distribution pattern of fatty acids in triglycerides: A comparative review of human, bovine, bubaline, caprine, and equine milk fat

Pavel Rout, Vivek Sharma and Sumit Arora 291

DAIRY PROCESSING

The effect of *Ruscus Hyracanus* extract on physicochemical, microbial and organoleptic properties of kefir

Mahsa Amir Sadeghi, Marjaneh Sedaghati and Mozhgan Emtiazjoo 303

Process optimization for the manufacturing of flax seed powder enriched *Kalakand*

Hemant Kumar, Ankita Hooda, Urvashi Vikranta, Himanshu Rai 310

Studies of physicochemical, microbiological and sensory characteristics of paneer (Indian Cottage Cheese) to check the efficacy of clove-oil nanoemulsion in enhancing its shelf-life

D Bhardwaj, B Mann, R Sharma, R. Kumar, Rashmi H.M., K. Gandhi, Sukthija M.P. and Minaxi Sharma 318

Development and experimental performance of a cleaning-in-place system for three stage scraped surface heat exchanger

Pooja N. Bhagat, P. Barnwal, Ankit Deep, Anup D. Gadwe and P. Behare 329

Preparation of synbiotic fermented milk and evaluation of short-chain fatty acids production during storage study

Mitali Makwana, JB Prajapati, Sreeja Mudgal and Subrota Hati 338

ANIMAL PRODUCTION & REPRODUCTION

Estrus intensity scoring and conception rate in postpartum anestrus buffaloes using estrus induction protocols

Renuka Mishra, Nitin Kumar Bajaj, Satya Nidhi Shukla, Madhuri Dhurvey, Abhishek Bisen, Pushpendra Maravi and Shashank Vishvakarma 349

Physical and morphometric characteristics of unidentified cattle breed of northern Karnataka region of India

Vijaylaxmi I Mundinamani, Mahadevappa D. Gouri, Kotresh Prasad C and Vivek M. Patil 354

Influence of calf-mother interaction on performance and behaviour of Murrah buffalo calves during heat stress

Nripendra Pratap Singh, ML Kamboj, Nishant Kumar and Sunil Dutt 362

Effect of non-genetic factors on production performance of Mehsana buffalo at organized farm

Rimee Dhakad, A. P. Chaudhary, J. P. Gupta and Sadhana Tiwari 372

DAIRY ECONOMICS & EXTENSION

Economic analysis of costs and returns of milk production in Andhra Pradesh

Konda R Reddy, Biswajit Sen, Udit Chaudhary, Unni Ravishankar and Ajmer Singh 379

SHORT COMMUNICATION

Elucidating the associations of polymorphism of growth hormone gene with milk production traits in Jamunapari goats of India

BK Prajapati, KP Singh, PK Rout, R Roy, Ishani Roy and Ajoy Mandal 387

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Stereospecific distribution pattern of fatty acids in triglycerides: A comparative review of human, bovine, bubaline, caprine, and equine milk fat

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Abstract: Major lipid fraction of milk fat is triacylglycerol (TAG) representing about 98% of total lipids. TAG contains a glycerol backbone and three fatty acids. These three fatty acids are positioned at sn-1, sn-2 & sn-3 position in glycerol backbone and the positioning is called as stereospecific positioning. This stereospecific positioning mainly effects the lipid metabolism of dietary lipids. During the digestion of TAG in human beings, lipase preferably attacks the TAG at sn-1 or sn-3 positions resulting in the release of free fatty acids along with sn-2 monoacylglycerol. It has been reported that saturated fatty acids are mainly esterified in sn-2 position whereas unsaturated fatty acids are esterified in sn-1 and 3 positions. The fatty acid distribution among the three sn-positions of the glycerol backbone is non-random. The non-random distribution is a result of the specificity of different enzymes during TAG biosynthesis. The distribution of fatty acids depends upon species, feed, season and lactation period. The stereospecific arrangement of fatty acids in fat also influences some physical properties of fats and oils like crystallization and melting properties. The stereospecific arrangement of milk fat is generally studied by using different lipases along with separation techniques and finally chromatography or spectroscopy. Understanding these aspects can aid in identifying the origin of the fat, detecting potential adulteration of different other species of milk, and predicting nutritional value of different species' milk based on differences in the form of sn-2-MAG and free fatty acids.

Keywords: Digestion; Fatty acids; Positional distribution; Milk fat; Triglycerides; Structure

Introduction

There are various major dairy animals in different regions of the globe. Cows are the most widely used dairy animals worldwide, accounting for 81.8% of all milk production (FAOSTAT, 2022). On the other hand, India and Pakistan are known for their buffalo milk consumption, these two countries produce more than 90% of the world's buffalo milk (Singh, 2023). In Asia, India and Bangladesh are also the primary producers of goat milk, accounting for 43.4% of the world's output (FAOSTAT, 2022). Central Asian nations such as Kazakhstan and Mongolia have the highest consumption of equine milk compared to other countries (Miraglia et al. 2020). In the high-altitude regions of like Tibet, the Qinghai region of China, and the Ladakh and north Sikkim regions of India yak milk is a popular food (Yang et al. 2018).

The nutritional properties of milk fat have garnered significant public interest. According to McSweeney et al. (2020) human and bovine milk fat comprise approximately 400 fatty acids. The fatty acids are categorized into three groups based on their chain length i.e., short- to medium-chain fatty acids (SMCFA) with a chain length of C4-C12, medium-chain fatty acids (MCFA) with chain length of C13-C16, and long-chain fatty acids (LCFA) with a chain length of C17-C23. The short-chain fatty acids and a portion of the medium-chain fatty acids in milk come from mammary cells' de novo synthesis, whereas long-chain fatty acids and remaining medium chain fatty acids are obtained from dietary sources (Chilliard et al. 2000).

Some saturated fatty acids (SFA) and trans-fatty acids have been linked to long-term health problems (Wales et al. 2009), which makes people more concern about milk fat. On the other hand, it has been observed that a reduced quantity of unsaturated fatty acids (UFA) in milk can positively impact human health. Specifically, the presence of Oleic acid (C_{18:1c9}) and linoleic acid (C_{18:2c9t11}) fatty acids in low concentrations has been linked to a decrease in plasma lipids and a hindrance in the development of cancer (Calder, 2014). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) play a crucial role in facilitating the development of the nervous system in infants (Guesnet & Alessandri, 2011). In addition to fatty acid composition the

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arrangement of fatty acids in the triglyceride also plays an important role in fat digestion and absorption (Mehrotra et al. 2019). This arrangement is called stereospecific arrangement or stereospecific distribution of fatty acids. Literature have shown that the distribution of fatty acids among the three sn (stereospecific numberings) positions of milk triacylglycerols (TAG) is non-random (Mugabo et al. 2016). The sn-system is a nomenclature recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) in 1967 to describe the stereochemistry of glycerol derivatives. It provides information about each fat, that the TAG fraction of each lipid matrix has a unique arrangement of fatty acids among positions of the glycerol backbone (Parodi, 1983). The stereospecific arrangement of fatty acids in fat also influences some physical properties of fats and oils like crystallization and melting properties. Stereospecific arrangement of fats and oils is generally studied by using different lipases along with separation techniques and finally chromatography or spectroscopy. Understanding these aspects can aid in identifying the origin of the fat, detecting potential adulteration of different other species milk, and predicting nutritional value of different milk based on differences in the form of sn-2-MAG and free fatty acids. The main aim of this review article is to compare different stereospecific distribution of fatty acids in triglycerides of bovine, bubaline, caprine and equine milk fat with human milk fat.

Fat digestion mechanism in human body

The digestion and absorption of lipids have some special challenges. Triglycerides are large molecules, but they are not water-soluble, like carbohydrates and proteins. Due to this, they tend to coalesce in huge droplets in a watery environment of the digestive tract. The digestive tract breaks these big fat droplets into smaller droplets and then uses enzymes called lipases to break down lipid molecules (Mu & Høy, 2004). The mouth and stomach play a little role in this process, but the small intestine is responsible for the most of fat digestion. After lipid digestion, the products of fat digestion are absorbed into circulation and spread throughout the body, which again requires for special treatment since lipids are not water-soluble and do not integrate with the watery element of blood (Mu & Høy, 2004).

The stereospecific position of fatty acids also plays an important role in nutrition and digestion of fats. Triacylglycerol breaks down with the help of lipase in the mouth, stomach, and small intestine. To hydrolyse triacylglycerol that has been emulsified by bile acids, pancreatic lipase works in cooperation with colipase. Pancreatic lipase is position specific, while hydrolysing the triglyceride, it attacks at sn-1 and sn-3 position and releases the fatty acids from sn-1 or sn-3 position, but the sn-2 positional fatty acid remains intact as 2-mono acyl glycerol (2-MAG) as depicted in Figure.1 (Akoh, 2017). Fatty acids and monoacylglycerols micelles migrate into enterocytes, and they undergo re-esterification to become triacylglycerols in small

intestine (Decker, 1996). These triacylglycerols are subsequently packed into chylomicrons along with lipoproteins and other components. Although this is how triacylglycerols are usually broken down and absorbed, the fatty acids released from the sn-1 and sn-3 positions often end up in different places in the body's metabolism. Short- and medium-chain fatty acids (5-10 carbons) can be dissolved in the intestine's aqueous content. They are absorbed, attached with albumin, and transported to the liver through the portal vein. Free long-chain fatty acids (palmitic and stearic) have low coefficients of absorption because their melting points are higher than body temperature, and they can form calcium soaps. So, fats with long-chain saturated fatty acids in the sn-1 and sn-3 positions of triacylglycerols may be absorbed differently than fats with palmitic or stearic acids in the sn-2 position (Bracco, 1994; Decker, 1996; Innis, 2011).

This process ensures that the fatty acids in the sn-2 position of the absorbed TAG molecules are retained, similar to those in the dietary TAG. The esterification of fatty acids in the glycerol backbone is significant for physiological and nutritional purposes. The study of intramolecular triacylglycerol (TAG) composition is particularly important due to its influence on various factors (like: melting property, crystallization property, digestion behaviour of fat). There are many studies on compositional differences in fatty acids in different milk species but limited study on positional distribution of milk fatty acids in different milch species are limited.

The fat composition of milk varies between different animal species

Milk of cow, buffalo, goat, and horse, has less saturated fat and more unsaturated fats like monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). It also has a higher ratio of omega-6 to omega-3 fatty acids (Table. 1). Typically, equine milk, has a reduced concentration of saturated fatty acids and cholesterol and an elevated concentration of polyunsaturated fatty acids in comparison to milks sourced from ruminant animals (bovine, bubaline and caprine). The quantity of conjugated linoleic acid is comparable between human and ruminant milks, although it is comparatively lower in non-ruminant milks, as seen in Table. 1.

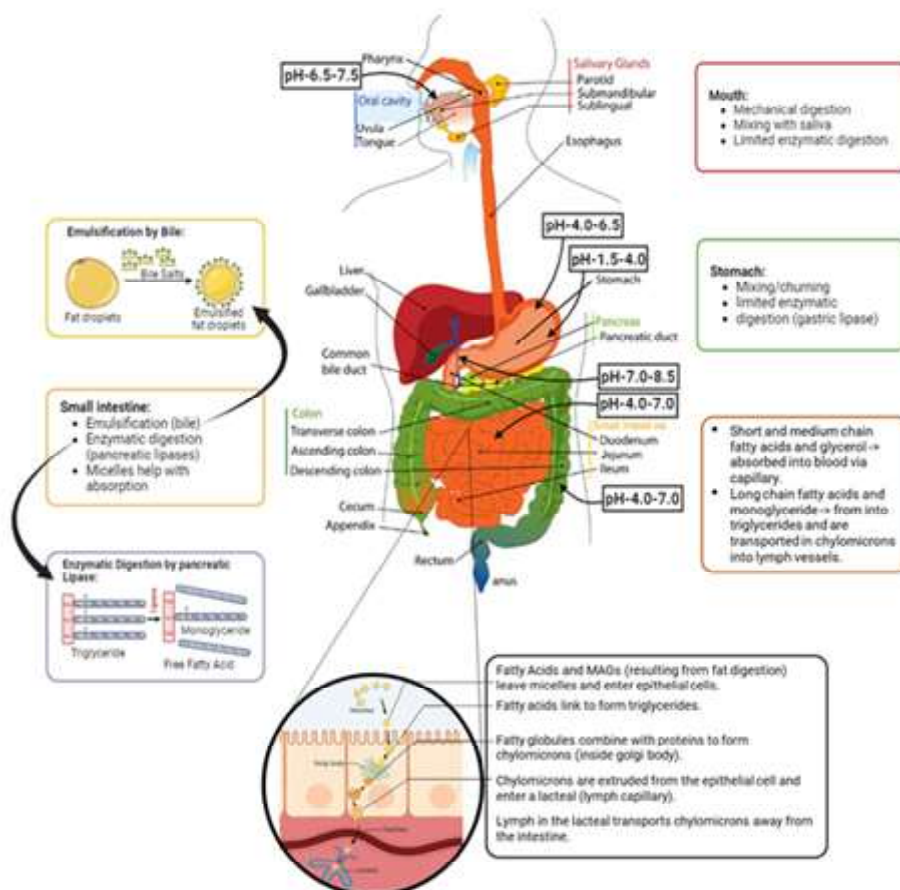
The fats found in caprine (goat) milk are recognized for their high content of short-chain and medium-chain fatty acids, which contribute to the unique flavor of its milk. Goat milk is also enriched with unsaturated fatty acids, such as oleic, linoleic, and linolenic acids (Ruiz Sala et al. 1996). Bubaline or buffalo milk has a higher percentage of medium-chain triglycerides compared to bovine milk. Bovine milk, on the other hand, is characterized by a higher concentration of long chain fatty acids (LCFAs) (Abd El-Salam & El-Shibiny, 2011; Park et al. 2007; Ruiz Sala et al. 1996). It is also reported that bubaline milk has a greater concentration of LCFAs and a comparatively less concentration of short chain fatty acids (SCFAs) when compared to bovine milk (Jilo & Tegegne, 2016).

From the above data, it is evident that among all species equine milk fatty acid composition is nearer to human milk. The variations mentioned above might potentially impact the digestive characteristics of milk fat across various species. This is because lipases are known to be more effectively break down short or medium-chain fatty acids in triglycerides (TAGs) (Lee et al. 2022).

Different techniques for determination of stereospecific arrangements of fatty acids in triglycerides

The study of triglyceride structure has two primary purposes: firstly, to detect the fatty acid present in the sn-2 position, without distinguishing between the sn-1 and sn-3 positions, and secondly, to identify each individual fatty acid on the triacylglycerol molecule by complete stereospecific analysis. Determination of stereospecific arrangement of fatty acids using these methodologies, involving the specificity of lipase enzyme, one can determine the impact of triglyceride composition on the reactions of lipid digestion, absorption, and metabolism.

Fig.1 Fat digestion in human body



(Source: Clier-Free-Vector-Images, 2012)

Table1: Lipid profile of equine, caprine, bubaline, bovine and human milk fat

Parameters	Species wise different types of milk fat				
	Equine	Caprine	Bubaline	Bovine	Human
Total Fat % (g/100g milk)	8-12	12-16	16-17	12-13	10-13
SFA% (% Total fatty acid)	40.5-67.7	59.9-73.7	62.1-74	55.7-72.8	39.4-45
MUFA% (% Total fatty acid)	15.3-35.0	21.8-35.9	24.0-29.4	22.7-30.3	33.2-45.1
PUFA% (% Total fatty acid)	14.17-30.5	2.6-5.6	2.3-3.9	2.4-6.3	8.1-19.1
ω-6: ω-3	0.9-6.1	3-4	2.5-3.0	2.1-3.7	7.4-8.1
CLA% (% Total fatty acid)	0.02-0.1	0.3-1.2	0.4-1	0.2-2.4	0.2-1.1
Cholesterol (mg/100ml milk)	5.8-8.8	10.7-18.1	4-18.0	13.1-31.4	14-20
% of C16:0 at sn-2	30-54	35-36	37-40	38-42	52-74

(Claeys et al. 2014; Gantner et al. 2015; Roy et al. 2020)

Enzyme-linked assays are often used for the analysis of the stereospecific arrangement of fatty acids on milk fat triglycerides. The diacylglycerol acyltransferase (DGAT) test is one example. It can be used to find out the enzyme activity that attaches fatty acids to the glycerol molecule at the sn-1 and sn-3 places (Liu et al. 2012). Although there are several isolation and quantification methods are reported in various studies for determining the positional distributions of fatty acids in triacylglycerol.

Stereospecific Position determining methods of fatty acids in Triglyceride

Stereospecific analysis of TAG can be obtained by the enzymatic reaction catalysed by porcine pancreatic lipase (EC 3.1.1.3) (Christie and Han., 2012) or by Grignard chemical diacylation (Blasi et al. 2008). The latter procedure is the preferred method for the analysis of milk TAG, because it does not show acylic specificity (Turon et al. 2002; Tzompa-Sosa et al. 2014). It allows the direct determination of FA composition of sn-2 position and the differentiation of FA esterified in the primary (sn-1,3) and secondary (sn-2) position of the glycerol backbone. This approach is based on 1,3 random, 2-random distribution theory (RR procedure). In TAG positional analysis, sn-2-monoacylglycerols (sn-2-MAG), obtained by enzymatic hydrolysis or chemical diacylation, are isolated by a preparative TLC and trans esterified as FAME for the subsequent analysis by GC-FID. It allows the determination of the FA composition (%) in the sn-2-position of the native TAG. The FA composition in the sn-1(3) positions can then be estimated from the composition of the sn-2-MAG and TAG, according to the following formula:

$$A_{(1,3)} = \frac{3 \times A_T - A_2}{2}$$

where $A_{1,3}$ = % FA in sn-1 and sn-3 positions; A_T = % FA in total TAG; A_2 = % FA in sn-2 position.

The pancreatic lipase technique does not differentiate between sn-1 and sn-3 positional fatty acids, so, it is mainly applicable to fats and oils with a restricted number of fatty acids that originate from symmetrical triglycerides, such as POP, SOS, and OOO (where P, O, and S, are palmitic, oleic, and stearic acids, respectively). Examples of this include cocoa butter and Borneo tallow.

In order to conduct a stereospecific analysis on a complex triacylglycerol mixture, such as milk fat, a more complex procedure is required. This procedure includes the use of the Grignard reagent to split the triglyceride into diglycerides, derivatization of that diglyceride to phospholipids, splitting with specific phospholipases and finally determination of fatty acids through gas chromatography. Figure.2. provides a concise summary of

the analytical procedures that are involved in the stereospecific analysis of complex triglycerides.

These procedures include both chemical and specific enzymatic processes. So, one can specify, for each kind of fat, the fatty acids that are located in the sn-2 position, as well as the overall composition of the triglyceride and the total fatty acid composition. The analytical approaches are based on enzymatic-instrumental and chemical-instrumental procedures. The use of sn-1,2 diglycerol kinase is more suitable for stereospecific analysis of vegetable fat (Han, 2016), whereas the most suitable for milk TAG, containing FA with a short chain, is the phospholipase A2 (PLA₂) procedure.

Another method for determining the positional distribution of fatty acids in triacylglycerols is high-resolution ¹³C nuclear-magnetic resonance spectroscopy, which is based on the chemical shift of esterified fatty acids in all three locations (Bunga et al. 2023; Edison, 2009; Hamilton, 1998) or by high-performance liquid chromatography (HPLC) of the diastereomeric derivatives (urethane) of partial glycerides (Takagi & Ando, 1991; Takagi & Suzuki, 1992).

a) Thin layer chromatography couples with GLC (Christie,1982)

For a long period, this method has been used in different studies with various modifications. The recently modified version was developed by Chen et al. (2020). They mentioned primary hydrolysis through pancreatic lipase and isolation of MAG through TLC for the determination of sn-2 positional FA. Then Grignard hydrolysis splits the triglyceride into diglycerides, next chemical derivatization of that diglyceride occurs to produce phosphatidylcholine. After that the fatty acids of phosphatidylcholine was splitter with the help of phospholipase A₂(PLA₂). Finally, determination through gas chromatography (GC-FID) of each fraction. The percent FA composition of sn-3 position can be calculated by applying the following formula:

$$A_3 = [3 \times A_T - A_2 - A_1],$$

where A_3 = % FA in sn-3 position; A_T = % FA in total TAG; A_2 = % FA in sn-2 position; A_1 = % FA in sn-1 position.

The most complete characterization of TAG by stereospecific analysis of donkey, cow, ewe, goat, and buffalo milk, carried out using the phospholipase A2 (EC 3.1.1.4) procedure, was obtained by Blasi et al. (2008). The main disadvantage of these methods is that it is time-consuming and not applicable for routine quality control.

b) High-Performance Liquid Chromatography method

One possible method for analysing TAG stereospecifically using chemical-instrumental methods involves the use of chiral HPLC

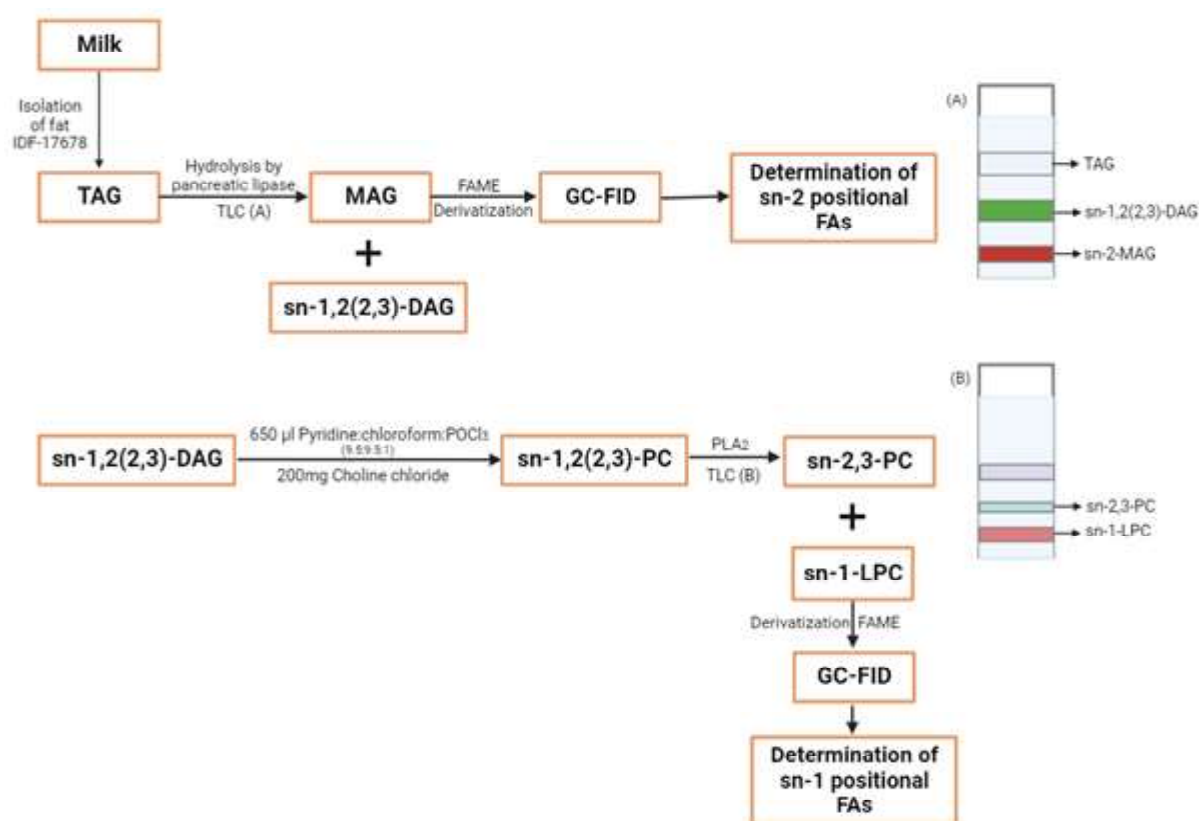


Fig.2 Schematic diagram of stereospecific analysis of fatty acids presents in milk fat TAG

to separate derivatized enantiomers. Unfortunately, only a few articles reported about the application of this approach to milk fat analysis. The first method of this kind to be talked about using the concept of resolution of diastereomeric diacylglycerol products (Figure 3. part a) (Christie, 1996; Laakso & Christie, 1990). The process began with the creation of sn-1,2-, 2,3-, and 1,3-diacylglycerols through a reaction with ethyl magnesium bromide. In the second step, these products were reacted with a chiral derivatizing agent, (S)-(+)-1-(1-naphthyl) ethyl isocyanate and the resulting diacyl-sn-glycerol urethane derivatives were isolated through chromatography on solid-phase extraction columns containing octadecyl silyl phase. The third and most crucial step involved the resolution of the diacylglycerol urethanes through HPLC on columns of silica gel. To achieve the best possible resolution, two columns of silica gel have been used (Hypersil TM 3-m, 250×4.6 mm i.d.) in series. The mobile phase consisted of 0.4 to 0.33% (v/v) 1-propanol (containing 2% water) in isooctane, and the flow rate was set to 1 mL/min. UV rays at 280 nm were used for analysis.

Takagi & Ando. (1991) reported a different but comparable approach (Figure. 3, parts b and c). They used high-performance liquid chromatography (HPLC), on a column that had a stationary phase with chiral moieties chemically bound to a silica gel base. Di- and monoacyl-sn-glycerols made from triacylglycerols,

converted to the 3,5-dinitrophenyl urethane (DNPU) derivatives. The 3,5-dinitrophenyl moieties of the urethanes help in charge-transfer with functional groups that have pi-electrons on the stationary phase (Christie, 1996; Takagi & Suzuki, 1992, 1993). After lowering the column temperature and slowing down the flow rate, the method could even be applied to such complex triacyl-sn-glycerols as fish oils. Following isolation of the various fractions, trans-methylation and gas chromatography, the distributions of fatty acids in each of positions sn-1, -2 and -3 can be calculated from the data (Vaille & Martin, 2004).

c) Mass spectrometry

Mass spectrometry is an effective method for examining the stereospecific positioning of fatty acids on triglycerides of milk fat. In 2005, Kuksis and Itabashi developed a method for MS analysis of regio-isomeric triacylglycerols (TAGs). Generally, ammonia negative-ion chemical ionisation (NICI) and collision-induced dissociation (CID) are used for investigating the regio isomerism of fatty acids in TAGs. The molecular ions, $[M-H]^-$, are allowed to hit an inert gas like argon or xenon. The fragment ions, $[M-H-RCOOH-100]^-$, $[M-H-RCOOH-74]^-$, and $[M-H-RCOOH-56]^-$, or $[M-H]^-$ molecular ions are all less abundant for fatty acids in the sn-2 position than for those located in the sn-1 and sn-3 positions. It is the $[M-H-RCOOH-100]^-$ ions and the $[RCOO]^-$ ions that are used to determine the TAG structures.

Positive NH_4^+ ESI-MS has not been shown to be very useful for identifying the difference between TAG regio isomers. An ion-trap device and ESI-MS in of $[\text{M} + \text{NH}_4]^+$ ions, on the other hand, have led to better results. It has been shown by Kalo et al. (2003) that successful separation of regio isomers of short-chain TAGs requires the use of positive NH_4^+ ion ESI-MS in combination with normal phase.

HPLC matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is also a technique that can precisely measure the fatty acid composition and distribution of milk fat triglycerides. MALDI-TOF MS is an extremely sensitive and precise technique capable of detecting even trace amounts of triglycerides and fatty acids (Christie, 1996; Cossignani et al. 2019).

d) Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is another technique that is utilized to analyse the stereochemistry of milk fat triglycerides. The locations of fatty acids on the glycerol backbone and other structural and kinetic characteristics of molecules may be thoroughly studied using NMR spectroscopy (Lopes et al. 2018). Tengku & Birch, (2014) reported in their study, fat sample of 180 mg was mixed with 700 ml of deuterated chloroform and put into a 5mm NMR tube using a syringe. NMR spectra was obtained on a Varian 500MHz VNMR device with a 5mm OneprobeTM working at 125.705 MHz. Peak recognition was done according to the work

of Aursand et al. (1995) and Suárez et al. (2010). The integrator response of the NMR spectra, which was done with Varian VnmrJ version 3.0 software, showed the percentage of fatty acids in the carbonyl region.

Mass spectrometry, nuclear magnetic resonance, and enzyme-linked assays are highly effective methods for analysing the stereospecific positioning of fatty acids on milk fat triglycerides. These techniques are used to determine the distribution of fatty acids on the glycerol backbone, which can be used to better understand and regulate the physical properties and nutritional quality of milk and dairy products.

Merits and demerits of different techniques used for positional analysis of fatty acid in triglyceride in milk fat

Multiple methods are employed to analyse the stereospecific positioning of fatty acids in milk fat triglycerides. Table.2 presents a compilation of various techniques utilized by different researchers, along with their respective findings and associated costs.

Mass spectrometry, nuclear magnetic resonance, and enzyme-linked assays are all powerful techniques for analysing the stereospecific positioning of fatty acids on milk fat triglycerides. These methods can provide detailed information about the positional distribution of fatty acids on the glycerol backbone, which can be used to understand and control the milk and dairy products' physical properties and nutritional quality.

Table 2: Merits and demerits of different techniques used for Stereospecific positioning of fatty acids in milk fat TAG

Author	Milk Species	Methodology	Findings	Cost involved
Parodi, 1979	Bovine milk	Pancreatic lipase diacylation and isolation through GLC	There is a change in medium chain fatty acids in sn-1(3), than sn-2 with season.	Low cost
Parodi et al.1983	Prepartum and Postpartum milk of Friesian cow	Pancreatic lipase diacylation and GLC	Prepartum mammary gland secretion has twice the amount of 16:0, than normal milk, and the amount of 14:0 is also higher.	Low cost
Andreotti et al. 2002	Cow, Sheep, goat	NMR-nuclear magnetic resonance	The short-chain fatty acids, 4:0 and 6:0, almost exclusively at the sn-3 position.	High cost
Blasi et al.2008	Donkey, cow, ewe, goat and buffalo milk	Phospholipase A2 hydrolysis, TLC and HRGC.	The FA distribution in glycerol backbone is non-random, SFA is esterified at sn-3 position, while MUFA are in sn-2. EFA as linoleic acid present in sn-2 at donkey milk.	High cost
Haddad et al. 2010	Camel milk	Pancreatic lipase + Grignard degradation + TLC + phosphor lipase A2 + GC	The sn-2 position is esterified with C16:0 (40.8% mol), C14:0(18.1% mol), C18:1(14% mol). LCFA are esterified at outer position (50.7% and 42.6% respectively in sn-1 & sn-3).	High cost

Haddad et al. 2012	Human colostum, transitional and mature milk	Grignard degradation, TLC and GLC.	Significant differences in fatty acid composition occurred between lactation times, quantities in each position can be changed.	Costly
Gotoh et al. 2012	Human, Rat, Cow, buffalo, goat and sheep milk	HPLC-UV-APCI-MS/MS	Palmitic acid in cow or buffalo milk cheese fat TAG is mainly present at sn-1, (3) position.	High cost
Tzompa-Sosa et al. 2014	HF cows	Grignard degradation +preparative TLC +GC-FID	GPAT indirectly controls the amount of FA that are esterified at the sn-2 position by selectively esterifying C 16:0 at sn-1.	High cost
Sun et al. 2018	Human milk fat and human milk formula	Pancreatic lipase hydrolysis, TLC and GLC.	Formula fat contains lower level of PA, SFA, LC-PUFA and higher level of Oleic, linoleic and α -linoleic Acid at sn-2 position.	Costly
Chen et al. 2020	Human milk	Pancreatic lipase and phospholipase A2 hydrolysis, TLC and GLC	MUFA are sn-1, sn-3 > sn-2, PUFA are sn-3 > sn-1 > sn-2. SCFA and MCFA are in sn-3.	Costly
Yener et al. 2021	Bovine milk	Candida antarctica lipase B Hydrolysis + SPE + GC-FID	Reported seasonal variation in the sn-2 and sn-1(3) FA compositions between summer and winter months.	Moderately Cost
Karrar et al. 2022	Camel, cow, donkey, goat, and yak milk	Pancreatic lipase hydrolysis + TLC + GC-FID	sn-2 position was mainly esterified by C18:1 n-9 and C16:0. Goat milk fat had a lower C18:1n-9/C16:0 ratio at the sn-2 position compared with milk fat from other species. Donkey and camel milk fat have more UFAs and less SFAs at the sn-2 and sn-1,3 positions than milk fat from other species.	Low cost
Pacheco-	Cow milk	Candida antarctica	Changes in FA positioning in different	Moderately

Stereospecific distribution of fatty acids in triglycerides of human, bovine, bubaline, caprine and equine milk fat

The positional distribution of fatty acids in milk fat has received considerable attention in recent years due to its large importance in understanding fat digestion, absorption, and metabolism in humans (Bakry et al. 2020; Cossignani et al. 2019; Karrar et al. 2022; L. Yang et al. 2022). Literature suggested that in milk fat of different species, the short-chain fatty acids are esterified in sn-1 or sn-3 position, medium-chain fatty acids like lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$) is esterified in sn-2 and sn-3 position. The long-chain fatty acids are mainly esterified at the sn-1 or sn-2 position.

Distribution of short chain saturated fatty acids and medium chain saturated fatty acids in human, bovine, bubaline, caprine and equine milk fat

Short chain fatty acids ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$ and $C_{10:0}$) are primarily esterified at either the sn-1 or sn-3 position, with a preference for the sn-3 position. sn-3 interpositional level (almost 90%) is far higher than sn-1 position. In human milk concentration of $C_{4:0}$, $C_{6:0}$, and $C_{8:0}$ can't be detected at sn-1 and sn-2 position, demonstrating that SCFAs are all distributed only at sn-3 position. In case of short chain fatty acids there are significant variation in between different species. Butyric acid ($C_{4:0}$) represents only 0.09-0.2% of total sn-3 fatty acids in the human milk, but almost 20% in buffalo milk (Blasi et al. 2008). Equine milk has a lower percentage (2.5-5%) of sn-3 position than bovine, bubaline, and caprine milk (Figure.3).

Human milk and equine milk possessed lowest sn-3 levels of caproic acid (0.03-0.08% and 0.9-1% respectively) and bovine milk possessed highest sn-3 concentration caproic acid (almost 8%). According to Blasi et al. (2008), amount of $C_{6:0}$ at sn-1/sn-2 position in bubaline milk was not detectable, so $C_{6:0}$ is mainly esterified at sn-3 position.

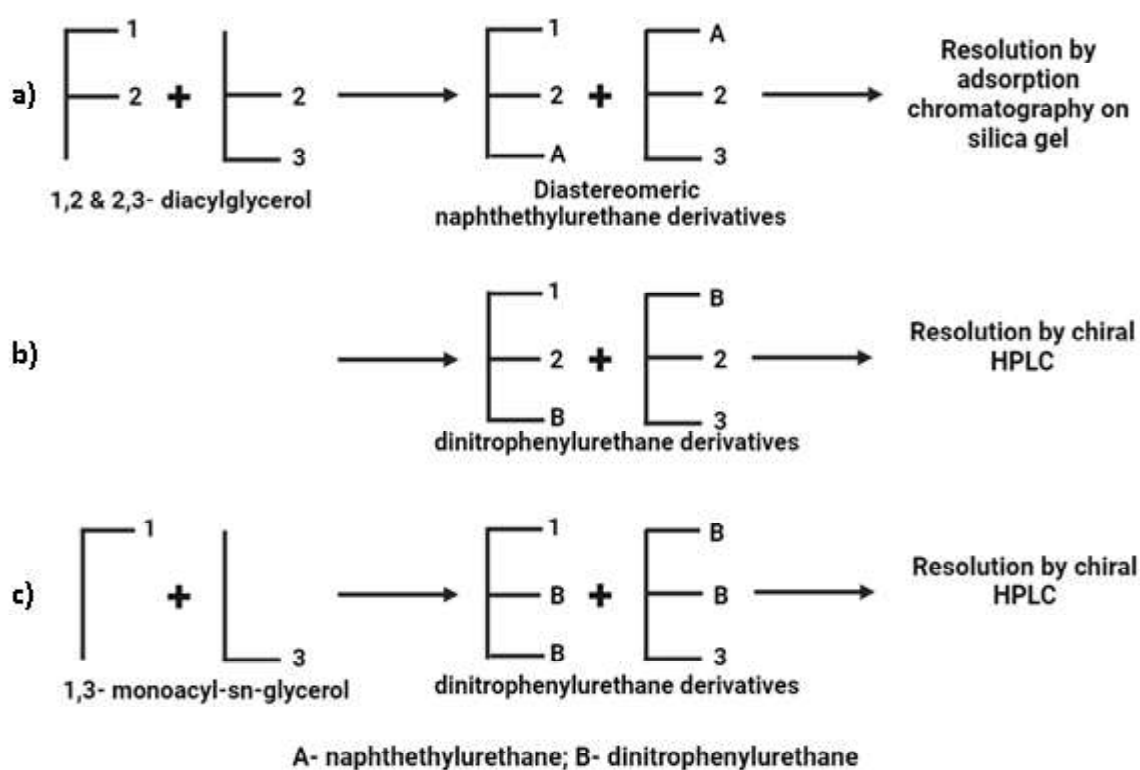


Fig 3. Stereospecific analysis of triacylglycerols via di- and monoacylglycerol for chiral chromatography. a) Preparation of diastereomeric naphthethyl urethane derivatives of diglycerol and resolution by HPLC in the adsorption mode. b) Preparation of dinitrophenyl urethane (DNPU) derivatives of diglycerol and resolution by chiral HPLC. c) Preparation of DNPU derivatives of monoacylglycerols and resolution by chiral HPLC

Equine milk has higher amount of caprylic acid ($C_{8:0}$) at sn-3 position, 9-10% of total sn-3 fatty acids, while in human milk its just 0.07-0.1% of total sn-3 fatty acids. At sn-1 and sn-3 positions concentration of $C_{8:0}$ is very minimal and almost similar in all species (Blasi et al. 2008; Karrar et al. 2022).

Capric acid ($C_{10:0}$) represented almost 18-28% in equine and 13-30% in caprine milk fat, where in human milk its only 1-2% of total sn-3 fatty acids. In bovine and bubaline milk, the percentage is 9% and 6% of total sn-3 fatty acids respectively. $C_{10:0}$ is also consists almost 8% of total sn-2 fatty acids in caprine milk fat but only 1% in bovine milk fat (Karrar et al. 2022).

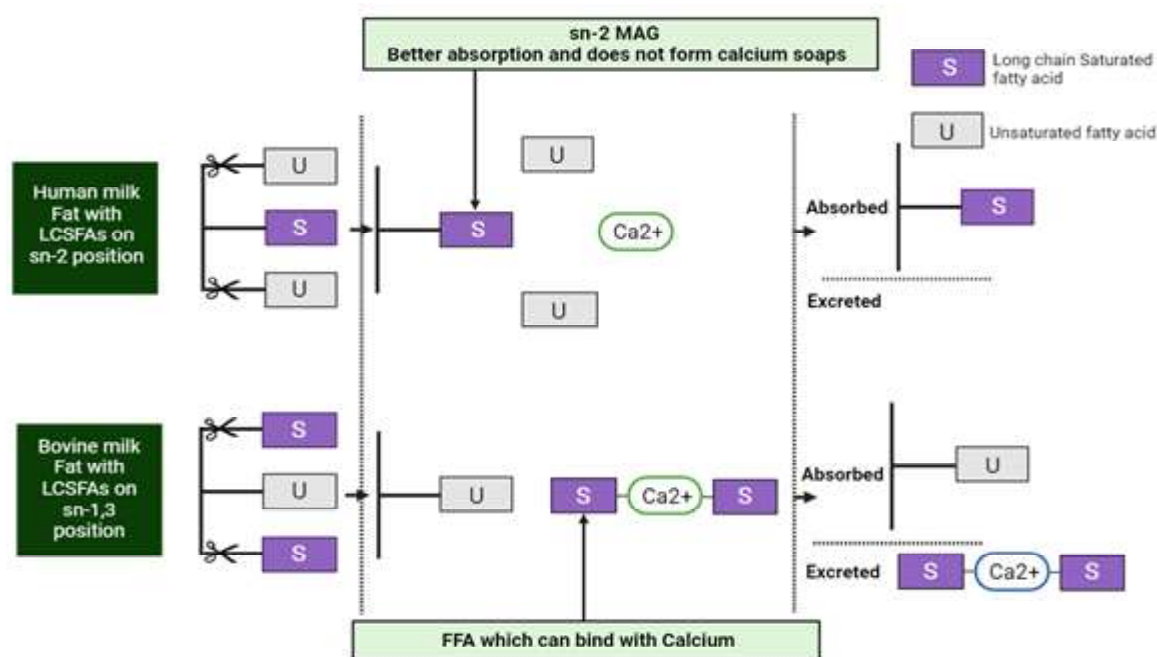
Lauric acid ($C_{12:0}$) in human milk first preferentially esterified at sn-3 position and second preferentially esterified at sn-2 position. Concentration of lauric acid in different species varies in sn-2 position. It represents almost 13% of total sn-2 FAs in Equine milk fat, where in bovine and bubaline milk it is only 3-4%.

Myristic acid ($C_{14:0}$) considerably higher in bovine milk (10-22%) but lower in human milk (7-9%) at sn-2 position (Blasi et al. 2008). At sn-3 position its percentage is too less in human milk, while in bubaline milk its almost 18% of total sn-3 fatty acids.

Distribution of long-chain saturated fatty acids in human, bovine, bubaline, caprine, and equine milk fat

Palmitic acid ($C_{16:0}$) is the main saturated fatty acid esterified at the sn-2 positions (Table.3). For $C_{16:0}$ at the sn-2 position, notable differences were observed between different species, ranging from 52-74% of total sn-2 fatty acids in human milk fat to 12.1-13.4% in equine milk fat. In human milk fat palmitic acid at the sn-1 position is only 9.7-10.2% where in case of bovine and bubaline milk fat palmitic acid is higher, 45.7-46.8% and 51.5% respectively, of total sn-1 fatty acids. In human body, when pancreatic lipase hydrolyses the milk fat, this sn-1 or sn-3 positional palmitic acid is easily separated, due to lipase's positional specificity. This separated palmitic acid exhibits poor absorption in its free form. May be because of their melting point is higher than the human body temperature. This palmitic acid reacts with the available calcium or magnesium in the intestine, forming insoluble soaps. These calcium or magnesium soaps cannot be absorbed by the human body and are excreted through the stool. This phenomenon leads to stool hardening or constipation and calcium excretion and increased stool fat, which can pose as a significant health challenge in infants (Linderborg & Kallio, 2005; Mehrotra

Fig. 4 The sn-2 positioning of fatty acids in TAG of human milk and bovine milk



et al. 2019). The effect of sn-2 positioning of fatty acids in human milk and bovine milk has been pictorially summarised in Figure.4. In a crossover trial in 12 preterm infants conducted by Carnielli et al. (1995), they compared two formulas with similar fatty acid composition and mineral content, but differing in the positional distribution of palmitic acid (in sn-1/3 or sn-2 positions). The study found that infants fed the sn-1(3) palmitic acid formula had higher levels of myristic, palmitic, and stearic acids and calcium, while exhibiting lower levels of oleic and linoleic acids in their feces compared to infants fed the sn-2 palmitic acid formula.

Human milk contains almost 70% of its palmitic acid in the sn-2 position. Pancreatic lipase mainly hydrolyses the fatty acids in the sn-1 and sn-3 positions, leaving palmitic acid as a sn-2 monoglyceride, which is easily absorbed in the body. The sn-2 MAGs are more soluble and polar, which allows for passive diffusion and prevents the formation of insoluble soaps with calcium and magnesium. As a result, human milk causes less constipation and improves the absorption of calcium and magnesium (Lasekan et al. 2017; Mehrotra et al. 2019).

It has been found that an infant's PUFA metabolism is influenced by the distribution pattern of saturated fatty acids. According to studies, linoleic acid increased, long-chain PUFAs in chylomicron phospholipids reduced, and the percentage of sn-2 palmitic acid increased (Innis et al. 1997). In another trial, the formula fat containing sn-2 palmitic acid caused lower proportions of arachidonic and docosahexaenoic acid in chylomicron TAGs, LDL phospholipids, and cholesteryl esters compared with the formula containing palmitic acid in the sn-1 and sn-3 positions (Innis & Dyer, 1997).

Stearic acid ($C_{18:0}$) is mainly found at the sn-1 position (13-16%) in human milk, other species also exhibits a similar percentage as human milk, among them caprine milk has the highest percentage of $C_{18:0}$ at the sn-1 position (>17%) and equine milk has the lower percentage of $C_{18:0}$ at sn-1 position (2-3%) (Karrar et al. 2022).

Distribution of Monounsaturated fatty acids (MUFA) in human, bovine, bubaline, caprine, and equine milk fat

As per different studies, in mammalian milk palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1\ n-9}$) are major MUFA. In equine milk, the percentage of $C_{16:1}$ is higher in sn-1 position, where in human milk it is only ~1% at sn-1 position, comparatively higher in sn-2 position (1.6-2.5%). The most abundant MUFA is oleic acid ($C_{18:1\ n-9}$), which is abundant in sn-1 or sn-3 position. In human milk it ranges in between 40-45% at sn-1 and sn-3 position and 8-9% in sn-2 position. Bovine and bubaline milk has very similar type of distribution, while in caprine milk its ranges 19.2-43.4% at sn-3 position (Table.3.).

Distribution of Polyunsaturated fatty acids (PUFA) within human, bovine, bubaline, caprine and equine milk fat

Human milk has highest amount of PUFA within following species. Equine milk is nearer to it. In human milk, PUFA (Linoleic and linolenic acid) are abundant in sn-3 position. In human milk at sn-3 position, linoleic acid is almost 30-39%, while bovine, bubaline, and caprine milk fat its ranges between 2-4%, equine milk is little higher, 10-10.5% at sn-3 position (Table.3) (Bakry et al. 2020; Blasi et al. 2008; Karrar et al. 2022).

Table 3: Stereospecific distribution of fatty acids in milk fat triglycerides of different milch species

Species	Human			Bovine (cow)			Bubaline (buffalo)			Caprine (goat)			Equine (donkey)		
	sn-1	sn-2	Sn-3	sn-1	sn-2	Sn-3	Sn-1	Sn-2	Sn-3	sn-1	sn-2	Sn-3	sn-1	sn-2	Sn-3
C4:0	ND	ND	0.09-0.2	1.3-1.6	0.3-0.4	17.9-18.3	1.2±0.4	1.4±0.5	20.5±1.0	1.4-1.5	0.2-0.3	13.0-15.9	1.0-1.1	0.6	2.5-5.1
C6:0	ND	ND	0.03-0.08	0.3-0.35	0.9-1.0	7.6-8.2	ND	ND	2.1±0.5	0.4-0.5	0.7-0.8	6.2-8.6	0.3-0.5	0.1-0.4	0.9-1.0
C8:0	ND	ND	0.07-0.16	0.3-0.32	0.2-0.3	4.5-4.7	ND	ND	3.4±0.1	1.2-1.7	0.1-0.3	1.1-10.6	0.9-1.1	0.9-1.5	9.2-10.6
C10:0	0.4-0.6	0.2-0.38	1.43-2.38	1.1-1.4	0.98-1.4	8.0-8.7	0.7±0.1	0.9±0.5	5.6±0.7	2.0-4.9	3-7.9	13.6-30.2	3.6-4.4	7.2-8.2	18.7-28.5
C12:0	2.8-3.5	3.7-6.9	4.1-7.08	2.3-2.5	2.02-4.8	4.7-5.1	1.6±0.1	3.1±0.7	4.3±0.7	2.2-3.3	5.9-6.35	2.2-3.3	6.1-7.2	3.19-12.7	8.3-8.6
C14:0	2.5-3.7	7.3-9.8	0.6-1.15	11.2-11.7	10.25-22.8	8.0-8.2	9.8±0.6	16.7±0.9	10.9±1.1	7.5-9.0	17.2-18.6	1.2-4.9	7.8-8.0	12.1-13.4	3.7-6.2
C16:0	9.7-10.2	52-74	0.8-1.6	45.7-46.8	38-42	11.0-12.0	51.5±2.0	37-40	17.8±1.8	38.4-45.4	31.2-36.0	1.4-3.2	23.2-28.6	30-54	2.1-7.9
C16:1	0.9-1.3	1.6-2.5	1.1-1.8	1.4-1.5	1.2-2.5	1.8-1.9	1.6±0.1	2.8±0.1	2.0±0.1	0.5-1.0	0.5-0.8	0.2-2.8	5.5-10.71	0.6-5.6	3.9-6.1
C18:0	13.0-16.0	2.2-4.9	0.8-1.06	11.1-11.6	5.3-6.12	5.2-6.1	13.8±0.6	10.2±1.0	9.9±1.5	17.6-17.9	7.9-12.0	7.1-13.1	2.0-3.0	1.7-2.3	0.9-1.6
C18:1	41.2-43.0	8.1-9.8	38.7-45.2	21.5-22.8	15.7-17.6	25.1-27.3	18.1±2.1	21.0±0.9	21.2±1.3	17.5-21.7	18.8-23.0	19.2-43.4	19.2-26.4	14.9-19.2	13.2-21.4
C18:2	21-22	14.5-16.9	30.9-39.0	1.5-1.7	1.9-4.35	2.5-2.7	1.6±0.2	2.7±0.1	2.0±0.1	0.7-0.9	2.2-4.2	1.6-6.8	9.0-13.7	9.1-9.7	10.0-10.4
C18:3	0.73-0.83	1.1-1.4	2.5-3.5	0.1-0.3	0.2-0.3	0.2-0.3	0.2±0.0	0.3±0.0	0.2±0.1	0.3-0.5	0.3-0.4	0.1-0.6	6.3-6.8	4.2-4.3	4.1-5.7

Source: (Blasi et al. 2008; Chen et al. 2020; Cossignani et al. 2011; Haddad et al. 2012; Karrar et al. 2022; Marai et al. 1969; Myher et al. 1986; Roy et al. 2020; Straarup et al. 2006; Sun et al. 2018; Zhang et al. 2020).

MUFA and some PUFA can help to prevent cardiovascular and inflammatory diseases (Blasi et al. 2008; Ulbricht & Southgate, 1991). Thus, equine milk fat provides have better health benefits than other milks.

Overall, there were significant ($p < 0.05$) variations in stereospecific positioning of fatty acids in TAG among milk fat from different species. The variations in positional distribution of fatty acids in TAG between species could be attributed to the differences in genetic background, animal species, feeding, lactation stage, and season (Karrar et al. 2022).

Conclusion

There is difference between the genetic structure, feed habit, season, region, lactation periods, infant requirement in different mammalian species. These differences affect the composition and distribution of fatty acids in different stereospecific position of fatty acids in milk fat triglycerides. This review also highlights the effect of stereospecific arrangements of fatty acids in triglyceride on nutrition and digestion of milk fat. In addition, various methods used to identify the position of fatty acids in triglyceride molecules are briefly described. Where, the most commonly reported Thin-Layer Chromatography (TLC) coupled with Gas-Liquid Chromatography (GLC) technique is maximum time-consuming, but the less explored technique using Nuclear Magnetic Resonance (NMR) required very minimal time. The bovine, bubaline and caprine milk have LCSFAs at sn-1 position at a major concentration, which can cause stool hardening or constipation problem in infant body. In equine milk long chain saturated fatty acids are mainly esterified at sn-2 position which is similar to the human milk. Equine milk's MUFA and PUFA distribution (especially linolenic acid) among the three position of triglycerides is also nearer to human milk. In spite of its low-fat percentage, equine milk is beneficial for human consumption due to its higher essential fatty acid (EFA) percentage and lower SFA percentage at sn-1/3 position than other milks; it has a higher EFA% in sn-2 position. Depending upon fat globule size also caprine and equine milk has the better digestion capability than other species.

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The effect of *Ruscus Hyracanus* extract on physicochemical, microbial and organoleptic properties of kefir

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Abstract: Kefir is a traditional functional fermented drink with acidic-alcoholic properties. In this study, the effect of *Ruscus hyracanus* extract (0, 0.25%, and 0.5%) on the *physicochemical*, *microbial*, and sensory properties of kefir during 20 days of storage was evaluated. Total phenolic content (TPC) and antioxidant activity (AO) were evaluated using Folin-Ciocalteu, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) techniques. The results revealed that the stability and viscosity of kefir samples containing *Ruscus hyracanus* extract were higher than control samples. Increasing the amounts of *Ruscus hyracanus* extract had a significant effect on the TPC, AO, and lactic acid (LAB) numbers of enriched kefir ($P < 0.05$). The presence of *Ruscus hyracanus* extract had a significant effect on the L^* values of kefir ($P < 0.05$). In general, a kefir sample containing 0.5% *Ruscus hyracanus* extract was more acceptable in comparison with the other samples on the 20th day of storage.

Keywords: Antioxidant, Kefir, Lactic acid bacteria, Phenol, *Ruscus Hyracanu*

Introduction

Kefir is a fermented dairy product with acidic-alcoholic properties that is related to people living in the Caucasus mountains. The Kefir idiom has its origin from the word “kef” which means ‘pleasant taste’ in Turkish. Generally, kefir is produced from the

acidic and alcoholic fermentation of cow’s milk using kefir seeds as a starter. Kefir grains are slimy, elastic, cauliflower-shaped, and in different sizes that are composed of *Kluyveromyces*, *Saccharomyces*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Acetobacter* species (Spizzirri et al. 2022).

Fermented kefir usually contains ethanol, lactic acid, and CO₂ as major components and acetaldehyde, acetoin, and diacetyl as aromatic ingredients. Also, nutritious components such as amino acids, folic acid, vitamin K, vitamin B₁, and B₁₂ increment over the fermentation period. Kefir generally contains 0.5% to 2% alcohol, a different amount of fat, with an acidic, slightly rough yeasty taste and pH of around 4.0 (Montanuci et al. 2012). These days, kefir is popular because of the presence of bioactive metabolites that confer health benefits. Lactose intolerance improvement, antimicrobial activity, antioxidant activity, anti-inflammatory effect, wound healing, antitumor activity, and immune system modulation are health-promoting properties that have been attributed to kefir consumption (Perna et al. 2019; Znamirowska et al. 2017).

Kefir fortified with plant extract rich in bioactive components can enhance nutraceutical benefits and promotes functional characteristics. Kim et al. (2017) reported beneficial effect of *Linum usitatissimum* (flaxseed) extract on the growth and viability of kefir-isolated Lactic acid bacteria. Similarly, Atalar (2019) presented that the presence of hazelnut milk in kefir improved bioactive properties, nutritional values and viability of LAB microorganisms. Also, Perna et al. (2019) stated that the total phenolic content (TPC) and antioxidant activity of donkey kefir increased in fortified samples with honey and *Rosmarium officinalis* essential oil.

The *Ruscus hyracanus* L is a rhizomatous, perennial, and treasureless shrub and is widely found in Mediterranean countries, north Africa, eastern Europe, southwest Asia, and different provinces of Iran (Shamalizade Baii et al. 2017). The *Ruscus hyracanus* extract contains flavonoids, phenolic, phytosterols, saponins, triterpenoids, and linalool compounds (Baharfar et al. 2016), which have high antimicrobial (especially antifungal) and antioxidant activities (Dehghan et al. 2016;

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Hadzifejzovic et al. 2013). The *Ruscus hyracanus* extract has diverse medicinal properties such as appetizing, diuretic, vasoconstrict, anti-laxative, antibleeding, anti-nephritis, and anti-infection properties (Dehghan et al. 2016).

Consumer demand for fortified kefir has increased due to benefit from its health-giving properties. The objective of the present study was to evaluate the effect

of *Ruscus hyracanus* extract addition on physicochemical properties and microbial quality of fortified kefir during storage.

Materials and Methods

Materials

Cow milk (2/5% fat) was purchased from Pegah company in Tehran (Iran). The DVS kefir starter culture in lyophilized form was supplied by Christian Hansen (Horsholm, Denmark). The *Ruscus hyracanus* leaf was purchased from the local market in Tehran, Iran. All chemicals used in this research provide from analytical grade and were prepared by Merck (Germany) company.

Preparation of *Ruscus hyracanus* extract and Kefir manufacture

For the *Ruscus hyracanus* extract preparation, the method of Ok and Jeong (2012) was applied with slight modifications. Briefly, *Ruscus hyracanus* samples were cut and put onto drying trays in a freeze-dryer (Biotron, Gyeonggi, Korea). Then, 10 g of ground *Ruscus hyracanus* mixed with 200 mL of distilled water and filtered through Whatman No. 1 filter paper. Next, the extract was centrifuged and supernatant was filtered using a 0.22 µm nylon filter. Finally, the *Ruscus hyracanus* extract was dried by freeze-dryer. Kefir was prepared according to Glibowski and Zielińska (2015) with some modifications. Briefly, *Ruscus hyracanus* powder (0, 0.25%, and 0.5% w/v) and DVS kefir culture was added at a level of 0.04 g into 4 L pasteurized and homogenized milk with 2.5% fat. The flasks were incubated for 24 h at 25 °C. Then, kefir samples were mixed with a laboratory spoon and filled into 250 mL PET bottles. Kefir samples were stored at 4 °C for 20 days. Kefir samples were assessed on the 1st, 10th, and 20th days of storage and tests have been performed in triplicates.

Physicochemical assessments

The pH value of the sample was determined with a calibrated pH meter by directly putting the probe into kefir samples. Titrable acidity was determined by titratable the samples with 0.1 N NaOH (Dewi et al. 2020). The viscosity was assessed using a Brookfield Viscometer (RVDV-III, Ultra, USA) with LV3 spindle. The samples were poured into the measuring vessel and sheared from 1.0 to 500 (1/s) at 20°C (Liu and Lin, 2000). The centrifugal method described by Bensmira and Jiang (2012) was applied for the assessment of syneresis of kefir samples. To evaluate color parameters, L^* , a^* , and b^* indexes in kefir samples, the Hunterlab

instrument (UltraScanvis, US-Vis 1,310, USA) was applied. Lightness was determined between zero (black) to 100 (white), a^* was evaluated from + 127 (red) to -128 (green), and b^* was determined from + 127 (yellow) to -128 (blue) (Zonoubi and Goli, 2020). All tests were performed in three replications.

Total phenolic content and antioxidant activity measurement

At first, kefir samples were centrifuged at 7690 g for 20 min. Syringes filter with 0.45 µm (MSCA, Shanghai, China) was used for sample filtration. The total phenolic content (TPC) and antioxidant activity (AO) of kefir samples were determined according to the method of Bensmira and Jiang (2015) with some modifications. The TPC was evaluated by Folin-Ciocalteu method, and AO was evaluated based on the free radical reduction of 2,2-diphenyl-1-picrylhydrazyl and tests have been performed in triplicates.

Microbial analysis

For the enumeration of the *Lactic acid bacteria*, the dilutions of samples were plated in the MRS agar using the *Pour Plate method for counting the number of colony-forming units present in the liquid samples*. The plates were placed in a CO₂ incubator for 72 h at 37°C. The results were expressed as Log cfu/g (Vasheghani Farahani et al. 2022). Kefir samples were assessed on the 1st, 10th, and 20th days of storage and tests have been performed in triplicates.

Sensory analysis

Sensory evaluation was performed by 12 panelists (6 women and 6 men, aged 20-30) from graduate students of Tehran Azad University's Food Science and Technology Department (Tehran, Iran) who were trained about the properties of kefir products. Five-point hedonic scale ranging from 1 (dislike extremely) to 5 (like extremely) was applied by the panelist for sensory evaluation on the 20th days of storage (Rojas-Torres et al. 2021). All tests have been performed in triplicates.

Statistical analysis

Experiments were performed in triplicate, and the significant differences between means were analyzed using one-way ANOVA and LSD post hoc tests (SPSS, version 22, 2016). The nonparametric data were analyzed by applying the Kruskal-Wallis tests.

Results and Discussion

pH and titrable acidity measurements

The pH values and acidity of the different kefir samples during cold storage were reported in Fig. 1 (a, b). A decrease in pH goes in hand with an increase in the acidity during fermentation and

these changes were significant ($p < 0.05$). However, statistical analysis cleared that the presence of *Ruscus hyracanus* extract decreased the pH of kefir samples insignificantly ($p > 0.05$). The kefir sample containing 0.5% *Ruscus hyracanus* extract had the highest acidity on the 1st day, and the lowest acidity belonged to the control sample on the 1st day. The previous studies have stated the increased trend in acidity due to the lactic acid bacteria growth, fermentation of lactose and production of lactic acid, and other organic acids (Vasheghani Farahani, 2022). Aiello et al. (2020) reported a reduction in the pH of lentil-supplemented kefir after 14 days of storage. Okur (2022) reported the increase in acidity in fortified kefir when olive leaf extract was used.

Viscosity and syneresis measurements

The viscosity of kefir samples was in the range of 4.017 to 6.387 (cP) which was higher than the 3 (cP) reported by Saygili et al. (2022) at 20°C. Kefir viscosity is influenced by milk physicochemical properties like dry matter, fat, protein concentration, pH, acidity, and temperature. Moreover, the assessment conditions of viscosity comprising shear rate, temperature, and spindle number are important (Haji Ghafarloo et al. 2019). As it is shown in Figure 2a, the addition of *Ruscus hyracanus* extract and the storage time caused significant increase in the viscosity of the kefir samples ($p < 0.05$). The viscosity of kefir may be influenced by carbohydrates contained in *Ruscus hyracanus* extract and the water absorption properties of these components (Edziri et al. 2020). Moreover, the protein-protein interaction and links between the proteins rearranging

due to the decrease in pH value during the storage period of kefir increased viscosity. Also, the viscosity might be raised due to the increment of the water-binding capacity of proteins after moving away from the isoelectric pH value (Bulut et al. 2021). As has been reported by Maleki et al. (2021), increasing the amount of free and microencapsulated extract of *Tragopogon collinus* caused a significant increment in the viscosity of probiotic yogurt. Also, Bulut et al. (2021) reported that the apparent viscosity of the fortified set-type yogurt with different plant extracts increased during storage.

The syneresis data for kefir samples are revealed in Figure 2b. Aqueous phase separation in fermented milk products occurs due to the accumulation of protein particles during storage and their deposition under gravity. Some other factors such as stabilizers, acidity, solids, and the type of milk and culture can be effective in the formation of the aqueous phase of fermented sweet drinks (Montanuci et al. 2012). In general, the addition of *Ruscus hyracanus* extract decreased the syneresis in kefir samples significantly ($p < 0.05$), but the difference between treatments on the 1st day was not significant ($p > 0.05$). Reduction in the syneresis level of *Ruscus hyracanus* extract in kefir samples was related to higher total solids and increased interactions between *Ruscus hyracanus* carbohydrate and protein particles. Also, *Ruscus hyracanus* extract contains higher phenolic components which react with casein and whey proteins and improve the kefir stability (Gomes et al. 2023). As has been reported by Znamirska et al. (2017), adding garlic powder (1% w/v) to kefir reduced syneresis significantly ($p < 0.05$). Similarly, Gomes et al.

Table 1: The effect of *Ruscus Hyracanus* extract on the color indexes (a) L* (b) b* and (c) a* of kefir during 20 days of storage ^{a,b,c}

Samples	L*		
	Day1	Day 10	Day 20
Control	88.33 ± 0.2 ^{Aa}	88.22 ± 0.06 ^{Aa}	85.17 ± 0.07 ^{Ab}
T ₁	85.53 ± 0.7 ^{Ba}	85.84 ± 0.3 ^{Ba}	84.87 ± 0.4 ^{Bb}
T ₂	85.53 ± 0.3 ^{Ba}	85.67 ± 0.05 ^{Ba}	84.2 ± 0.5 ^{Bb}
b*			
Control	2.37 ± 0.1 ^{Aa}	2.33 ± 0.05 ^{Aa}	1.29 ± 0.3 ^{Ab}
T ₁	2.67 ± 0.06 ^{ABa}	2.48 ± 0.09 ^{Aa}	1.43 ± 0.08 ^{Ab}
T ₂	3.04 ± 0.04 ^{Aa}	2.96 ± 0.2 ^{Aa}	1.8 ± 0.05 ^{Ab}
a*			
Control	0.456 ± 0.08 ^{Aa}	0.303 ± 0.3 ^{Ab}	-0.553 ± 0.1 ^{Ac}
T ₁	0.826 ± 0.06 ^{Aa}	0.363 ± 0.05 ^{Aab}	-0.253 ± 0.4 ^{Ab}
T ₂	1.28 ± 0.02 ^{Aa}	0.91 ± 0.1 ^{Aab}	0.273 ± 0.08 ^{Ab}

^aSamples were included (Control (0% *Ruscus Hyracanus* extract), T₁ (0.25 *Ruscus Hyracanus* extract), and T₂ (0.5% *Ruscus Hyracanus* extract))

^bMeans within each column followed by different letters (A–D) show significant difference ($P < 0.05$) between treatments at the same time

^cMeans within each row followed by different letters (a–b) show significant difference ($P < 0.05$) at a treatment during storage period

(2023) reported that adding *Moringa oleifera* leaf extract to the yogurt matrix diminished the increased level of syneresis significantly ($p < 0.05$).

Color analysis

The basis of colorimetry is the measurement of L^* , a^* , and b^* indexes which indicate white to black, green to red, and blue to yellow respectively. Table 1 shows the effect of different concentrations of *Ruscus Hyracanus* extract and storage time on the investigated color parameters. By increasing the amount of *Ruscus hyracanus* extract, a significant decrease was observed in the brightness of kefir samples ($p < 0.05$). Presumably, the presence of dark compounds in the *Ruscus hyracanus* extract could diminish the brightness of the product. According to Table 1, with the increase of storage time, the L^* index of the kefir samples decreased significantly ($p < 0.05$). The reduction of the L^* index during storage may be related to increasing proteolysis and lipolysis reactions and changes in the structure of kefir protein and fat globules as well as color substances during the storage period. Following the trend as observed by Znamirska et al. (2017), who reported L^* value decreased in kefir in the presence of garlic powder (1% w/v).

Table 3 shows the effect of *Ruscus hyracanus* extract and storage time on the b^* parameter. By increasing the amount of *Ruscus hyracanus* extract, no significant effect was observed in the yellowness of kefir samples. As results revealed the b^* value was decreased significantly during storage ($p < 0.05$). Presumably, the reduction in the intensity of the yellow color during storage was due to the degradation of yellow pigments of kefir samples. Contrary to these results Ardalanian and Fadaei (2018) reported all probiotic dough samples containing ginseng extract exhibited higher b^* values. Table 3 shows the effect of different percentages of *Ruscus hyracanus* extract and storage time on a^* parameter. The results showed that only on the 1st day with the increase of *Ruscus hyracanus* extract to 0.5%, the a^* parameter tended towards the red color, but on the 20th day with the increase of *Ruscus hyracanus* extract, this parameter tended towards the green color, which was not significant ($p > 0.05$). Probably, the *Ruscus hyracanus* extract contains compounds producing red color, but these pigments are not stable over time and only increase the redness on the first day of storage. Similarly, Felfoula et al. (2017) observed regardless of the ginger addition intensity of the bovine milk increased.

Total phenolic content and Antioxidant activity

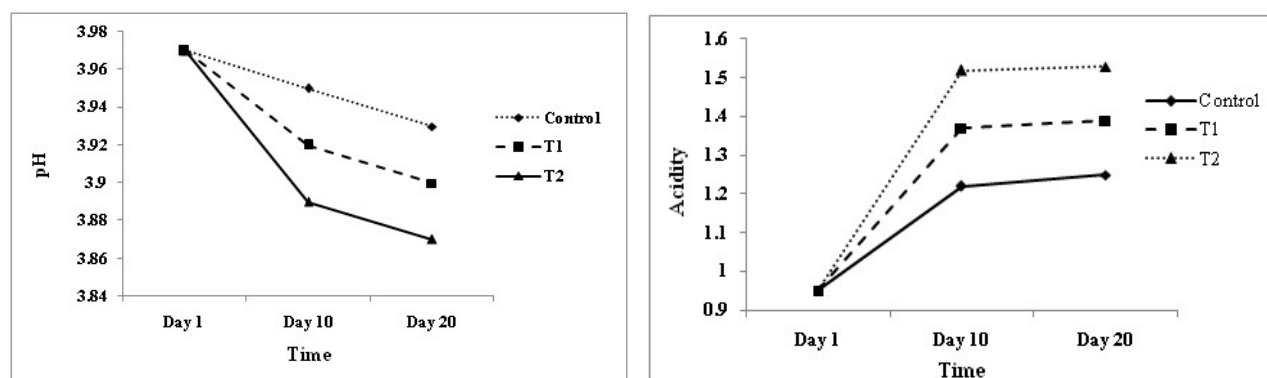


Fig. 1 The effect of *Ruscus Hyracanus* extract on pH (a) and acidity (b) of kefir during on 20th day of storage

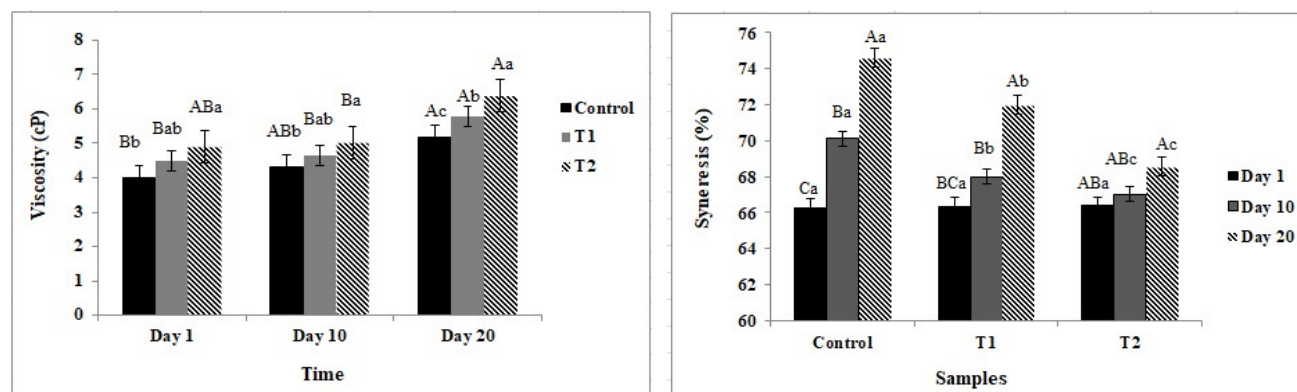


Fig. 2 The effect of *Ruscus Hyracanus* extract on the viscosity (a) and syneresis (%) (b) of kefir during 20 days of storage

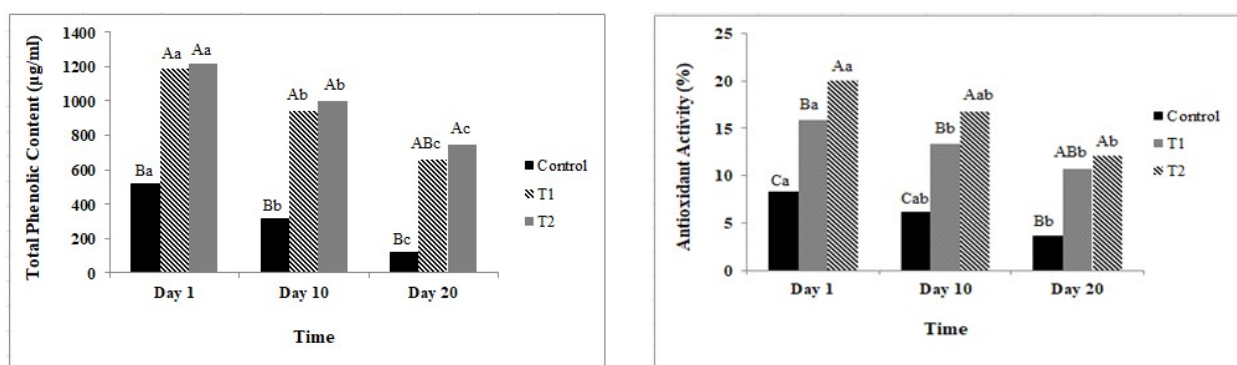


Fig. 3. The effect of *Ruscus Hyracanus* extract on total phenolic count (mg GAE/g) (a) and antioxidant activity (DPPH (%)) of kefir samples on 20th days of storage

Table 2. The effect of *Ruscus Hyracanus* extract on the Lactic Acid Bacteria (LAB) count of kefir during 20 days of storage ^{a,b,c}

Samples	Viable Lactic acid bacteria count (Log cfu g ⁻¹)		
Day	Day1	Day 10	Day 20
Control	7.2 ± 0.2 ^{ABa}	7.3 ± 0.08 ^{ABa}	7.36 ± 0.2 ^{Ba}
T ₁	7.38 ± 0.09 ^{Aa}	7.43 ± 0.3 ^{Aa}	7.46 ± 0.04 ^{ABa}
T ₂	7.44 ± 0.3 ^{Aa}	7.49 ± 0.07 ^{Aa}	7.53 ± 0.07 ^{Aa}

^aSamples were included (Control (0% *Ruscus Hyracanus* extract), T₁ (0.25 *Ruscus Hyracanus* extract), and T₂ (0.5% *Ruscus Hyracanus* extract))

^bMeans within each column followed by different letters (A–D) show significant difference ($P < 0.05$) between treatments at the same time

^cMeans within each row followed by different letters (a–b) show significant difference ($P < 0.05$) at a treatment during storage period

The AO and TPC of fortified kefir in the presence of *Ruscus hyracanus* extract are presented in Fig. 3 (a, b). It can be observed that using *Ruscus hyracanus* extract in kefir increased the TPC and AO of kefir samples, and these increments were related to increasing in *Ruscus hyracanus* concentration. As presented in Fig. 3 (a, b), the incorporation of *Ruscus hyracanus* extract significantly enhanced the AO activity from 8.3 in the control sample to 20.1 in the kefir containing 0.5% *Ruscus hyracanus* extract. Antioxidants as a chemical ingredient could maintain the human body from progressive diseases by preventing oxidative damage through scavenging free radicals. *Ruscus hyracanus* extract as natural antioxidant could absorb free radicals and improve the antioxidant properties of kefir. The presence of flavonoid glycosides, p-coumaric acid, amides of hydroxycinnamic acids, and phenolic acids in *Ruscus* species led to increasing in AO activity of fortified kefir (Hadzifejzovic et al. 2013). Hadzifejzovic et al. (2013) found that the methanolic extract of *Ruscus aculeatus* L. and *Ruscus hypoglossum* revealed antioxidant activity and there is a relatively strong correlation between the total phenolic content and the antioxidant capacity of plants' extracts. Jakovljević et al. (2016) reported the antioxidant activity of *Ruscus* species and their potential for inhibiting lipid peroxidation. Moreover, the results showed that the AO activity decreases significantly in all fortified kefir treatments over storage.

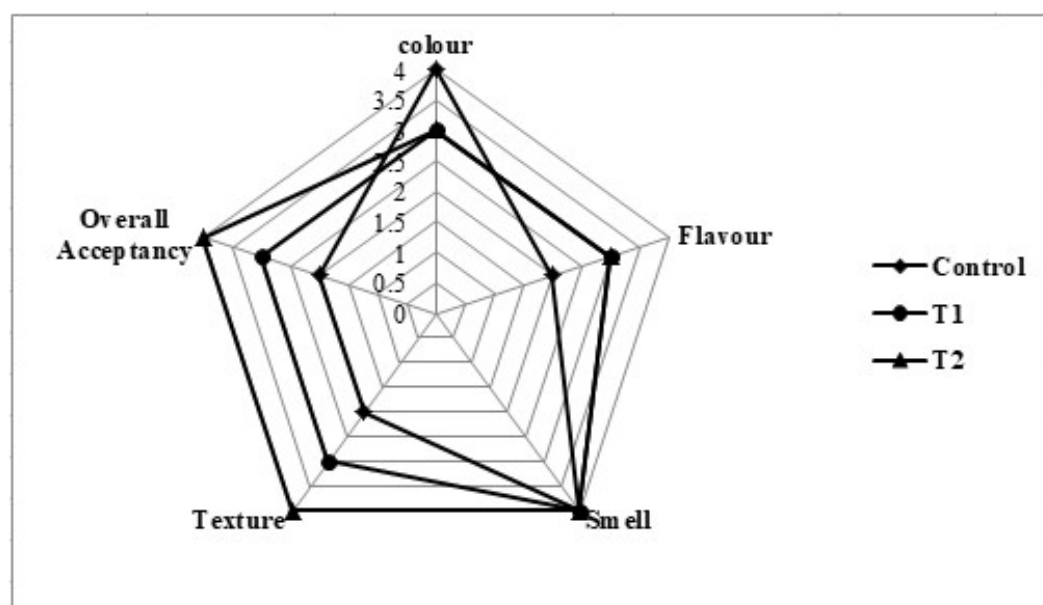
Presumably, the phenolic component in plant extract was changed through oxidation reactions during storage and decreased the AO activity. Similarly, Soliman and Nasser (2022) found that the AO activity reduced significantly in stirred yogurt samples with the increment in the storage period.

Changes in survivability of Lactic Acid Bacteria

Table 2 shows the effect of *Ruscus hyracanus* extract and the storage time on the variation of LAB numbers in kefir samples. The results showed that the presence of *Ruscus hyracanus* extract improve the viability of LAB significantly compare to the control samples ($p < 0.05$). As seen in Table 2, the highest and lowest population of LAB was related to the sample containing 0.5% *Ruscus hyracanus* extract on the 20th day and the control sample on the 1st day, respectively. From these observations, it can be concluded that LAB used *non-digestible carbohydrates* for their growth and development, which caused their population to increase. According to the present study, some researchers investigated that *Ruscus hyracanus* extract increased LAB microorganisms in dairy drinks. The obtained results of LAB survivability were consistent with the acidity results. Ghosi Hoojaghan et al. (2022) reported that fennel extract could improve the growth of LAB in Iranian doogh. Similarly, Ardalanian and

Fig.4. The effect of *Ruscus Hyracanus* extract on sensory properties of kefir samples on 20th

days of storage



Fadaei (2018) reported that the viability of *Lactobacillus acidophilus* (La5) and *Bifidobacterium lactis* (Bb12) increased by the addition of Ginseng extract in the probiotic Doogh. According to Table 2, with increasing storage time, the population of LAB had insignificant changes ($p>0.05$). It was observed that the highest number was on the 20th day and the lowest was on the 1st day of storage. Contrary to these results Haji Ghafarloo et al. (2019) stated that the number of *Bifidobacterium Bifidum* decreased significantly during 30 days of storage of symbiotic doogh.

Sensory properties

Figure 4 reveals results of comparing the sensory properties of kefir reported by panelists on the 20th day of storage for color, flavor, smell, texture, and overall acceptability. Regarding the smell parameters, all samples revealed the same acceptance and had no significant difference on the 20th day. The T₁ treatment with 0.25% *Ruscus hyracanus* extract and T₂ treatment with 0.5% *Ruscus hyracanus* extracts had higher flavor scores compared to control samples. The presence of *Ruscus hyracanus* extract increased the flavor score significantly ($p<0.05$). This revealed that *Ruscus hyracanus* extract covers undesirable flavor created in the kefir over storage due to the presence of phenolic compounds in *Ruscus hyracanus* extract. Similarly, Haji Ghafarloo et al. (2019) stated that doogh samples with 0.25% ginger extract obtained higher flavor scores than the samples without extract. Regarding the color parameters, the highest color score was related to the control sample without *Ruscus hyracanus* extract ($p<0.05$). It seems that reducing brightness, yellowness, and redness in the treated kefir samples is effective factor in reducing the color score. Our findings are in agreement with the research of Ghosi Hoojaghan et al. (2022) who noted that the presence of fennel extract decreased the color score of treated doogh. As

Figure 5 revealed adding *Ruscus hyracanus* extract increased the texture score significantly ($p<0.05$). The highest texture score was related to the T₂ sample while the control sample had the lowest texture score. Presumably, the distinction of panelists related to the changes in the viscosity characteristics of samples. Similarly, Glibowski and Zielińska (2015) stated the texture score between set-type kefir with and without inulin did not differ. The overall acceptance score of kefir samples revealed that there was a significant difference between samples ($p<0.05$). The highest overall acceptance score belong to T₂ treatment with 0.5% *Ruscus hyracanus* extract and control samples had the lowest overall acceptance. Ardalanian and Fadaei (2018) reported an increase in the overall acceptance score of doogh enriched by ginger extract ($p<0.05$).

Conclusions

In this research, a new functional kefir by applying *Ruscus hyracanus* extract in kefir formulation was assessed. The results of this study showed a significant increment in the TPC and AO activity of kefir in the presence of *Ruscus hyracanus* extract. According to the results, adding *Ruscus hyracanus* extract at the level of 0.5% increased the number of LAB significantly. The kefir sample containing 0.5% *Ruscus hyracanus* extract had the highest number of LAB and overall acceptability score in treated samples. Generally, our results recommended the consumption of this enriched kefir as a functional product for progress in consumer health. Also, syneresis of enriched kefir decreased, kefir stability increased, and the viscosity of treated samples improved. Moreover, adding *Ruscus hyracanus* extract had a proper effect on the sensory properties of the kefir samples.

Acknowledgments

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Process optimization for the manufacturing of flax seed powder enriched *Kalakand*

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Abstract: *Kalakand* is made traditionally, with no concern for the quality of the raw materials as well as no functional properties. This product is consumed by a large sector of population, hence enriching it with flaxseed could impart many functional properties. Flaxseed is well-known for its nutritional benefits. It is recognized for its high quantities of alpha-linolenic acid, lignan, a phytoestrogen molecule, and soluble as well as insoluble fibre. Jaggery includes 60-85% sucrose, 5- 15% glucose and fructose, 0.4 % protein, 0.1 g fat, 0.6 to 1.0 g minerals (8 mg calcium, 4 mg phosphorus, and 11.4 mg iron), traces of vitamins and amino acids, and around 383 Kcal of energy per 100 g of jaggery. Flax seed is a functional food and generally help in treatment of numerous diseases. In terms of sensory qualities compared to the other treatments, T1 (with 1% flaxseed powder) was observed to have the required quality. The results show that enriched *Kalakand* was analyzed to have decreased moisture, carbohydrate, and pH while increased content of protein, fat, ash, total solids, acidity, and antioxidant activity as compared to the control. The heart and circulatory systems benefit from flaxseed in all forms. The another important component was jaggery, which is not only a natural sweetener but has been shown to reduce the risk of heart disease, cancer, control blood pressure, liver detoxification, cataracts, and many inflammatory changes, also.

Keywords: Antioxidant, flaxseed, jaggery, *Kalakand*, fatty acid

Introduction

Milk and milk products have long been recognised as important sources of key nutrients in the human diet. Milk products are very suitable vehicle for delivery of bioactive ingredients (Kaur et al. 2018). *Kalakand* is an important indigenous milk product that is appealing to all consumer classes. One of the native milk products, *Kalakand*, is made by heating milk, adding sugar, and finally using the right coagulant. Even though both types of milk had the same nutritional value, it was observed that *Kalakand* made from buffalo milk was more popular than *Kalakand* made from cow milk and acidic milk. Due to its higher fat content, buffalo milk is preferred to cow milk when making *Kalakand*, which is traditionally made with danedar *Khoa*. Furthermore, since citric acid and sugar have such a big impact on the product's texture and flavor, it is crucial to add the right amount to the mixture (David, 2015). Danedar *Khoa* is used in the preparation of *Kalakand* and *gourd burfi*, where granulation is highly valued (Keerthi et al. 2018). *Kalakand*, a whole milk concentrate, contains a good amount of lactose, minerals, fat, and energy-giving proteins. On a per-unit weight and calorific value basis, it is 4-6 times more nutrient-denser than milk (Arora & Chandra, 2015). One of the most well-known sweets made from *Khoa* is called *Kalakand*; it is made from *Khoa* and sugar and has a flavour that is mildly caramelised and pleasant. The sensory qualities, nutritive values, and physico-chemical properties of *Kalakand* are impacted by the addition of flaxseed (Marpalle et al. 2014).

Flaxseed (*Linum usitatissimum*), also known as linseed, is frequently referred to as *Alsi*, *Jawas*, or *Aksebjia* in India (Anonymous, 2000) and is one of the most important oilseed crops for industrial applications (Singh et al. 2011; Chishty and Bissu, 2016). Flaxseed is an excellent source of fat (40 % of the content), fibre (28 % of the content), and proteins (10–30 % of the content) (Singh et al. 2011; Shim et al. 2014; Tripathi et al. 2013; Bartkowski, 2013; Soni et al. 2016). The addition of flaxseed components to food products has been shown to provide numerous health benefits (Kristina et al. 2018; Ambuja and Rajkumar, 2018). The high concentration of bioactive ingredients

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found in α -linolenic acid (ALA), lignans, phenolic compounds, and soluble fibre makes flaxseed a functional food (Gutte et al. 2015; Mishra and Verma, 2013; Tavarini et al. 2019). Previous research has shown that flaxseed has the potential to prevent a variety of illnesses, including colon and breast cancer, rheumatoid arthritis, obesity, cardiovascular disease (CVD), and osteoporosis. It can also strengthen the immune system (Bartkowski, 2013; Soni et al. 2016; Ambuja and Rajkumar, 2018; Campos et al. 2019). An anti-cancer phytoestrogen called lignans aids in reducing cell division (Singh et al. 2011; Chishty and Bissu, 2016; Mishra and Verma, 2013). In addition it has been proved to have anti-inflammatory, anticoagulant, and antiarrhythmic properties, ALA helps reduce blood lipid levels and promote infant brain development (Kajla et al. 2015; Gutte et al. 2015). Flaxseed currently has new opportunities as a functional food due to consumers' growing interest in foods that have outstanding health benefits. It has gained popularity as a desirable ingredient in diets created specifically for potential health benefits owing to its excellent nutritional profile (Oomah, 2001). Flaxseeds have been incorporated several dairy products in differential forms (Kaur et al. 2018). Microencapsulated flaxseed oil was used to be prepare *dahi* by Goyal et al. 2016. Ice cream was fortified by flaxseed oil by Goh et al. 2006 and the properties of oil were intact after freezing too. Milk, whey and butter have also been supplemented by flaxseed extracts or additives successfully at Industrial processing conditions (Hyvarinen et al. 006, Ivanov et al. 2011). Therefore, the present study has been undertaken to manufacturing of flax seed powder enriched *Kalakand* and to identify the most appropriate inclusion rate for flaxseed for an organoleptically acceptable product.

Materials and Methods

Materials

The whole fresh clean buffalo milk required for this investigation was collected from the Dairy farm under the Department of Dairy Science and Food Technology, Institute of Agriculture Science, Banaras Hindu University Varanasi and standardized to 6% fat and 9% SNF. Superior quality of flaxseeds and jaggery were procured from the local market of Varanasi. For the manufacture of *Kalakand*, Anhydrous Citric acid (SRL) was utilised as a coagulant was provided from the Department of Dairy Science and Food Technology, Institute of Agriculture Science, Banaras Hindu University Varanasi. A control sample was also prepared using sugar purchased locally.

Preparation of flaxseed powder

Flax seeds powder was prepared by manually cleaning dust and other extraneous particles. The cleaned flax seeds were processed in a dry mixer (Make: Philips) to make powder. Flax seed powder enriched *Kalakand* was made using this powder made from flax seed.

Treatment combination

For preparation of enriched *Kalakand* by using flaxseed powder, the treatment combinations were finalized on weight basis such as, the control (T0) was having 100 % Milk, T1 was having 1% flax seed, T2 was standardized to contain 2 % flax seed. T3 was having 3% flax seed while the amount of jaggery was kept constant in all the 3 treatments (6% jaggery along with milk).

Preparation of flax seed powder enriched *Kalakand*

The flax seed powder enriched *Kalakand* was prepared as per the method suggested by Aneja et al. (2002) and was followed with slight modification. A clean, dry iron karahi was filled with one litre of Buffalo milk that had been precisely measured. For direct heating, it was placed over a brisk, smoke-free fire on a pressure stove. Khunti was used to stir the milk continuously in a circular motion. After 10 to 15 minutes of boiling, 0.02 % solution of citric acid was slowly poured, which caused the milk to partially coagulate. To produce a product of high quality at this stage, vigorous stirring was necessary. To stop the charring of milk solids, solid material stuck to the pan's side had to be scraped off frequently with the sharp edge of the khunti. The Karahi's contents were heated and stirred continuously until it reached a semi-solid consistency of *danedar Khoa*. At this point, 6% of jaggery was added, thoroughly mixed, and then the addition of flax seed powder (@ 1, 2, and 3%). For four to five minutes, the Karahi's contents were heated on low heat while being constantly stirred. After that, the finished product was taken out and placed in a greased aluminum tray and then it was left to cool at room temperature. Once the finished product had cooled, it was weighed and stored in a refrigerator (Figure 1).

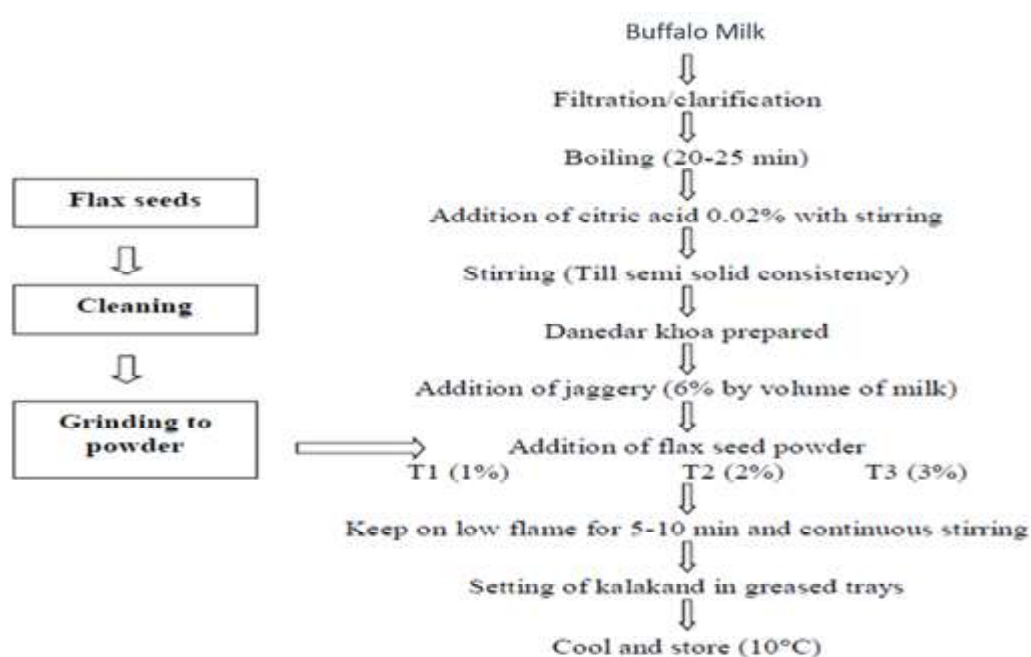
Optimization of flax seeds powder levels in *Kalakand*

Flax seeds powder levels were optimised by the sensory evaluation of *Kalakand* based on a panel of seven judges' approval on a 9-point hedonic scale score card. According to the sensory parameters, it was found that *Kalakand* with 1.0%, 2.0% and 3.0% of flaxseeds powder on 6% jaggery equivalence milk basis was acceptable. Sensory, physico-chemical, textural and microbiological analyses were examined for each of these three treatment combinations. Based on the product's sensory quality, the optimum product combination was chosen.

Physico-chemical analysis

The moisture content of *Kalakand* was determined using the gravimetric method outlined in the IS Handbook (SP: 18 (part XI) 1981). *Kalakand* fat content was calculated using the Soxhlet technique as described by AOAC (1995). The total protein content of *Kalakand* samples was determined by Micro Kjeldahl method as described by IS Handbook (SP: 18 (part XI) 1981). The ash content was determined as per AOAC, (2005). The total solid content of *Kalakand* was assessed using the IS Handbook (1479

Fig. 1 Flow diagram for Preparation of Flax seed powder enriched *Kalakand*



(Part-II) 1961) recommended method. The total carbohydrates content was calculated by subtracting the sum of the total major constituents such as moisture, protein, fat, and ash from 100. The titratable acidity of *Kalakand* was determined using the procedure outlined in the IS Handbook (SP: 18 (part XI) 1981). *Kalakand* sample was grated properly in pestle and mortar after which it was diluted using distilled water and the pH of *Kalakand* was determined at 25 °C using a digital pH meter (Elico digital pH meter).

Antioxidant activity

The Antioxidant activity was determined by DPPH method described in Ranganna (1986). The DPPH radical scavenging method is commonly used to assess the potential of natural antioxidants to scavenge free radicals. The determining factor for the reduction was the decrease in absorbance at 517 nm. The scavenging activity was determined as a portion of the scavenging impact using the formula:

$$\% \text{ RS Activity} = \frac{Ab - As}{Ab}$$

Where,

RS = Radical scavenging activity or % inhibition of DPPH

Ab = Absorbance of blank.

As = Absorbance of sample.

Texture profile analysis (TPA)

A texture profile analyser (Brookfield TA. XT Plus, UK) was used to assess the hardness, cohesiveness, adhesiveness, springiness, gumminess, and chewiness of *Kalakand*. The textural parameters were calculated using the force-time curve acquired for each sample, with the probe force on the Y-axis and time on the X-axis. The sample was compressed by the plunger twice (like two bites) and the force exerted back by the sample on the plunger was sensed by the machine, yielding a two-peak force – time curve. The following were the Texture Analyzer conditions for measuring textural properties: Pre-Test Speed: 1 mm/s; Post-Test Speed: 5 mm/s; Test Speed: 5 mm/s; Trigger Force: 10 g; Time: 5 sec. Each evaluation used a sample weighing between 100 and 150 g for texture examination. The temperature of the materials was kept at 25°C for the textural examination.

Microbial analysis

Control and flaxseed powder enriched *Kalakand* samples were kept in refrigerator (10°C) and samples were tested at 0, 2, 4, 6 and 8 days of storage for the following analytical parameters: microbiological examination including standard plate count, coliform count, and yeast and mould counts by IS: (SP-18, Part XI) (1981). For standard plate count using Plate Count Agar; incubation of the plates was done at 37 °C for 24-48 hours. For coliform count using Violet Red Bile Agar; incubation of the plates was done at 37 °C for 24 hours. For yeast and mould count using Potato Dextrose Agar (PDA); incubation of the plates was done at 30 °C for 48-72 hours.

Sensory analysis

The samples of *Kalakand* were judged by a sensory panel composed of seven semi-trained panelists selected from the staff and students of the Dairy Science and Food Technology, Institute of Agricultural Sciences, BHU, Varanasi. The product was presented to the panelists at 30°C (room temperature) in portions of 40–50 g. The samples were assessed using a nine-point hedonic scale (range from 9—like highly to 1—dislike exceedingly) for a number of characteristics, including colour and appearance, flavour, body and texture, taste and overall acceptability.

Statistical Analysis

The results of preliminary studies were expressed as mean. Statistical significance was tested by employing analysis of variance (ANOVA). For computation of data, software application programmes like Microsoft Excel and OPSTAT were used.

Results and Discussion

Kalakand was made using the flaxseeds powder at the rate of 1, 2 and 3%. Flaxseed powder enriched *Kalakand* was examined for physico-chemical analysis, antioxidant activity, textural profile analysis (TPA), microbial analysis and sensory analysis.

Optimization of flaxseeds powder in *Kalakand*

According to the sensory evaluation, flaxseeds powder significantly lowers sensory scores ($p \leq 0.05$) than the control when employed at levels of 1, 2 and 3 % in *Kalakand*. Flaxseeds powder in *Kalakand* with a concentration of 1 % scored better overall in terms of perceived taste and resemblance the control. Sensory evaluation includes the analysis such as colour and appearance, body and texture, flavor, taste and overall

acceptability. On the basis of sensory evaluation treatment T1 with 1.0 % flaxseed powder was finalized as the optimized product. In a study, it was found that addition of flaxseed powder in yogurt decreased the sensory parameters as compared to control (Mousavi et al. 2019).

Physico-chemical analysis

The data obtained for physico-chemical analysis of *Kalakand* are displayed in Table 1. The moisture content of experimental *Kalakand* samples decreased significantly ($p \leq 0.05$) when the level of flax seed powder was increased. The control T0 sample had significantly ($p \leq 0.05$) higher moisture content among all the *Kalakand* samples. T1 showed the highest moisture level among all the treatments but was lower than that of the control sample. This resulted from the inclusion of flaxseed powder, which contained less moisture. T3 showed the lowest moisture level. The fat content of experimental *Kalakand* samples increased significantly ($p \leq 0.05$) when the level of flax seed powder increased. T3, *Kalakand* made from 3 % flax seed powder, had a maximum fat content. This is due to the addition of flaxseed powder as it is an excellent fatty acid source; with high polyunsaturated fatty acids (PUFA) levels (73 %) especially Omega 3 fatty acids. T0 had the least amount of fat content as it does not contain flaxseed powder. The protein content of the *Kalakand* samples significantly increased in the following order: T0 < T1 < T2 < T3. T0 (control) had the lowest protein level. Treatment T3 had the highest protein level compared to the rest of the treatments. The carbohydrate level of *Kalakand* differed significantly ($p \leq 0.05$) and decreased in the order T0 > T1 > T2 > T3. The maximum total carbohydrate content in *Kalakand* was found in T0 with the addition of 100 % buffalo milk *Khoa* and the lowest total carbohydrate content was found in T3 with the addition of flax seed powder. The significant decrease in carbohydrate content with increasing flaxseed powder could be

Table 1: Physico-chemical analysis of *Kalakand*

Parameter	T0	T1	T2	T3
Moisture	24.1 ± 0.70**	23.16 ± 0.65**	21.90 ± 0.62*	21.70 ± 0.59*
Fat	20.77 ± 0.64*	21.46 ± 0.15*	21.87 ± 0.59*	22.63 ± 0.45**
Protein	16.69 ± 0.52*	16.75 ± 0.47*	17.68 ± 0.36**	17.97 ± 0.64**
Carbohydrate	39.02 ± 0.57**	37.85 ± 0.70*	37.45 ± 0.48*	37.14 ± 0.84*
Ash	3.23 ± 0.13*	3.61 ± 0.15**	3.88 ± 0.13**	3.95 ± 0.07**
Total solid	75.90 ± 0.7*	76.83 ± 0.65*	78.01 ± 0.62**	78.29 ± 0.57**
pH	6.39 ± 0.05**	6.20 ± 0.10**	5.90 ± 0.14*	5.730 ± 0.09*
Acidity	0.346 ± 0.03*	0.45 ± 0.04*	0.51 ± 0.02**	0.53 ± 0.02**
Antioxidant	10.12 ± 0.98 ^{NS}	23.85 ± 0.88 ^{NS}	31.86 ± 0.39*	43.49 ± 0.37**

**=0.001, *=0.05 Level of significance NS= Non-Significance, S.D. = Standard Deviation, Values are means of triplicate ± S.D.

T0- Control Sample; T1- 1.0% Flaxseed powder; T2- 2.0% Flaxseed powder; T3- 3.0% Flaxseed powder

attributed to the *Kalakand*'s slightly higher fat, protein and ash content. Masoodi and Bashir, 2012 also reported that there was a decrease in carbohydrate content on addition of flaxseed flour. The ash content of the *Kalakand* samples differ significantly ($p \leq 0.05$) and increased in the following order: $T0 < T1 < T2 < T3$. The treatment T0 having the lowest ash content while T3 having the highest ash content. With increasing ash content in *Kalakand*, the incorporation of flax seed powder in different treatments increased. This could be because flax seed powder has more ash content than buffalo milk *Khoa* and jaggery is also a source of iron. The ash, protein, fat and crude fiber increased in cookies on addition of flaxseed flour (Kaur et al. 2018). The total solid content of enriched *Kalakand* significantly differ in the increasing order such as $T3 > T2 > T1 > T0$. The highest total solid content in *Kalakand* was found in T3 with a combination of buffalo milk *Khoa* and 3% flax seed powder, whereas the lowest total solid content was found in T0 without flax seed powder (control). The variance in total solid content among the enriched *Kalakand* samples is closely related to their moisture content. The pH content of *Kalakand* was dramatically altered by the addition of flax seed powder. It was discovered that adding flax seed powder to *Kalakand* significantly lowers the pH level in the order of $T0 > T1 > T2 > T3$. The pH of T0, a *Kalakand* made from 100% buffalo milk *Khoa* without the addition of flax seed powder (control) was higher than that of T3, a *Kalakand* made from 100% buffalo milk *Khoa* with the addition of 3 % flax seed powder which had the lowest pH content. The acidity content of *Kalakand* was dramatically altered by the addition of flax seed powder. The acidity content of *Kalakand* was shown to increase as the amount of flax seed powder was increased in the order of $T3 > T2 > T1 > T0$. T0 (control) has the lowest acidity content and T3 has a higher acidity level. This could be because flax seed powder has a higher acidity level than buffalo milk *Khoa* *Kalakand*. A study says that using 0.25% flax seed powder enhances physicochemical, rheological as well as sensorial attributes of formulated yogurt (Kalyas & Urkek, 2022).

Antioxidant activity

The antioxidant status of the *Kalakand* samples increased significantly ($p \leq 0.05$) in the order $T0 < T1 < T2 < T3$, respectively. Samples T3 and T0 (control) had maximum and minimum antioxidant content, respectively. T1 had the least amount of antioxidant in treated samples. This could be due to the fact that T3 had more flax seed powder, which improved anti-oxidant activity. The main source of antioxidants in flax seeds is p-coumaric acid, a polyphenol. Ferulic acid, another antioxidant found in flaxseed, may help prevent a number of chronic diseases. Lignans, which are found in almost all plants and function as both antioxidants and phytoestrogens, are also present in flax seeds. When compared to other foods, flax seeds have up to 800 times as much lignan. This could be the cause of the increased anti-oxidant activity observed when flaxseed concentration rises relative to control. A similar finding was seen in a flax seed fortified yogurts

that states that the fortified yogurts had a higher antioxidant activity compared to the controls (Marand et al. 2020). Antioxidant activity increases due to high phytochemical content of flaxseeds (Kaur et al. 2018). Phytochemical properties include both antioxidant and total phenol content which increases significantly on addition of flaxseed in any forms (Kaur and Das, 2014, Rao et al. 2013).

Textural profile analysis (TPA)

Kalakand texture is a significant feature that influences consumer acceptance. Using the texture expert exceed software (v 2.55) provided by the manufacture along with the instrument, different textural qualities like as hardness, cohesiveness, adhesiveness, springiness, gumminess and chewiness were estimated for T0, T1, T2, T3, from the resulting force time curves. The texture profile analysis (TPA) of enriched *Kalakand* as well as control is illustrated in Figure 2. The most often used criterion for determining the texture of *Kalakand* is its hardness. It was observed that, when compared to other samples, sample T3 had the highest hardness which could easily be correlated to the lowest moisture and high total solid content of this sample. This indicates that adding flax seed powder to *Kalakand* boosts its hardness (Mousavi et al. 2018). *Kalakand*'s hardness is determined by several elements, including moisture content and mineral content. The ratio of the area under the second bite curve before reversal compression to the area under the first bite curve is known as cohesiveness. In terms of cohesiveness, the T3 *Kalakand* sample outperformed the other *Kalakand* samples, with the highest cohesion. The reason in support could be that the flaxseed shows influence on internal bonds like it did in case of yogurt structure, thus resulting in increased cohesiveness (Bhat et al. 2018). Adhesiveness is measured as a negative peak following the first peak and is connected to sensory stickiness. Sample T0 has the least amount of adhesion (control sample). The degree of springiness is determined by elements such as heat treatment and hardness. The results were obtained in terms of the springiness of association with an increase in flax seed powder content. However, in general the springiness of T3 samples was higher than T0 based on *Kalakand* sample. The addition of flaxseed to yoghurt enhances its textural integrity, which is why the increasing trend in springiness observed in the *Kalakand* containing flaxseed in comparison to the control sample (Mudgil et al. 2017). Gumminess is determined by multiplying the two fundamental characteristics of hardness and cohesion. The highest gumminess was found in the T3 sample, while the lowest values were found in the sample T0 (control). One of the most essential textural features of *Kalakand* is chewiness. The chewiness of *Kalakand* was greatly improved by adding flax seed powder. T1 had the least chewiness of all the samples, whereas T3 had the most. A study in which fortification was studied states that the addition of flax seed powder influences the textural attributes of the formulated product (Reeta et al. 2018). Similar results of texture analysis i.e. increase in

Fig. 2 Texture profile analysis of *Kalakand*. Values are mean of 3 Samples $\pm$ S.D.

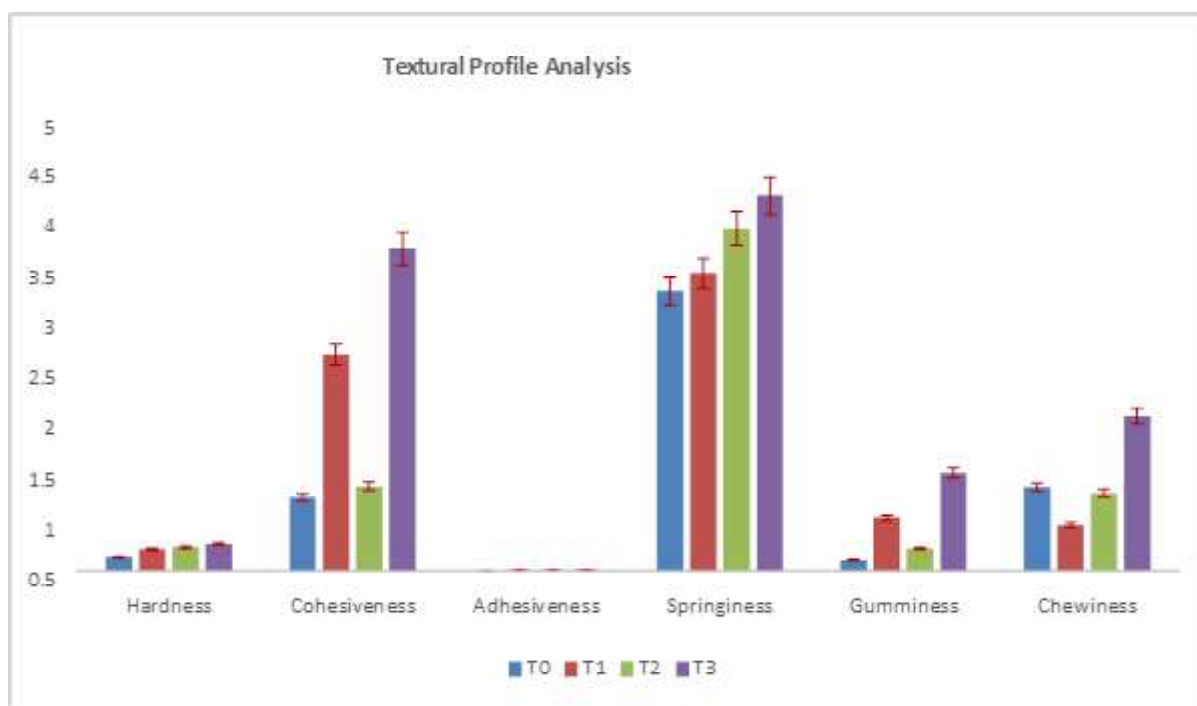


Table 2: Microbial analysis of *Kalakand*

Parameters (cfu $\times 10^3$ / gm)	Treatments			
	T0	T1	T2	T3
SPC	11.00 $\pm$ 0.68**	4.33 $\pm$ 0.57*	6.33 $\pm$ 0.87*	8.33 $\pm$ 0.45*
Yeast & Mould	6.66 $\pm$ 0.57**	2.33 $\pm$ 0.65*	3.66 $\pm$ 0.73*	4.33 $\pm$ 0.84*
Coliform	ND	ND	ND	ND

**=0.001, *=0.05 Level of Significance NS= Non-Significance, S.D. = Standard Deviation, ND = Not Detectable

The values shown are the average of 8 days of storage $\pm$ S.D.

Table 3: Sensory analysis of *Kalakand*

Treatments	T0	T1	T2	T3
Colour and Appearance	8.4 $\pm$ 0.50**	7.5 $\pm$ 0.64**	6.7 $\pm$ 0.71*	5.7 $\pm$ 0.92*
Body and texture	8.2 $\pm$ 0.63**	7.4 $\pm$ 0.67**	6.5 $\pm$ 0.56*	4.9 $\pm$ 1.30*
Flavour	8.5 $\pm$ 0.45**	7.5 $\pm$ 0.58*	6.6 $\pm$ 0.87*	5.3 $\pm$ 0.64 ^{NS}
Taste	8.3 $\pm$ 0.48**	7.6 $\pm$ 0.46*	6.5 $\pm$ 0.85*	5.2 $\pm$ 0.89 ^{NS}
Overall Acceptability	8.3 $\pm$ 0.11**	7.5 $\pm$ 0.03*	6.5 $\pm$ 0.04*	5.2 $\pm$ 0.28 ^{NS}

**=0.001, *=0.05 level of significance NS= Non-significance, S.D. = Standard Deviation The values mean of 7 replicates $\pm$ S.D.

hardness have been reported by various other researchers (Kaur et al. 2018).

Microbial analysis

Due to the entire dependence of product shelf life on the growth of microorganisms in the product during storage, the microbiological quality of dairy products like *Kalakand* becomes more substantial. As a result, microbiological analysis of the flaxseed blended *Kalakand* samples as well as untreated *Kalakand* (control *Kalakand*) was performed. Table 2 displays

the microbial analyses of the enriched *Kalakand* and the control sample. According to Table 2, both the control and the enriched *Kalakand* had higher total plate counts, yeast and mould counts, and coliform counts at storage days, although these numbers remained within the acceptable range until the 8th day of storage and this finding is in accordance to the results concluded by Jain et al. (2015). In samples T0 (control) and T1, the highest and lowest standard plate count (SPC) count mean values were 11.00 $\times 10^3$ CFU and 4.33 $\times 10^3$ CFU, respectively. The proportion of untreated samples in T1 was the lowest. This could be because the amount of flaxseed powder in T3 enhanced the number of

SPC in the treated samples. Yeast and Mould count were 6.66 and 2.33×10^2 CFU per g, individually in samples T0 and T1 (control). T1 revealed that treated samples had the lowest yeast and mold count. This could be because T3 contained more flaxseed powder, which increased the Yeast and Mould count. The Coliform test for both the control and experimental samples was 100% negative. It reveals that there were no gram-negative bacteria, indicating that high hygienic standards were followed during the preparation.

Balasova et al. 2018 studied the influence of flaxseed oil and meal on the activity and growth of yogurt culture. The researchers concluded that oil did not influence the growth of *Lactobacillus acidophilus* while the activity of *Streptococcus acidophilus* was affected as clear from lack of acidification. Whereas the addition of meals stimulated the growth of both microorganisms.

Sensory analysis

Table 3 displays the sensory analysis of the enriched *Kalakand* and control. The sensory scores for colour and appearance, body and texture, flavor, taste and overall acceptability of the *Kalakand* samples decreased in the following order: T0 > T1 > T2 > T3. The treatment T0 (control) received the highest rating for colour and appearance, body and texture, flavor, taste and overall acceptability. The treatment T3 received the lowest rating for colour and appearance, body and texture, flavor, taste and overall acceptability. Among the treated samples, T1 received the highest rating for colour and appearance, body and texture, flavor, taste and overall acceptability. The judging panel determined that Treatments T1, T2, and T3 were all suitable in terms of colour and appearance, body and texture, flavor, taste and overall acceptability of the product. The score for colour and appearance, body and texture, flavor, taste and overall acceptability decreases as the addition of flaxseed increased. A study had shown that the yogurt fortified with flaxseed powder was found to be acceptable in terms of organoleptic properties (Marand et al. 2020). Various studies are in line with current finding that although sensory scores decrease due to flaxseed fortification but the product is acceptable overall (Ramacharitar et al. 2005, Rangrez et al. 2014, Masoodi and Bashir, 2012, Bashir et al. 2012). The reason may be that the flaxseed impart a slightly nutty flavor to the product which increases its acceptability to certain extent (Ivanov et al. 2011).

Conclusion

Flaxseed powder can be successfully used to make *Kalakand*, according to the results of this study. T1 with 1 % flaxseed powder was determined to be the desirable in terms of sensory qualities among the other treatments. According to the results, addition of flaxseed powder in *Kalakand* reduced moisture, carbohydrate and pH while increasing protein, fat, ash, total solids, acidity and antioxidant activity in the treated product as compared to the

control. In treatment, *Kalakand* prepared with flaxseed powder T1 had the best organoleptic qualities and scored the highest score (colour & appearance, body & texture, flavour & taste, overall acceptability). Flaxseed in any form is beneficial to heart and circulatory health. The addition of flax seed powder in *Kalakand* is generally an increase in omega-3 fatty acid content of products and made the omega-3 fatty acid enriched product because flax seed is a great source of omega-3 fatty acid. Jaggery was the key ingredient, a natural sweetener that has been demonstrated to lower the risk of heart illness, cancer, control blood pressure, detoxification of liver, cataracts and several inflammatory changes.

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RESEARCH ARTICLE

Studies of Physicochemical, Microbiological and Sensory characteristics of Paneer (Indian Cottage Cheese) to check the efficacy of clove-oil nanoemulsion in enhancing its shelf-life

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Abstract: Paneer is a popular dairy product in India and is highly vulnerable to spoilage due to its high moisture and nutrient content. In the presented study paneer samples were treated with clove-oil nanoemulsion and its effectiveness in enhancing the shelf-life of paneer was studied by analyzing the physicochemical, microbiological and sensory characteristics of the stored samples. Paneer treated with clove-oil nanoemulsion showed the lesser significant increase ($P<0.05$) in extent of proteolysis and free-fatty acid content while preventing reduction in moisture and pH of treated samples in comparison to control. Microbiological analysis revealed that paneer samples treated for 15 min with clove oil nanoemulsion followed by packing in nanoemulsion treated High Density Polyethylene Film (HDPE) packaging film) showed reduction in Standard plate count, coliform and Yeast and mold count by 2.02, 2.43 and 1.88 log cycles respectively in comparison to untreated control paneer samples on 21st day of storage. Treated paneer samples were observed to elicit almost constant sensory scores throughout the storage period as compared to control which showed significant reduction ($P<0.05$) in sensory scores throughout the storage period.

Keywords: Clove-oil; Nanoemulsion; Paneer, Shelf-life

Introduction

India has grown tremendously in the field of dairy sector after the success of the world's largest dairy development program 'Operation-Flood'. Now India is not only self-sufficient but also the largest producer of milk in the world with the production of 230.58 million tonnes in the year 2022-23 (<https://pib.gov.in/PressReleasePage.aspx?PRID=1988609>). The surplus milk is being converted into wide range of value added dairy products such as curd, cheese, paneer, yoghurt and sweets etc. Paneer is one of the most popular and widely consumed traditional Indian dairy product (Kapoor et al. 2021). It is an un-ripened cheese produced by the method of acid and heat coagulation and almost 5% of the total milk produced in India is being converted into paneer. In 2021, International Market Analysis Research and Consulting (IMARC) group reported that market value of paneer was 365.5 billion INR and it was estimated to grow at the rate of 15% in the future. Paneer is manufactured by the method of acid and heat coagulation. It is a rich source of nutrients like proteins, vitamins, minerals and fat. Due to globalization as well as its increasing use as a meat substitute, the demand for paneer is continuously increasing. However, the rich moisture and nutrient content of paneer makes it highly vulnerable to spoilage. The spoilage of paneer is mainly attributed to growth of surface-tainting and off-flavor producing microorganisms such as coliforms, psychrotrophs, yeast and molds (Varalakshmi and Leysen, 2020).

Presently, preservation methods like brining, storage at refrigeration temperature, vacuum- based and addition of synthetic preservatives (benzoates, sorbates, H_2O_2 etc.) and antimicrobial packaging systems are being used to enhance the shelf-life of paneer (Sharma et. al, 2019). Due to the increasing awareness of consumer and their concern towards a healthy lifestyle, the demand for plant-based preservation methods is increasing day by day. Therefore, the urge to develop preservation methods that are safer, more sustainable and includes minimum use of preservatives is there (Shashikumar et al. 2011). In this view, US code of Federal Regulations has provided GRAS (Generally Regarded as Safe) status to a number of essential oils and associated bioactive components with them. Different research investigations have shown effective results in

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enhancement of shelf-life of paneer by the addition or incorporation of essential-oils for preservation (Badola et al. 2018). Clove oil extracted from the bud, leaf or stem of the spice *Syzygium aromaticum* is one of the most popular essential oil inherited with a wide range of therapeutic properties like antibacterial, antifungal, antiviral, anti-oxidative, anti-inflammatory and analgesic properties (Takahashi et al. 2021 ; Raju and Sasikala, 2016). Therefore conventional methods of preservation could be efficiently replaced by addition of antimicrobial clove essential oil and it could prove to be comparatively better, safer and more sustainable approach for the preservation of paneer. However, several challenges limit its usage when it is directly added to a food system like high volatility, effect on organoleptic properties, high sensitivity towards light, low solubility and destruction of their bioactive component when exposed to different processing conditions (Rani et al. 2014). Nanoencapsulation can prove to be an effective approach to deal with these challenges. It results in improvement of bioavailability due to large surface area of small particles, increases the stability, results in masking the unpleasant taste and also leads to proper release of bioactive component (Chawla et al. 2021). Therefore, to explore the potential of nanoencapsulated clove oil in enhancing the shelf-life of paneer, the present storage study was carried out-(Liao et al. 2019).

Materials & Methods:

Preparation of clove-oil nanoemulsion:

Clove-oil nanoemulsion of oil in water (O/W) type was prepared using non-ionic surfactant 'Tween-80' and 'Clove-oil'. Both the ingredients Tween-80 and clove-oil were added at the rate of 1% followed by making up the volume using distilled water. Mixing of appropriate concentration of ingredients was followed by homogenization using pilot-scale homogenizer with pressure of 2000 psi at first stage and 500 psi at second stage. The prepared clove-oil nanoemulsion was further diluted to 1:1 using distilled water for treatment of paneer.

Treatment of paneer samples:

Paneer blocks of 100 g each with uniform dimensions were obtained from Model dairy plant, ICAR-NDRI Karnal before dipping stage of manufacturing and were treated with prepared clove-oil nanoemulsion. Three different batches of paneer samples were prepared by treating paneer and its packaging material (HDPE) with clove oil nanoemulsion and chilled water for different intervals keeping the total time of dipping constant which was 40 min. First batch was prepared by dipping the paneer samples in chilled water for 40 min, 30 min and 25 min followed by treating with clove-oil nanoemulsion for 0 min, 10 min and 15 min respectively. Second batch was prepared by packing untreated paneer samples in packaging material dipped in clove-oil nanoemulsion (PM). Third batch consisted of paneer samples

dipped in chilled water for 30 min and 25 min and then treating with clove-oil nanoemulsion for 10 min (PM+10) and 15 min (PM+15 min) followed by packing in packaging material treated with clove-oil nanoemulsion. Untreated sample of paneer was kept as control.

Analysis of paneer samples treated with clove-oil nanoemulsion:

Paneer samples treated with clove-oil nanoemulsion were stored at 4°C and were analyzed for different physicochemical, microbiological and sensory characteristics at interval of every three days which was on 0th, 3rd, 6th, 9th, 12th, 15th, 18th and 21st day. All the experiments were performed in triplicates.

Storage Study: Analysis of physicochemical and Microbial parameters of prepared paneer samples treated with clove oil nanoemulsion:

Moisture content:

Moisture content of paneer samples was analyzed using the method of IS: SP 18 (Part XI) (BIS, 1981). About 2.0 g of paneer was weighed in an aluminium dish with a glass rod followed by complete mashing with 4.0 mL of distilled water. Another 1.0 mL distilled water was added to wash-off adhered particles and the aluminium-dishes were kept in hot air oven maintained at 102 ± 1°C for about 4 h. After 4 h, the dishes were transferred to desiccator then cooled and were weighed. Cooling and weighing cycles were repeated after every 30 min until a constant reading was obtained. Moisture content was calculated using the formula:

$$\text{Moisture (\%)} = (w1 - w2) / (w2 - w) \times 100$$

where,

w1 = mass in g of the dish with material before drying

w2 = mass in g of the dish with the material after drying

w = mass in g of the empty dish

pH:

To analyze pH of paneer samples 10.0 g of paneer was taken and was converted into paste using 10.0 mL distilled water and then pH was determined using calibrated pH meter.

Free-fatty acids:

Free-fatty acid content was ascertained using the method developed by Deeth and Fitz-Gerald (1975). About 5.0 mL distilled water was added in 3.0 g grated paneer sample taken in 60 mL stoppered test-tube. Followed by addition of 10 mL extraction mixture (isopropanol, petroleum ether and 4 N sulphuric acid in the proportion 40:10:1) and then 6.0 mL petroleum ether. This

was followed by tempering at 40! for 10 min and the two layers were allowed to separate. 5.0 mL of upper layer was taken and was titrated against 0.02 N methanolic KOH after addition of few drops of phenolphthalein indicator. The formula used to calculate the free-fatty acids is as given below:

$$\text{TFFA } (\mu\text{eq/g of fat}) = \frac{V \times N}{P \times W} \times 1000$$

where,

V= Net titration volume in mL,

N= Normality of methanolic potassium hydroxide solution

P= Proportion of upper layer of aliquot titrated (i.e. volume of aliquot withdrawn / total volume of upper layer)

W = Weight of the sample in g

TFFA=

Extent of proteolysis:

Extent of proteolysis that the paneer samples went through was determined by preparing water soluble extract by method developed by Kuchroo and Fox, (1982). Sixty mL distilled water was added to 20.0 g of paneer followed by sonication. The homogenous solution was centrifuged at 1400 g for 10 min and the upper layer was removed. Followed by this, the pH was adjusted to 4.6 using 1 N HCl and the extract was filtered through 0.22 µ filter. Further protein content was analyzed using the method given by Lowry et al. (1951).

Effect of treatment of clove-oil nanoemulsion on Microbiological-parameters of paneer:

Standard plate count (SPC):

SPC count of paneer samples was taken by pouring 1 mL paneer sample diluted from 10^{-1} to 10^{-5} in a petri plate followed by layering of nutrient agar under aseptic conditions and incubating at 37! for 24 h. Followed by this, colony count was taken and was recorded in log cfu/ mL (Donnelly, 1976).

Coliform count:

Coliform count of paneer samples was taken by pouring 1 mL paneer sample diluted from 10^{-1} to 10^{-3} in a petri plate followed by layering of violet red bile agar under aseptic conditions and incubating at 37! for 24 h. Followed by this, dark red coloured colonies were counted and were recorded in log cfu/ mL (Houghtby et al. 1992).

Yeast and mold count:

Yeast and mold count of paneer samples was taken by pouring 1 mL paneer sample diluted from 10^{-1} to 10^{-3} in a petri plate followed by layering of potato dextrose agar under aseptic conditions and incubating at 25! for 3-5 days. Followed by this, colony count was taken and was recorded in log cfu/ mL (Marshall, 1992).

Storage Study: Analysis of sensory characteristics of prepared paneer samples treated with clove oil nanoemulsion

Paneer samples were cut into cubes of size 2.0 cm x 1.5 cm x 1.5 cm and were evaluated by trained panelists comprising of scientists of NDRI using the 9 point hedonic scale.

Results and Discussion

Analysis of physicochemical and Microbial parameters of prepared paneer samples treated with clove oil nanoemulsion

Moisture content

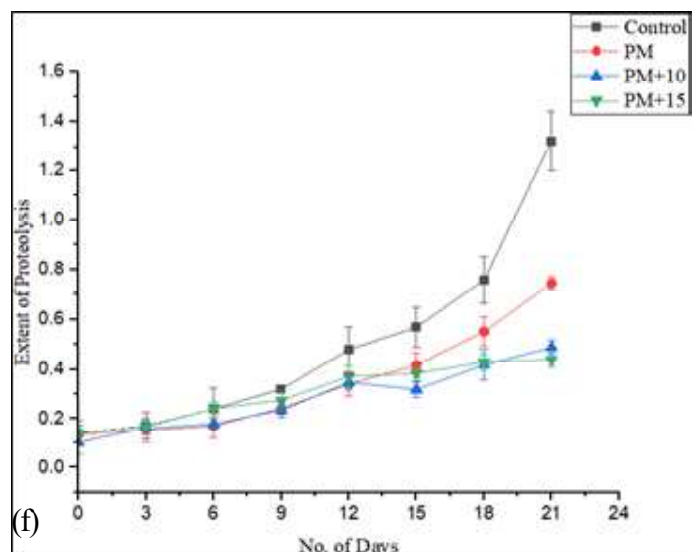
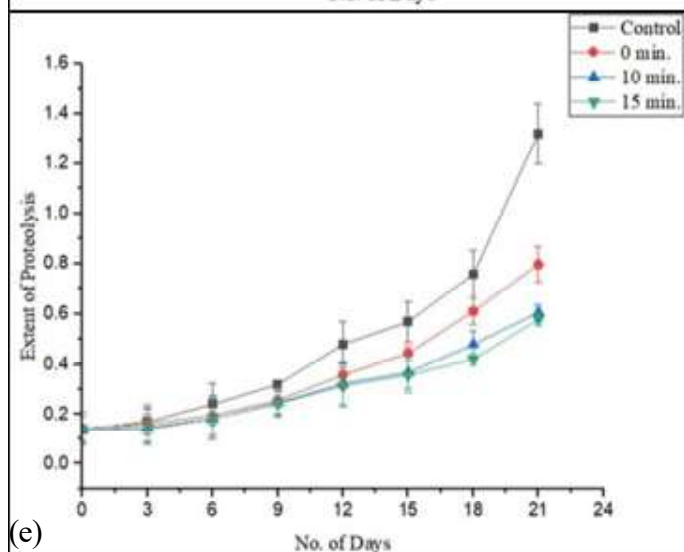
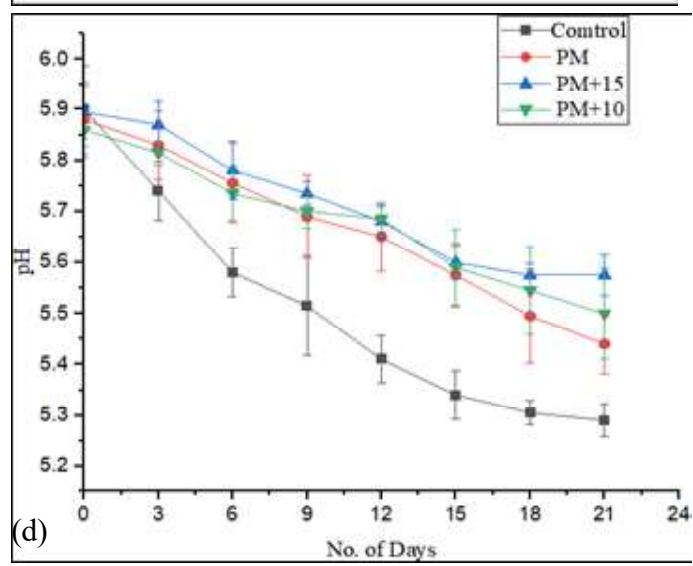
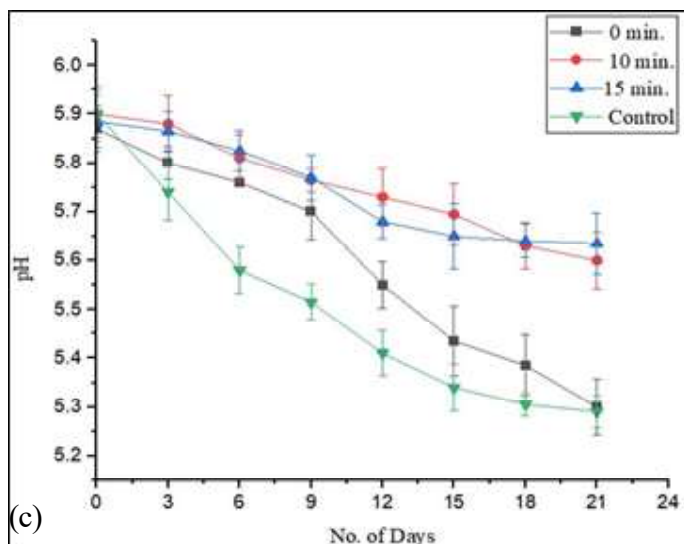
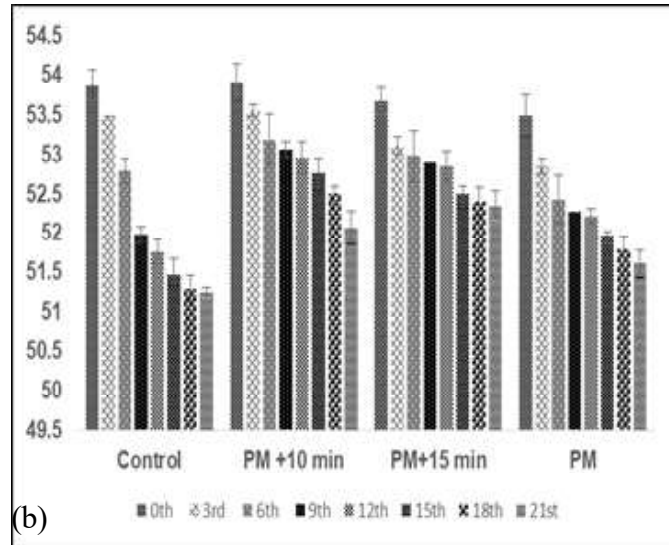
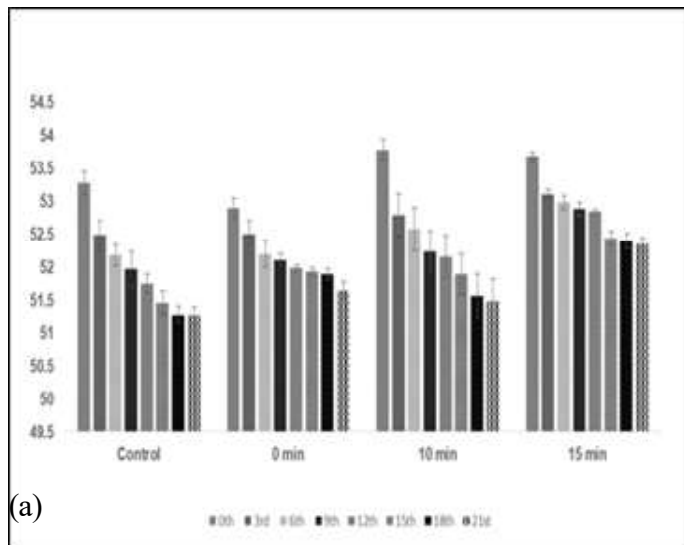
Moisture content of paneer samples treated with clove-oil nanoemulsion for 0 min, 10 min, 15 min and control were observed as $53.78 \pm 0.16\%$, $52.9 \pm 0.15\%$, $53.90 \pm 0.07\%$ and $53.27 \pm 0.18\%$ on the 0th day of storage and got reduced to $51.49 \pm 0.33\%$, $51.65 \pm 0.13\%$, $52.24 \pm 0.18\%$ and $51.27 \pm 0.13\%$ on the 21st day of storage as shown in Fig. 1 (a). While the paneer samples coded as PM, PM+10 and PM+15 showed reduction from $53.49 \pm 0.17\%$, $53.21 \pm 0.00\%$ and $53.68 \pm 0.07\%$ to $51.60 \pm 0.18\%$, $52.07 \pm 0.19\%$ and $52.35 \pm 0.09\%$ respectively as depicted in Fig. 1 (b). Paneer samples were taken as control showed significant reduction ($P < 0.05$) from $53.27 \pm 0.18\%$ to $51.27 \pm 0.13\%$ during 21 days of storage at 4°C. While no significant reduction ($P < 0.05$) was observed in moisture content of paneer samples treated with clove oil nanoemulsion. It was observed that moisture content decreased as the storage period of the paneer was increased which might be because of evaporation of moisture during storage. Due to the treatment with clove oil nanoemulsion, there might be formation of oil layer on the surface of the paneer samples which acted as a barrier and prevented the evaporation of moisture from the paneer. Several other research workers such as Khatkar et al. (2017a) and Masud et al. (2007) also documented similar results of reduction in moisture content during storage of paneer.

pH:

The pH of stored paneer samples was observed to reduce as the storage period was increased. On the 0th day of storage, pH of paneer was 5.90 ± 0.07 , 5.87 ± 0.02 , 5.90 ± 0.03 and 5.89 ± 0.03 for untreated control, samples treated for 0 min, for 10 min and for 15 min respectively. On the 3rd day of storage, the pH of control samples reduced down to 5.74 ± 0.06 , while all the treated paneer samples showed pH values higher than 5.80 ± 0.01 . Further on

the 6th day of storage, there was drastic reduction in pH down to the value 5.58 ± 0.05 while the treated paneer samples continued

to show comparatively much higher values as shown in Fig. 1. Similar trend was observed throughout the storage period when



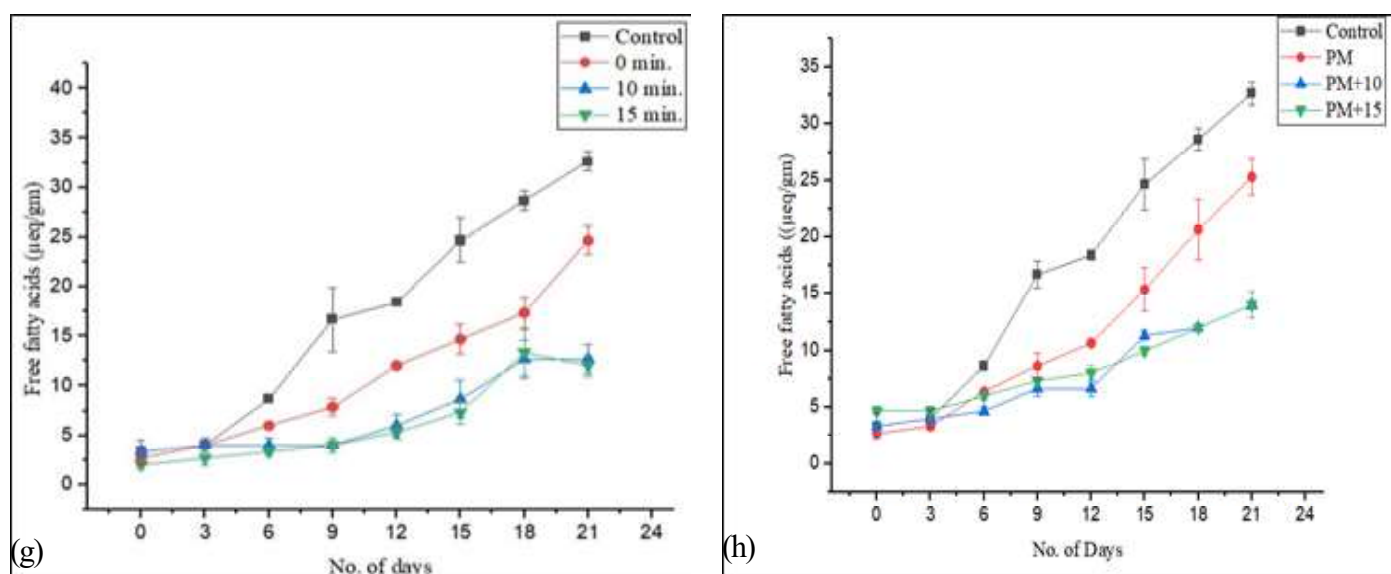


Fig. 1: Physicochemical analysis of paneer samples treated with clove oil nanoemulsion for different intervals of time such as 0 min, 10 min and 15 min in comparison to untreated control during storage a) Moisture content c) pH e) Extent of proteolysis g) Free fatty acids; Paneer samples treated with clove oil nanoemulsion for different intervals of time such as 10 min and 15 min and packed in packaging material treated with clove essential oil nanoemulsion in comparison to untreated control during storage b) Moisture content d) pH f) Extent of proteolysis h) Free fatty acids

the pH of paneer samples was analyzed on the 9th, 12th, 15th and 18th day of storage. On the 21st day of storage, it was observed to get reduced to 5.29 ± 0.03 , 5.30 ± 0.06 , 5.60 ± 0.06 and 5.65 ± 0.07 for untreated control, samples treated for 0 min, for 10 min and for 15 min as shown in Fig. 1 (c). Similarly reduction in pH from 5.88 ± 0.07 to 5.44 ± 0.06 in case of PM, from 5.90 ± 0.02 to 5.58 ± 0.04 in case of PM+10 and from 5.86 ± 0.03 to 5.50 ± 0.09 for PM+15 samples was recorded as observed in Fig. 1 (d). The reduction in pH in control paneer samples was observed to be significantly higher ($P < 0.05$) in comparison to paneer samples treated with clove-oil nanoemulsion for 10 min, 15 min, PM+10 and PM+15 paneer samples.

The observed reduction in pH could be well-correlated with the SPC and yeast and mold count of paneer samples. On 21st day of storage, the pH of control sample reduced down to minimum value of 5.29 ± 0.03 and showed the highest SPC count of 5.31 ± 0.03 log cfu/g and yeast and mold count of 4.55 ± 0.00 log cfu/g. Likewise pH of paneer samples treated with clove oil nanoemulsion for 10 min and 15 min was recorded as 5.60 ± 0.06 and 5.65 ± 0.06 respectively and the SPC count of 3.92 ± 0.13 and 3.71 ± 0.08 log cfu/g and yeast and mold count of 3.61 ± 0.05 and 2.76 ± 0.13 log cfu/g respectively. Similar pattern was observed in the samples treated with clove oil nanoemulsion and packed in treated packaging material which showed pH value of 5.58 ± 0.04 and 5.5 ± 0.09 and SPC count of 3.34 ± 0.05 and 3.29 ± 0.05 log cfu/g and yeast and mold count of 2.67 ± 0.08 and 2.23 ± 0.10 log cfu/g for samples treated for 10 min and 15 min respectively. The decrease in pH was lesser in case of clove oil nanoemulsion

treated samples because of its preservative effect which inhibited the growth of microbes which ultimately led to reduction in pH of paneer (Khatkar et al. 2017 ; Karunamay et al. 2020). While in case of untreated samples, there was growth of acid producing microbes and other spoilage causing bacteria due to which higher reduction in pH was observed (Mishra et al. 2016 ; Dwiwedi et al. 2014).

Extent of proteolysis:

The freshly prepared paneer samples on 0th day of storage were recorded to have water soluble protein content of 0.14 ± 0.06 , 0.14 ± 0.07 , 0.14 ± 0.06 and 0.15 ± 0.05 mg/mL for samples treated with clove oil nanoemulsions for no time (control), 0 min, 10 min and 15 min respectively as mentioned in Fig 1. On 3rd day of storage, the stored paneer samples started undergoing proteolysis, with the value increasing to 0.17 ± 0.10 mg/mL in case of control samples while it was observed to be 0.14 ± 0.02 mg/mL and 0.15 ± 0.03 mg/mL for samples treated for 10 min and 15 min respectively. Similar pattern of control samples undergoing extensive proteolysis as compared to those given treatment with clove oil nanoemulsion was observed on 6th, 9th, 12th and 15th day of storage. On 18th day of storage, the control samples were recorded to undergo proteolysis as high as 0.76 ± 0.44 mg/mL while those which were treated for 10 min and 15 min was 0.48 ± 0.01 mg/mL and 0.42 ± 0.00 mg/mL treated for 0 min, 10 min and 15 min respectively. On 21st day of storage, the soluble nitrogen content was observed to be 1.32 ± 0.12 , 0.78 ± 0.07 , 0.61 ± 0.03 and 0.58 ± 0.03 mg/ml for untreated, treated for 0 min, 10 min and 15 min respectively as shown in Fig 1 (e). While soluble nitrogen

content was found to increase from 0.13 ± 0.04 to 0.74 ± 0.03 mg/mL for PM+10 samples and from 0.10 ± 0.05 to 0.49 ± 0.03 mg/mL for PM+15 paneer samples as indicated in Fig. 1 (f). The results obtained by analysis of extent of proteolysis showed that paneer samples taken as control underwent significant increase ($P < 0.05$) in soluble nitrogen as compared to the paneer samples treated with clove oil nanoemulsion for 10 min and 15 min. Degradation of proteins during storage was mainly associated with secretion of enzymes like proteases by the microorganisms (Khatkar et al. 2017b). Similar findings were reported by Sharma (2017) who studied the effect of treatment of clove oil nanoemulsion on extent of proteolysis of paneer during its storage, it was reported that the protein content of water soluble extract was found to reduce from 0.48 ± 0.01 , 0.48 ± 0.01 , 0.49 ± 0.01 , 0.50 ± 0.01 and 0.51 ± 0.01 mg/mL to 0.97 ± 0.02 , 0.76 ± 0.01 , 0.67 ± 0.04 , 0.65 ± 0.02 , 0.80 ± 0.04 and 0.88 ± 0.02 mg/mL for samples treated for 0 min, 10 min, 15 min and 30 min respectively with clove oil nanoemulsion stabilized with milk proteins. Comparable results were pointed by another research study that extent of proteolysis in samples

treated with clove oil nanoemulsion as compared to the control samples increased at a slower rate (Khadka et al. 2021).

Free fatty acid content:

Lipases produced by microorganisms results in hydrolysis of lipids which led to formation of free fatty acids. It was observed that free fatty acid (FFA) content on 0th day of storage was 3.33 ± 0.47 , 2.67 ± 0.39 , 3.33 ± 0.27 and 2.00 ± 0.27 $\mu\text{eq/g}$ of paneer for samples taken as control, treated with clove oil nanoemulsion for 0 min, 10 min and 15 min respectively. On 3rd day of storage, the FFA content of all the paneer samples remained almost same. While on 6th day of storage, a major increase was observed, the FFA content of control samples got increased to 8.67 ± 0.27 $\mu\text{eq/g}$ and those of paneer samples treated for 0 min to 6.00 ± 0.27 $\mu\text{eq/g}$. Similarly, on 9th, 12th, 15th and 18th day of storage, the FFA content of paneer samples taken as control continued to increase at much higher rate as compared to the paneer samples treated with clove oil nanoemulsion. On 21st day of storage, it was observed to increase to 32.67 ± 0.98 , 24.67 ± 0.82 , 12.67 ± 0.81 and

Table 1: Standard plate count, Coliform count and Yeast and mold count (log cfu/g) of different paneer samples treated with clove oil nanoemulsions for different intervals

Day	Microbiological parameters	Control	0 min.	10 min.	15 min.	PM	PM+10	PM+15
0	Standard plate count	4.62 ± 0.14^D	4.63 ± 0.14^C	4.68 ± 0.10^A	4.58 ± 0.03^A	4.55 ± 0.09^{AB}	4.69 ± 0.01^A	4.45 ± 0.09^A
	Coliform count	1.60 ± 0.09^C	1.58 ± 0.11^C	1.36 ± 0.03^B	1.48 ± 0.10^{AB}	1.47 ± 0.03^C	1.45 ± 0.13^B	2.24 ± 0.01^B
	Yeast & mold count	1.74 ± 0.09^E	1.51 ± 0.01^D	1.36 ± 0.00^D	1.46 ± 0.02^C	1.49 ± 0.23^D	1.53 ± 0.07^{DE}	1.70 ± 0.11^B
3	Standard plate count	4.92 ± 0.05^{CD}	4.66 ± 0.06^C	4.62 ± 0.05^C	4.54 ± 0.06^A	4.65 ± 0.01^{AB}	4.62 ± 0.08^A	4.55 ± 0.06^A
	Coliform count	2.08 ± 0.19^{BC}	1.62 ± 0.08^C	1.84 ± 0.15^{AB}	1.00 ± 0.24^B	1.37 ± 0.03^C	0.61 ± 0.01^C	0.95 ± 0.12^B
	Yeast & mold count	1.57 ± 0.14^E	1.49 ± 0.01^D	1.49 ± 0.10^D	0.69 ± 0.15^D	1.27 ± 0.01^D	1.27 ± 0.11^E	1.01 ± 0.03^E
6	Standard plate count	5.31 ± 0.04^{AB}	4.62 ± 0.01^C	4.40 ± 0.05^C	4.31 ± 0.05^A	4.34 ± 0.02^B	4.56 ± 0.02^A	4.48 ± 0.13^A
	Coliform count	2.13 ± 0.00^{BC}	1.62 ± 0.21^B	0.85 ± 0.16^C	0.63 ± 0.13^C	1.21 ± 0.11^B	0.60 ± 0.12^C	0.90 ± 0.09^{BC}
	Yeast & mold count	2.34 ± 0.06^{DE}	1.15 ± 0.02^D	1.05 ± 0.06^E	0.43 ± 0.03^D	1.48 ± 0.05^D	0.87 ± 0.08^{DE}	0.72 ± 0.11^F
9	Standard plate count	5.43 ± 0.06^A	4.97 ± 0.05^B	3.58 ± 0.10^B	3.81 ± 0.02^A	4.39 ± 0.18^B	3.45 ± 0.13^B	3.29 ± 0.12^B
	Coliform count	2.43 ± 0.00^B	2.36 ± 0.10^B	1.25 ± 0.07^C	1.08 ± 0.10^{AB}	2.14 ± 0.13^B	1.19 ± 0.09^B	1.45 ± 0.06^B
	Yeast & mold count	2.71 ± 0.04^{CD}	1.97 ± 0.05^C	1.39 ± 0.01^D	1.19 ± 0.03^C	2.02 ± 0.12^C	1.41 ± 0.11^D	1.11 ± 0.04^E
12	Standard plate count	5.21 ± 0.16^{ABC}	5.15 ± 0.02^A	3.23 ± 0.08^A	3.99 ± 0.12^A	4.75 ± 0.01^{AB}	3.28 ± 0.01^B	3.05 ± 0.01^{BC}
	Coliform count	2.60 ± 0.04^B	2.45 ± 0.14^B	1.37 ± 0.02^B	1.22 ± 0.10^{ABC}	2.20 ± 0.12^{BC}	1.47 ± 0.07^B	1.80 ± 0.10^{AB}
	Yeast & mold count	3.26 ± 0.11^{BC}	2.78 ± 0.00^B	2.26 ± 0.02^C	1.49 ± 0.02^C	2.59 ± 0.14^B	2.18 ± 0.08^C	1.37 ± 0.10^D
15	Standard plate count	5.03 ± 0.04^{BC}	5.68 ± 0.06^C	3.84 ± 0.08^C	3.93 ± 0.14^A	4.93 ± 0.06^A	3.27 ± 0.12^B	2.97 ± 0.10^B
	Coliform count	2.59 ± 0.16^B	2.52 ± 0.12^B	1.61 ± 0.16^A	1.23 ± 0.16^A	2.46 ± 0.19^{AB}	2.36 ± 0.04^{AB}	2.04 ± 0.07^A
	Yeast & mold count	3.29 ± 0.00^B	2.77 ± 0.01^B	2.05 ± 0.03^C	2.00 ± 0.04^B	2.92 ± 0.08^B	2.20 ± 0.07^B	1.60 ± 0.04^{BC}
18	Standard plate count	5.16 ± 0.12^{ABC}	4.49 ± 0.12^{BC}	3.86 ± 0.02^C	3.74 ± 0.16^A	4.36 ± 0.13^B	3.69 ± 0.03^B	3.43 ± 0.02^B
	Coliform count	3.17 ± 0.18^A	2.83 ± 0.13^{AB}	1.88 ± 0.11^A	1.25 ± 0.09^{AB}	2.92 ± 0.00^A	1.82 ± 0.10^A	1.35 ± 0.01^B
	Yeast & mold count	3.62 ± 0.00^B	3.60 ± 0.01^A	2.78 ± 0.03^B	2.24 ± 0.01^B	3.58 ± 0.00^A	2.49 ± 0.11^B	1.83 ± 0.07^B
21	Standard plate count	5.31 ± 0.03^{AB}	4.42 ± 0.12^C	3.92 ± 0.13^C	3.71 ± 0.08^A	4.54 ± 0.01^B	3.34 ± 0.05^B	3.29 ± 0.05^B
	Coliform count	3.38 ± 0.00^A	3.04 ± 0.05^A	2.11 ± 0.14^A	1.17 ± 0.10^{AB}	2.77 ± 0.10^A	1.49 ± 0.08^A	0.95 ± 0.03^B
	Yeast & mold count	4.55 ± 0.03^A	3.78 ± 0.01^A	3.61 ± 0.05^A	2.76 ± 0.13^A	3.76 ± 0.03^A	2.73 ± 0.08^A	2.12 ± 0.10^A

12.00 $\pm$ 0.67 $\mu\text{eq/g}$ for paneer samples taken as control, treated with clove oil nanoemulsion for 0 min, for 10 min and for 15 min respectively as shown in Fig. 1 (g). Similar trend was observed for paneer samples packed in treated packaging material after treating with clove oil nanoemulsion such as 25.33 $\pm$ 0.94 $\mu\text{eq/g}$ in PM, 14.00 $\pm$ 0.47 $\mu\text{eq/g}$ for PM+10 and 14.00 $\pm$ 0.47 $\mu\text{eq/g}$ for PM+15 paneer samples as depicted in Fig. 1 (h).

FFA content showed direct relation with the SPC and yeast and mold count of paneer samples. It was observed that paneer samples taken as control showed maximum increase in SPC count to 5.29 $\pm$ 0.03 log cfu/g and yeast and mold count got increased to 4.55 $\pm$ 0.00 log cfu/g. While paneer samples which were treated with clove oil nanoemulsion showed a very low SPC count of 3.92 $\pm$ 0.13 log cfu/g and 3.71 $\pm$ 0.08 log cfu/g and yeast and mold count of 3.61 $\pm$ 0.05 log cfu/g and 2.76 $\pm$ 0.13 log cfu/g for samples treated for 10 min and 15 min with clove oil nanoemulsion. This clearly indicated that increase in FFA content was directly related with the microbial growth in the paneer samples which secreted the enzymes like lipases and ultimately resulted in lipolysis of paneer samples. Treatment of paneer samples with clove oil nanoemulsion for 15 min reduced the FFA content by 63.2% as compared to control.

Similar results were reported, when Sharma (2017) studied the effect of treatment of clove oil nanoemulsion in reducing the FFA content of the paneer samples. It was reported that FFA content was significantly lower in those samples which were treated with the nanoemulsion for 10 min. and 15 min. as compared to those which were kept untreated. In another study, it was observed that when the efficiency of turmeric was tested for its ability to enhance the shelf life of paneer, FFA content of the untreated samples increased at a much faster rate as compared to samples treated with turmeric. Also it was documented that addition of 0.6% turmeric in paneer samples resulted in reduction of its FFA content (Buch et al. 2014).

Effect of treatment of clove-oil nanoemulsion on Microbiological-parameters of paneer:

Standard plate count:

Steep rise was observed in SPC count of paneer samples with the increase in storage time. SPC count of the clove oil nanoemulsion treated paneer samples was observed to increase at a gradual rate as compared to the untreated samples. On 0th day of storage, it was observed as 4.62 $\pm$ 0.14, 4.63 $\pm$ 0.14, 4.68 $\pm$ 0.10 and 4.58 $\pm$ 0.03 log cfu/g for samples taken as control, treated with clove oil nanoemulsion for 0 min, for 10 min and for 15 min. respectively as shown in Table 1. It remained almost same on 6th day of storage. On 9th day of storage, the SPC count of the control paneer samples increased abruptly to 5.43 $\pm$ 0.06 log cfu/g while a sharp decrease in SPC count to 3.58 $\pm$ 0.10 and 3.81 $\pm$ 0.02 log cfu/g was recorded for samples treated with clove oil

nanoemulsion for 10 min and 15 min. respectively. It was notified that the antimicrobial effect of added clove oil became evident at the end of the lag phase of microbial growth, when the exponential phase of growth of microbes just got started.

On further storage of paneer samples, the SPC count of all the samples observed to increase when assessed on 12th, 15th and 18th day of storage as shown in Table 1. On 21st day of storage, the SPC count was recorded as 5.31 $\pm$ 0.03, 4.42 $\pm$ 0.12, 3.92 $\pm$ 0.13 and 3.71 $\pm$ 0.08 log cfu/g for paneer samples taken as control, treated with clove oil nanoemulsion for 0 min, 10 min and 15 min respectively. Similar pattern was observed in paneer samples packed in treated packaging material after the treatment of samples with clove oil nanoemulsion like SPC count remained static in case of untreated samples packed in treated packaging material, reduced from 4.69 $\pm$ 0.01 to 3.34 $\pm$ 0.05 log cfu/g when packed after treatment for 10 min and reduced from 4.45 $\pm$ 0.09 to 3.29 $\pm$ 0.05 log cfu/g when treated with clove oil nanoemulsion for 15 min as shown in Table 1. The observed significant difference ($P < 0.05$) between the increase in SPC count of treated and untreated samples could be justified by the antimicrobial effect associated with clove oil. Devaki et al. 2021, reported that star anise incorporated paneer samples showed an increase from 12x10² cfu/g to 51x10² cfu/g in 30 days while for control samples it was reported to increase from 28x10³ to 75x10³ cfu/g after 30 days. It was observed that paneer incorporated with spices showed lower microbial count as compared to the control samples. Sharma (2017) reported that shelf-life of nanoemulsion treated paneer increased to more than 15 days as compared to 6 days in case of control. It was reported that paneer samples treated for 10 min and 15 min with clove essential oil showed four log cycle lower counts for SPC as compared to the control when stored for 20 days.

Coliform count

On the 0th day of storage study, coliforms count were recorded to be 1.60 $\pm$ 0.09, 1.58 $\pm$ 0.11, 1.36 $\pm$ 0.03 and 1.48 $\pm$ 0.10 log cfu/g for control samples, samples treated for 0 min, for 10 min and for 15 min respectively. It was observed that on 6th day of storage of paneer samples, there was a steep rise in coliform count from 1.60 $\pm$ 0.09 to 2.13 $\pm$ 0.00 log cfu/g in case of control while the paneer samples treated with clove oil nanoemulsion showed a reduction in coliform count from 1.36 $\pm$ 0.03 to 0.85 $\pm$ 0.16 log cfu/g for samples treated with clove oil nanoemulsion for 10 min and from 1.48 $\pm$ 0.10 to 0.63 $\pm$ 0.13 log cfu/g for samples treated for 15 min with clove oil nanoemulsion as observed from the Table 1. Increase in coliform count was observed, when the paneer samples were analyzed further during the storage period on 9th, 12th, 15th and 18th day of storage. However, increase in coliform count in treated paneer samples was lesser significant ($P < 0.05$) compared to the untreated samples as depicted in Table 1. Coliform count on 21st day of storage was reported to be 3.38 $\pm$ 0.00, 3.04 $\pm$ 0.04, 2.11 $\pm$ 0.14 and 1.17 $\pm$ 0.10 log cfu/g for control

samples, for samples treated for 0 min, for 10 min and for 15 min respectively as shown in Table 1.

Similar trend was observed, when paneer samples were packed in treated packaging material after being treated with clove oil nanoemulsion such as coliform count on 21st day of storage was observed to be 2.77 ± 0.10 log cfu/g for untreated paneer samples packed in treated packaging material, 1.49 ± 0.08 log cfu/g for samples treated with clove oil nanoemulsion for 10 min before packaging. The results obtained during the study showed that treatment of paneer with clove oil nanoemulsion reduced the coliform count by 1.27 and 2.21 log cycles respectively for samples treated for 10 min and 15 min respectively. While for samples packed in treated packaging material the reduction was estimated to be 1.89 and 2.43 log cycles for samples treated for 10 min and 15 min respectively.

Similarly, Khatkar et al. 2017 reported that coliform count increased from 1.17 to 1.84 log cfu/g during the storage period of 15 days. Another research study pointed that cardamom treated paneer samples showed a sharp decrease in coliform count after 14 days of storage while it was reported to increase abruptly in case of control samples. Shelf-life of paneer samples was reported to increase upto 28 days after treatment of paneer (Eresam et al. 2015).

Yeast and mold count:

Yeast and mold count was 1.74 ± 0.09 , 1.51 ± 0.01 , 1.36 ± 0.00 , 1.46 ± 0.02 and 1.49 ± 0.23 log cfu/g for untreated paneer samples, samples treated for 0 min, for 10 min, for 15 min and packed in treated packaging material respectively on 0th day of storage as shown in Table 1. Initially there was reduction in yeast and mold count on 3rd day of storage and then yeast and mold count started to increase. On 6th day of storage, it was observed that the count increased to 2.34 ± 0.06 log cfu/g in case of control while it got reduced to 1.05 ± 0.6 log cfu/g and 0.43 ± 0.03 log cfu/g in samples treated with clove oil nanoemulsion for 10 min and 15 min respectively. Increase in yeast and mold count was observed when paneer samples were further analyzed on interval of every 3 days like on 12th, 15th and 18th day of storage as observed from Table 1.

On 12th day of storage, black coloured spots which suggested the growth of *Aspergillus spp.* or *Cladosporium spp.* were visible on the control samples. No such growth was witnessed on paneer samples treated with clove oil nanoemulsion as shown in Plate 1. On 21st day of storage, the count was reported to be 4.55 ± 0.03 , 3.78 ± 0.01 , 3.61 ± 0.05 , 2.76 ± 0.13 and 3.76 ± 0.03 log cfu/g for control samples, samples treated for 0 min, for 10 min, for 15 min and for the packaging material dipped in clove oil nanoemulsion as shown in Table 1. While paneer samples dipped in clove oil nanoemulsion for 10 min and 15 min followed by packing in treated packaging material were ascertained to have yeast and

mold count of 2.73 ± 0.08 and 2.12 ± 0.10 log cfu/g respectively as shown in Table 1. On 21th day of storage, spots of pink, green, black and brown colour were observed on paneer samples taken as control, dipped in clove oil nanoemulsion for 0 min and packed in packaging material dipped in the nanoemulsion as shown in Plate 1. The spots of pink, green, black and brown might be because of growth of yeast *Rhodotorula*, yeast *Candida albicans*, mold *Aspergillus* and mold *Penicilli* respectively (Khatkar et al. 2017 ; Goyal and Goyal 2016).

Sharma (2017) documented similar results that treatment of paneer samples with clove oil nanoemulsions prepared using whey protein concentrate and maltodextrin conjugate resulted in reduction in yeast and mold count by one and three log cycles after storing the samples in refrigerator for three and twelve days respectively as compared to control. Also it was reported that shelf-life of treated paneer samples increased to 15 days as compared to 6 days in case of control. Eresam et al. 2015 reported that yeast and mold count increased sharply in case of control samples and no such steep rise in cardamom treated paneer samples was observed.

Storage Study: Analysis of sensory characteristics of prepared paneer samples treated with clove oil nanoemulsion

Flavor:

Paneer samples treated with clove oil nanoemulsion elicited comparatively lower scores of 7.33 ± 0.13 and 7.10 ± 0.11 for samples treated for 10 min and 15 min in comparison to the untreated sample which received the scores of 8.33 ± 0.18 on 0th day of storage. Clove-oil flavor was prominent in samples treated with clove-oil nanoemulsion for longer time and therefore these samples received lower scores. However there was reduction in clove-oil flavor with time and the treated samples received higher scores on 3rd day of storage of 7.47 ± 0.11 and 7.20 ± 0.11 for samples treated for 10 min. and 15 min. which may be because of volatile nature of clove-oil.

While the sensory scores of untreated sample got diminished to 8.27 ± 0.20 . On 6th day of storage, there was a tinge of acidic flavor and the overall acceptability score was recorded as 7.73 ± 0.31 which might be because of the growth of lactic acid bacteria. No such flavour was perceived in paneer samples treated with clove oil nanoemulsions for different intervals of time and sensory scores remained the same. The reason may be preservative action due to antimicrobial activity of clove essential oil which inhibited the growth of lactic acid bacteria and prevented paneer from spoilage. The overall acceptability score of control samples diminished drastically on 9th day of storage to 6.27 ± 0.87 while the paneer samples treated for 10 min received 7.37 ± 0.13 and those treated for 15 min received 7.17 ± 0.13 as sensory scores. Paneer samples treated for 0 min. were given 7.03 ± 0.94 scores.

Similarly, it was observed that the acceptability scores of control samples diminished continuously while those of paneer samples treated with clove oil nanoemulsion remained almost constant when assessed on 12th, 15th and 18th day of storage as shown in Fig. 2 (a). On 21st day of storage, it was observed that paneer samples taken as control and dipped in nanoemulsion for 0 min. became unacceptable on the basis of their organoleptic characteristics with the sensory scores of 0.67 ± 0.31 and 4.30 ± 0.69 respectively. On the other hand, paneer samples treated for 10 min and 15 min remained acceptable with sensory scores of 6.43 ± 0.20 and 6.63 ± 0.15 respectively and no change in flavour or taste was perceived by the panelists. Similarly, paneer samples

packed in treated packaging material became unacceptable while those packed after giving treatment for 10 min and 15 min remained acceptable with sensory scores of 6.30 ± 0.14 and 6.30 ± 0.13 respectively as shown in Fig. 2(b).

Body and Texture

Textural characteristics of treated paneer samples were ascertained to be similar to the control sample because the total time of dipping the paneer samples collectively in water and clove oil nanoemulsion was kept constant. No deterioration in texture of

Plate 1: Appearance of paneer samples treated with clove essential oil nanoemulsions for different intervals of time on 21st day of storage

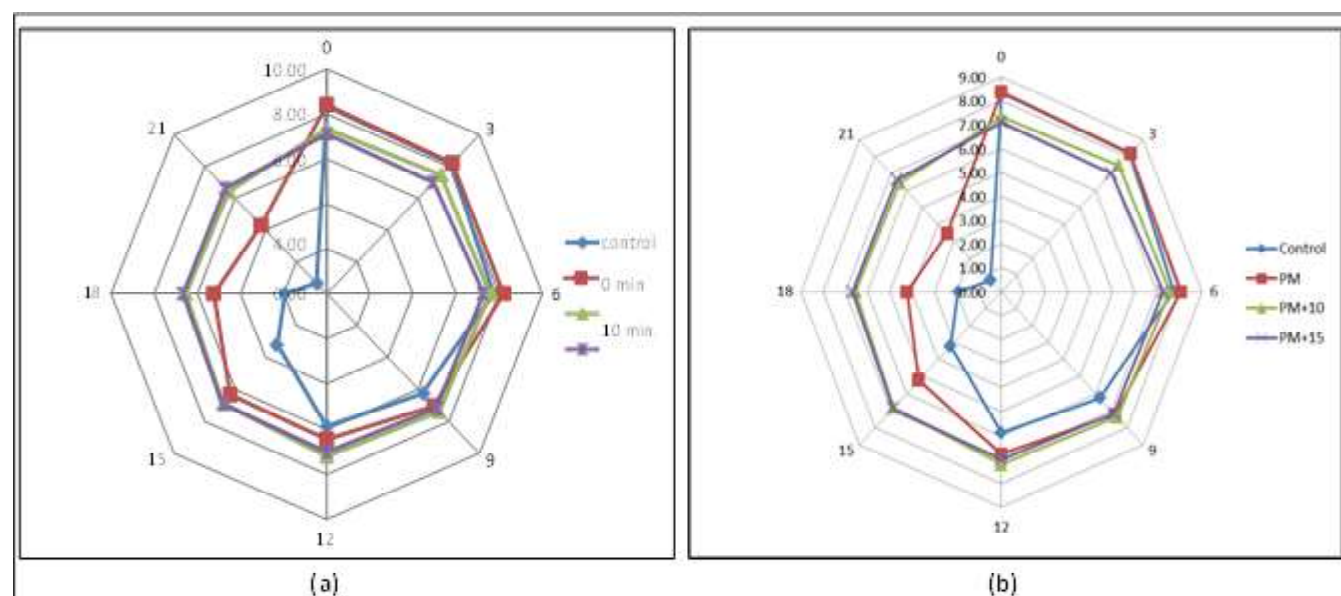
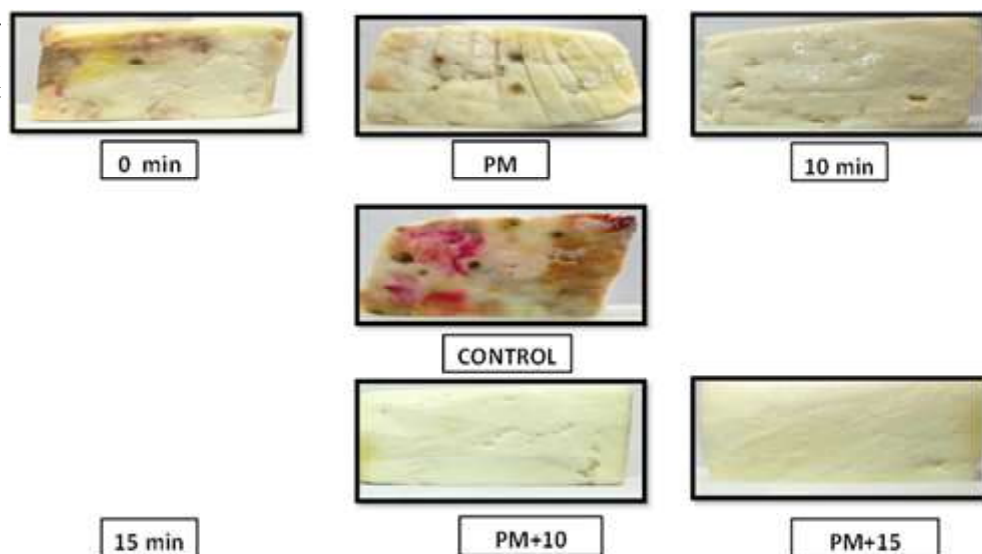


Fig. 2 Change in sensory scores of paneer samples treated with clove oil nanoemulsion for a) different intervals of time such as 0 min, 10 min. and 15 min. in comparison to untreated control during storage b) different intervals of time such as 10 min. and 15 min. and packed in packaging material treated with clove essential oil nanoemulsion in comparison to untreated control during storage

the treated paneer sample was noticed on 3rd and 6th day of storage. On the other hand, greenish-yellow colored slime formation became evident on 9th day of storage which might be because of the growth of slime secreting bacteria such as *Escherchia spp.*, *Streptococcus spp.* and *Staphylococcus spp.* (Yashwantha et al. 2020). Similar slime growth was observed in paneer samples treated for 0 min and samples packed in treated packaging material with clove oil nanoemulsion on 15th day of storage. Body and texture of paneer samples treated for 10 min. and 15 min. remained almost similar and was acceptable by all the panellists.

Appearance

Appearance of different paneer samples was also recorded during the storage study and it was observed that in case of control samples black coloured spots became visible on 12th day of storage. Growth of different molds which could be ascertained by different pink, yellow, brown and green coloured spots became evident on subsequent days of storage. Appearance of these spots could be correlated with growth of different yeast and molds such as yeast *Rhodotorula*, yeast *Candida albicans*, mold *Aspergillus* and mold *Penicillium* (Yashwantha et al. 2020).

No such development of coloured spots was witnessed in case of paneer samples treated for 10 min. and 15 min. and also in samples followed by packing in treated packaging material as evident from Plate 1. Due to which the paneer samples treated for longer time elicited higher scores as compared to other mentioned samples on the 21st day of storage as shown in Fig. 2 (a) and Fig. 2 (b). Based on the sensory characteristics of paneer in terms of flavour, texture and appearance of paneer samples, it was observed that the paneer samples treated with clove oil nanoemulsion for 10 min. and 15 min. and those packed in nanoemulsion treated packaging material after treating the paneer samples with clove oil nanoemulsion for 10 min and 15 min were found to be shelf-stable during twenty-one days of storage.

Paneer spread containing 1% salt with sodium benzoate preservative reported that acceptability score diminished from 8.20 to 4.00 over a period of 20 days of storage. It was reported that sensory characteristics of paneer spread remained almost unaltered when preservatives like sodium benzoate and potassium bisulphate were added (Dongare et al. 2019; Singh et al. 2020).

Conclusion

Findings of the present study revealed that treatment of paneer with clove oil nanoemulsion resulted in enhancement of shelf-life of paneer by efficiently inhibiting the growth of coliforms, yeast and molds and other microbes without having negative effect on sensory characteristics of treated samples. Physicochemical characteristics such as moisture content, pH, free fatty acid content and extent of proteolysis were ascertained to be $52.35 \pm 0.09\%$, 5.50 ± 0.09 , 14.00 ± 0.47 $\mu\text{eq./g}$ and 0.44 ± 0.04

mg/ mL for paneer samples treated with clove-oil nanoemulsion for 15 min. followed by packing in treated packaging material while $51.27 \pm 0.13\%$, 5.29 ± 0.03 , 24.67 ± 0.82 $\mu\text{eq./g}$ and 1.32 ± 0.76 mg/ mL respectively in case of untreated control samples on 21st day of storage. Similarly studies of microbiological parameters revealed reduction in SPC, coliform and Yeast and mold count by 2.02, 2.43 and 1.88 log cycles in comparison to control. It was observed that as the time of treatment of paneer samples was increased, there was improvement in physicochemical, microbiological and sensory characteristics of paneer and hence it could be prevented from spoilage for longer time. Clove-oil nanoemulsion treatment of paneer resulted in increase in its shelf-life by almost two-weeks. Therefore, it may be stated that clove oil, a natural antimicrobial essential oil in its nanoemulsion form may prove to be an efficient method in replacing the existing chemical methods of preservation of paneer.

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RESEARCH ARTICLE

Development and experimental performance of a cleaning-in-place system for three stage scraped surface heat exchanger

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Abstract: Aim of present study was to develop an innovative Cleaning-in-place (CIP) system, having control system with instrumentation, for dairy process equipments. Its performance was evaluated for three-stage thin film scraped surface heat exchanger (TS-TFSSHE). CIP system consists of multi-partition tank, control system with instrumentation and fluid flow system. Experiments were conducted with three independent variables (scraper speed: 300, 225 and 150 RPM; solution temperature: 80, 70 and 60°C; solution concentration: 2%, 1.375% and 0.75%) using response surface methodology. CIP performance responses were chemical rinsing duration, total CIP time, total plate count and coliform count. Optimized solution was obtained as 0.85% sodium-hydroxide concentration, 72.7°C CIP solution temperature and 150 RPM scraper speed. Validation of optimized solution showed that predicted response values were comparable with mean experimental values and found non-significant ($p > 0.05$). This CIP system is movable (wheel mounted) and may be used for cleaning of other dairy process equipments at other places as well.

Keywords: Cleaning; CIP; Process equipment; Scraped surface heat exchanger

Introduction

Cleaning-In-Place (CIP) is unit operation for cleaning or removal of fouling deposits and product traces from surfaces of equipment and pipelines which helps in production of quality products. It is defined as “cleaning of complete items of plant any pipeline

circuits without dismantling or opening of equipment and with small or no manual involvement on part of operator” (Romney, 1990). In dairy and food processing industries, re-use and multi-use of cleaning-in-place (CIP) system is practiced without taking equipment apart (Gésan-Guizieu et al. 2002) for maintaining high level of hygiene. Industries such as dairy, beverage, brewing, processed foods etc. require high level of hygiene and rely on CIP.

The fouling of heat exchangers is a major concern in dairy industry, with a negative impact on operational costs and product quality (Andritsos *et al.*, 2002). Fouling reduces heat transfer and capacity of the heat exchanger and consequently the process fluids may be affected adversely. In general, for plate heat exchangers, increase in temperature resulted in substantial reduction in cleaning time and increase in flow rates had very significant effect on cleaning rates (Timperley and Smeulders, 1988). For CIP, chemicals should be selected on the basis of their ability to remove organic and inorganic fouling layers (Chisti and Moo-young, 1994). Alkaline detergent should be circulated to remove organic soil or fouling layers e.g. proteins and fats. It helps in lifting of the soil from the surface and holds it in suspension or dissolved in the alkaline detergent solution (Memisi et al. 2015). Temperature and flow velocity of CIP solution had an evident effect on effectiveness of CIP (Tuladhar et al. 2002). Kumari and Sarkar (2014) reported the use of 1.5% concentration of sodium hydroxide solution at 65°C for 30 minutes followed by use of 1% concentration of nitric acid with water rinsing step (at start, in between and end) for optimum removal of biofilm formation. Burfoot et al. (2017) suggested that the use of air bubbles in water could provide small improvements in cleaning surfaces or potentially similar contamination removal using less water. Various researchers have studied on different aspects of CIP of process equipments (Bava et al. 2011; Chisti and Moo-young, 1994; Gésan-Guizieu et al. 2002; Khalid et al. 2015; Mattila et al. 1990; Kumari and Sarkar, 2014; Sundberg et al. 2011; Timperley and Smeulders, 1988). Van Asselt et al. (2002) reported the use of electrochemical sensors for monitoring of system for improving cleaning efficiency of CIP processes. Khalid et al. (2015) developed a test rig for cleaning studies and evaluated laboratory-scale experiments.

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In industries, scraped surface heat exchangers (SSHEs) are widely used for the processing of dairy and food products with high viscosity (Rao and Hartel, 2006). For continuous manufacture of Indigenous milk products of India, a thin film scraped surface heat exchanger could be very effectively used (Abichandani and Sarma, 1991). Three-stage thin film SSHE (TS-TFSSHE) is a versatile dairy process equipment in which high temperature, pressure, flow rate of product and high heat transfer rates co-exist during processing operations (Dodeja and Deep, 2012). These harsh conditions are conducive to form tenacious layer of milk solids on the surface of equipment. Proper cleaning of product surfaces of the heat exchanger is essential for production of the quality product. Hence three stage SSHE was selected as model equipment for testing of proposed CIP system.

Considering the hygienic importance of CIP and human interactions involved in traditional or conventional cleaning process (time consuming and risk prone), the objective of this study was to develop an innovative CIP system for dairy process equipments and it was evaluated for cleaning of three-stage scraped surface heat exchanger.

Materials and Methods

Development of CIP system

This study was conducted at Dairy Engineering Division, ICAR-NDRI, Karnal, Haryana, India. The existing three stage thin film scraped surface heat exchanger (TS-TFSSHE) has three heat exchangers, each identical in length. The scraper assembly consisted of solid SS shaft of 2.5×10^{-2} m diameter. The first and second stages of TS-TFSSHE had four variable clearance blades each of 1.332 m length, 0.005 m thickness and 0.04 m width and are hinged between cross supports in each scraper at 1.405 m distance from front end and 0.356 m from rear end. Control system consists of variable frequency drives, process controller, control switches/buttons, MCBs, digital displays for process parameters. Its instrumental parts consist of pressure gauge, magnetic flow meter, I/P converter, pressure transmitters, pneumatic valves, air pressure indicators. Some ancillary equipments associated with TS-TFSSHE consisted of balance tank, feed pump and valves for steam supply (Dodeja and Deep, 2012).

To design proposed CIP system, experiments were conducted to determine the hold-up volume for TS-TFSSHE at fixed parameters i.e. steam pressure, flow rate and scraper speed for water as a process fluid. It was found as 10 litres (approx.) for operating parameter range (scraper speed 200, 175, 40 rpm; flow rates 350 to 528 kg/h and steam pressures 3.5, 2.5, 1 kgf/cm² for first, second and third stage respectively). The following equations were used for determination of the holdup volume and volumetric flow rate during steady state flow

$$\text{condition: Hold up volume} = \frac{\pi d^2 \times (H_i - H_f)}{4} \dots \dots \dots (1)$$

$$\text{Volumetric flow rate} = \frac{\text{volume}}{(T_f - T_i)} \dots \dots \dots (2)$$

Where d is diameter of balance tank; H_i is initial level of balance tank; H_f is final level of balance tank; T_i is initial time in minutes at H_i level of balance tank; T_f is final time in minutes at H_f level of balance tank.

The developed CIP system consists of CIP multi-partition tank and pipelines, control panel and its components, direct steam Injection and inline filter and instrumentation.

CIP tank and pipelines

Important considerations for CIP multi-partition tank (capacity: 700 L; five partitions) were savings of resources such as space, water, cleaning time, heat energy etc. The volume of multi partition CIP tank (200 L) was intended for circulation of CIP solution four times (4 times hold up volume: 50 L approx.) in its CIP procedure. The tank along with mobile stand (wheels) was fabricated which consists of five partitions on the basis of hold-up volume. The five partitions were made for conc. Acid (50 L), conc. sodium hydroxide (50 L), dilute Acid (200 L), dilute sodium hydroxide (200 L) and water (200 L). These concentrated solutions were intended to be stored for making up the required concentration of CIP solutions as and when needed. During manufacturing of product using TS-TFSSHE, some milk particles falls out from vapour ducts on to nearby area including CIP tank which will make recovered and fresh solutions in tank unsuitable for re-use. To prevent it, a stainless steel (SS 304) cover (1.5 mm thick sheet) for the CIP tank was fabricated. The forward line was fabricated using 38 mm pipe (SS 304) along with two different sensors i.e. temperature (Pt-100) and pH sensors. The CIP return line was made to recover the CIP solution when it was passed through the electrical conductivity sensor (Figure 1). The valves were operated manually to recover the solution after the pump for recovery was started.

Control panel and its components

The control panel was designed to house main circuit breaker, direct on line motor starters for all four pumps (conc. acid, conc. sodium hydroxide, CIP forward, and CIP return pumps), Arduino Uno microcontroller for pH sensing system, electrical conductivity monitor, temperature indicator and level indicators for all partitions. It was positioned above the forward and return CIP pipelines for easy operation.

Direct steam Injection and inline filter

Direct steam injection (DSI), having non-return valve (NRV), was used to effectively heat the fluid either water or CIP solution. The pipeline after NRV was welded inclined in order to save inner surface of the opposite side of pipeline from high pressure steam.

The in-line filter (stainless-steel cylindrical shape) is connected in the recovery line of CIP next to the recovery pump.

Instrumentation for CIP system

The level of prepared solution was examined visualising by light emitting diode (LED). There are three different levels i.e. lower, medium, higher are indicated by LED lights (located at control panel). One resistance temperature detector (Pt-100) sensor device was used to measure the temperature of cleaning solution. The pH sensor was calibrated by the use of different buffer solutions, having the pH 4, 7, 9.2 by Arduino programming. The outgoing solutions pH was monitored by the calibrated pH sensor. Electrical conductivity sensor was fitted in the CIP return line for conductivity monitoring of recovering material. If the conductivity is matched with the inline flow, the recovery valve was open manually. Conductivity measurement is suitable for this application as only one solution was flowing at a time.

Materials required for experimental trials

Fresh buffalo milk was procured from Experimental Dairy (a Unit for teaching, and training of UG/PG students), at ICAR-NDRI, Karnal. *Khoa* manufacturing using TS-TFSSHE was selected for soiling because the process equipment (TS-TFSSHE) was primarily developed for continuous manufacture of *khoa* (Dodeja and Deep, 2012). In TS-TFSSHE, most of mineral deposits were not expected to be deposited on heat transfer cylindrical surface due to scrappers use. For CIP, sodium hydroxide is the most widely and regularly used CIP chemical in dairy industries. The *khoa* manufacturing process was used for simulating actual process plant condition soiling after production where milk fat and proteins are the major factors contributing to soiling deposit on process equipment/heat transfer surface. Sodium hydroxide flakes (having 80% assay) were diluted in potable water and a solution of 0.750 to 2.000 % strength was prepared. SS vessels and trays were used for *khoa* collection.

Experimentation

Initiation of Experiment: For each trial 40 litre buffalo milk (20-25°C) was procured from Experimental Dairy, ICAR-NDRI, Karnal, Haryana. Initially, TS-TFSSHE was rinsed with the potable water. After this, *khoa* was prepared (for soiling of equipment) from milk concentration by using TS-TFSSHE and it was collected in tray.

Product recovery and gross debris removal: After complete milk was utilised for *khoa* production, the TS-TFSSHE was flushed with water through balance tank to recover milk solids and also removal of gross debris.

Initial water flushing: For conducting CIP trials, the main balance tank was filled with hot water (60/70/80°C). The feed pump was then switched on manually and flow rate was set at 8.4-8.5 lit/min

and setting the CIP return line to drain. The scraper speed was kept fixed and same (150/225/300 rpm) for all three different stages, respectively as soon as the water started to come in the shell.

Chemical rinsing: After water started coming clear (seen visually) then sodium hydroxide solution at selected concentration (0.750/1.375/2.000%) was forwarded from CIP system to TS-TFSSHE manually. When Electrical conductivity (EC) reading matched with forward EC, time taken (chemical rinsing) was recorded.

Final water flushing: Then water was circulated through the TS-TFSSHE until EC reading in the return line matched with forward water EC reading. During CIP, the steam supply to TS-TFSSHE was not closed.

Performance testing: A swab was wiped on the marked surface area after CIP was completed.

In swab test, a cotton swab moistened with quarter strength ringer's solution is applied over the known area of equipment and in this process, micro-organism adhered to the surface of the equipment are transferred to the swab. Generally, two areas admeasuring 7cm×7cm each are randomly selected at the outlet/product discharge end (third or final stage) on the test equipment. Being concentrated product with least mechanical agitation in third stage, the chances of most tenacious soil formation will be more as compared to second and first stages. Sterilized petri dishes, test tubes, sterilized cotton swab, nutrient agar (NA), violet red bile agar (VRBA), phosphate buffer solution were used for swab method. The colony forming unit (cfu/cm²) was computed from following Equation

$$\text{Colony forming unit } \left(\frac{\text{cfu}}{\text{cm}^2}\right) = \text{Average} \left(\frac{\text{colony count on petriplate} \times 10^{(\text{dilution used})}}{\text{area from which swab was wiped, cm}^2} \right) \dots\dots\dots (3)$$

Mainly swab test is conducted as performance determination parameter as it would indicate the efficacy of cleaning in terms of microbiological analysis after CIP was done. Target for cleanliness using developed CIP system was to assure NIL counts from microbial analysis (swab test) and minimising time taken for circulation of CIP solutions along with visual cleanliness of all product contact surfaces.

The terms used for performance analysis of the developed CIP system are:

- (i) **Chemical rinsing duration Y₁:** Time taken by the system during which sodium hydroxide solution was in circulation
- (ii) **Total CIP time Y₂:** Total time taken by the CIP system to complete CIP process
- (iii) **Total plate count, Y₃:** Total colony forming units as calculated by using Equation (3) on data obtained from nutrient agar plates and

(iv) **Coliform count, Y_4** : Total colony forming units as calculated by using Equation (3) on data obtained from VRBA plates.

Experimental design and analysis

For experimental trials, three independent variables (Table 1) were chosen and responses (dependent variables) were chemical rinsing duration (Y_1), total CIP time (Y_2), total plate count (Y_3), coliform count (Y_4) (Table 1). Response Surface Methodology (RSM) was used to optimize performance of the system. It was assumed that independent variables (X_1, X_2, X_3) affect the performance responses.

After experimental trials, the values of performance responses may be defined in terms quadratic model by following equation (Yadav et al. 2012):

$$Y_k = \beta_{k0} + \sum_i^3 \beta_{ki} X_i + \sum_{i=1}^3 \beta_{kii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{kij} X_i X_j + e_k \quad \dots$$

(4)

Where Y_k is the performance response (Y_1 = Sodium hydroxide time, Y_2 = Total CIP time, Y_3 = Total plate count and Y_4 = Coliform Count); β_{k0} is the regression coefficient (value of fitted response at centre point of the design); β_{ki} , β_{kii} , β_{kij} are linear, quadratic, interaction regression coefficient; X_i , X_j are independent variables ($i = 1, 2, 3$, respectively where X_1 = concentration of sodium hydroxide solution, %; X_2 = temperature of solution, °C; and X_3 = scraper speed, rpm) and e_k is unexplained error.

A face centred central composite design (FCC) with three variables was used and experimental design is presented in Table 1. There may be effect of unexpected variability in performance responses due to extraneous factors, so experiments were performed in random order. Based on result of preliminary trials, the experimental range of independent variables was identified for experimental design. In present study, the optimization was carried out using Design-Expert v.8.0.7.1 software (Stat Ease Inc., Minneapolis) to obtain the optimized conditions on suitable criteria (Table 3).

Experimental Validation of optimized solution

Responses were numerically optimized using design expert software which provides optimum conditions (sodium hydroxide concentration, X_1 ; temperature of CIP solution, X_2 and scraper speed, X_3) under criteria listed in Table 3 within range of experimental data. The experiments were conducted in triplicates at optimum conditions. To test the significance difference between experimental and predicted value at optimum parameters, student's t-test ($p < 0.05$) was used. The Equation (5) was used for computing p values (Lamauro et al. 1985):

$$P = \frac{100}{n} \sum_{i=1}^n \left| \frac{Q_{i\text{exp}} - Q_{i\text{pre}}}{Q_{i\text{exp}}} \right| \dots \dots \dots (5)$$

Where, P is p value, $Q_{i\text{exp}}$ is experimental value of response and $Q_{i\text{pre}}$ is predicted value of response.

Results and Discussion

Figure 1 shows the developed CIP system (isometric drawing and assembled unit) for cleaning of three stage thin film scraped surface heat exchanger. It consists of mainly multi-partition tank along with mobile stand (on wheels), CIP fluid flow system, and Instrumentation and control system.

Performance Evaluation of CIP system

Table 2 shows ANOVA using quadratic (second-order) response surface model which describe influence of independent variables on the performance responses. The model was significant ($p \leq 0.05$) and the lack of fit was non-significant ($p > 0.05$). The negative sign of a regression coefficient, at linear level, showed reduction in response value with an increase in level of independent variable whereas at interactive level, level of one variable decreased while that of other variable increased to give similar response values (Table 2). The 3-D response surface plots were created (Figures 2-3) to visualize the combined effect of two variables on a specific response.

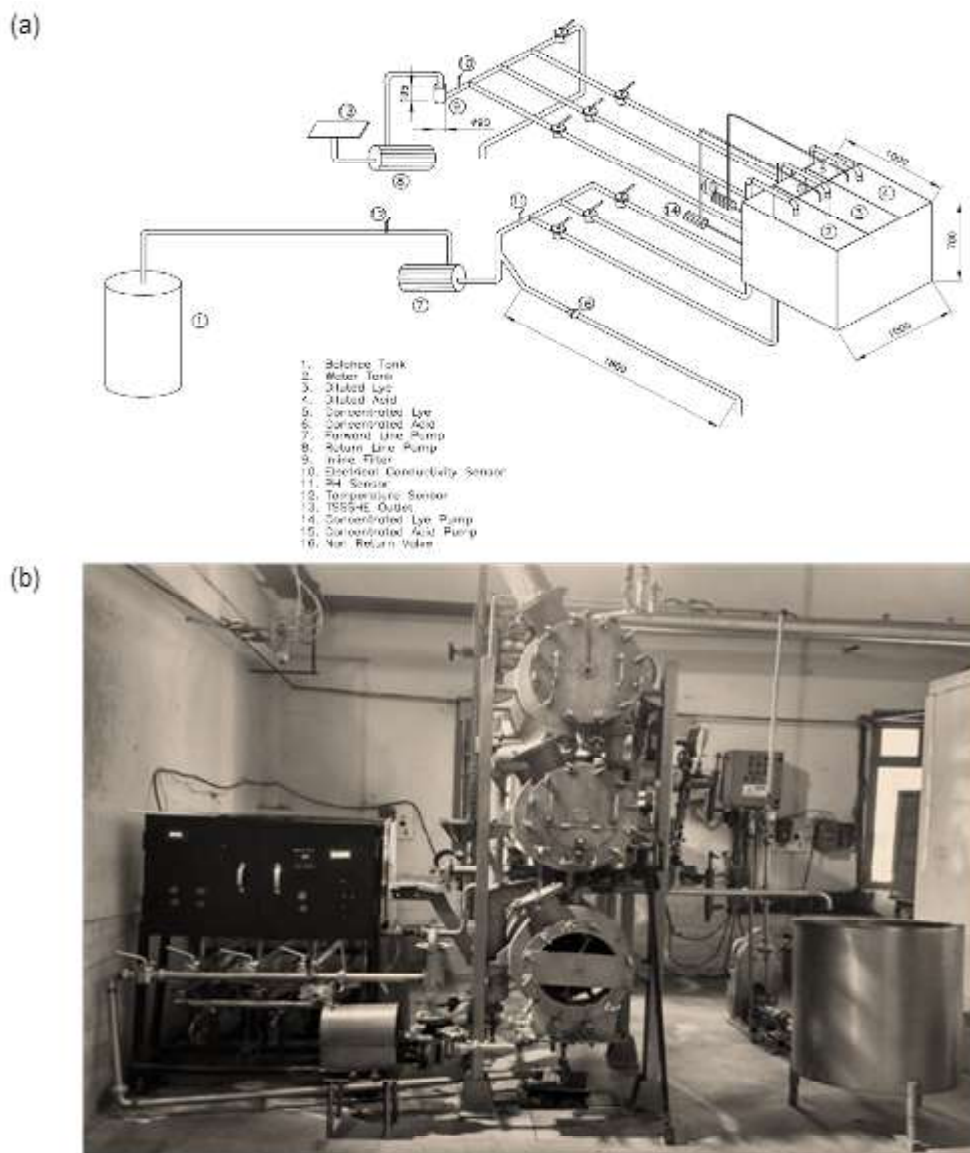
Influence of process parameters on chemical rinsing duration

The maximum and minimum chemical rinsing duration were 740 s and 333 s, respectively (Table 1). With increase in temperature from 60° C to 80° C at constant concentration and speed, chemical rinsing duration decreased more rapidly ($p \leq 0.01$), which may be due to decrease in viscosity of hot solution (along with soil material) and the more cleaning effect at higher temperature and hence some increase in its flow velocity. However, as the speed increased, there was a continuous increase ($p \leq 0.05$) in the chemical rinsing duration at a specific concentration and temperature, which may be due to obstruction by scraper against flow of solution leading to increased residence time of the solution in the equipment (Table 2). Non-significant effect was observed for chemical rinsing duration with quadratic levels of concentration, temperature and scraper speed (Table 2). However, with increase in time and temperature of exposure by CIP solution, a linear increase in removal of bacterial load was observed (Kumari and Sarkar, 2014). The response surface and contour plots for chemical rinsing duration scores in relation to concentration-temperature, speed-concentration and speed-temperature have been presented in Figure 2. The coefficient of determination

Fig.1 Developed CIP system.

(a) schematic sketch, (b)

Assembled unit



($R^2=0.86$) was in reasonable agreement with adjusted R^2 -value of 0.74. The interaction effect of concentration and temperature showed a rapid increasing effect of chemical rinsing duration ($p \leq 0.01$). The decreasing effect ($p \leq 0.05$) of chemical rinsing duration was observed with interaction of concentration and scraper speed as well as temperature and scraper speed (Figure 2, Table 2).

Influence of process parameters on Total CIP time

The maximum total CIP time observed was 2004 s, while the minimum was 1549 s (Table 1). With increase in temperature at constant concentration and speed, total CIP time decreased more rapidly ($p \leq 0.01$), which may be due to decrease in viscosity of hot solution and hence some increase in its flow velocity (Table 2). However, no significant effect was observed for total CIP time with quadratic levels of concentration, temperature and scraper

speed (Table 2). The response surface and contour plots for sodium hydroxide time scores in relation to concentration-temperature, speed-concentration and speed-temperature have been presented in Figure 2. The coefficient of determination ($R^2=0.86$) was in reasonable agreement with adjusted R^2 of 0.73. The interaction effect of concentration and temperature showed an increasing effect of total CIP time ($p \leq 0.05$). The rapid decreasing effect ($p \leq 0.01$) of total CIP time was observed with interaction of concentration and scraper speed as well as temperature and scraper speed (Figure 2, Table 2). Khalid et al. (2015) reported that process of fouling deposit removal increased with increasing chemical concentration, temperature and velocity.

Influence of process parameters on Total plate count (TPC)

The maximum TPC observed was 1.15 cfu/cm², while the minimum was 0 cfu/cm² (Table 1). At linear level, CIP solution temperature

was having highly significant negative effect ($p \leq 0.01$) for TPC of bacterial load with time and temperature of exposure by CIP solution. At quadratic level, temperature showed highly positive significant effect ($p \leq 0.01$) on TPC (Table 2). However, the regression coefficient value of temperature was very less. The Kumari and Sarkar (2014) also reported a linear increase in removal response surface and contour plots for sodium hydroxide time

Table 1: Experimental design and data for the response surface analysis (FCC)

S.No.	Factors (X)									Responses (Y)			
	X ₁ : Sodium Hydroxide Concentration (%)			X ₂ : CIP Solution Temperature (°C)			X ₃ : Scraper Speed (rpm)			Time (s)	Microbiological Testing (cfu/cm ²)		
Coded Value	-1	0	+1	-1	0	+1	-1	0	+1	Y ₁ : Chemical Rinsing Duration	Y ₂ : Total CIP	Y ₃ : Total Plate Count	Y ₄ : Coliform Count
Actual Value	0.750	1.375	2.000	60	70	80	150	225	300	Duration	Count	Count	(TPC)
1		0.750			60				300	740	2004	0.900	3.500
2		1.375			70				225	414	1739	0.000	0.000
3		1.375			70				225	414	1738	0.000	1.800
4		0.750			80				300	401	1695	0.700	2.261
5		2.000			60				150	357	1663	1.150	0.145
6		2.000			80				300	356	1549	0.214	0.000
7		1.375			70				225	432	1782	0.020	0.020
8		2.000			70				225	422	1747	0.031	0.000
9		2.000			60				300	399	1729	0.600	0.000
10		0.750			80				150	333	1617	0.500	0.200
11		1.375			80				225	348	1685	0.200	0.100
12		1.375			70				225	415	1772	0.000	0.000
13		1.375			70				300	365	1748	0.032	0.900
14		1.375			70				225	363	1687	0.020	0.000
15		1.375			60				225	438	1762	0.850	0.000
16		1.375			70				150	349	1667	0.000	0.000
17		0.750			60				150	423	1735	0.900	0.000
18		0.750			70				225	365	1685	0.100	1.030
19		2.000			80				150	440	1741	0.041	1.600
20		1.375			70				225	379	1686	0.800	0.020

Table 2 Analysis of variance for Y₁: chemical rinsing duration, Y₂: total CIP time, Y₃: total plate count (TPC) and Y₄: coliform count using quadratic response surface model

Source	df	Y ₁ : Chemical Rinsing Duration				Y ₂ : Total CIP time				Y ₃ : Total Plate Count (TPC)				Y ₄ : Coliform Count			
		(R ² = 0.86, R ² _{adjusted} = 0.74)				(R ² = 0.86, R ² _{adjusted} = 0.73)				(R ² = 0.80, R ² _{adjusted} = 0.61)				(R ² = 0.85, R ² _{adjusted} = 0.71)			
		SS	MS	F	p	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Model ^a	9	1.19×10 ⁵	13189	6.85**	0.003	1.23×10 ⁵	13623	6.65**	0.003	2.4	0.27	4.31*	0.016	15.59	1.73	6.15**	0.005
X ₁	1	8294.4	8294.4	4.31 ^{NS}	0.065	9424.9	9424.9	4.60 ^{NS}	0.056	0.11	0.11	1.83 ^{NS}	0.206	2.75	2.75	9.78*	0.011
X ₂	1	22944	22944	11.91**	0.006	36724	36724	17.93**	0.002	0.75	0.75	12.19**	0.006	0.027	0.03	0.09 ^{NS}	0.765
X ₃	1	12888	12888	6.69*	0.027	9120.4	9120.4	4.45 ^{NS}	0.061	2.09×10 ⁻³	2.09×10 ⁻³	0.03 ^{NS}	0.856	2.22	2.22	7.90*	0.019
X ₁₂	1	27495	27495	14.27**	0.004	13203	13203	6.45*	0.029	0.1	0.1	1.62 ^{NS}	0.232	0.78	0.78	2.76 ^{NS}	0.128
X ₁₃	1	22791	22791	11.83*	0.006	27966	27966	13.65**	0.004	0.04	0.042	0.67 ^{NS}	0.431	6.67	6.67	23.70**	0.001
X ₂₃	1	17578	17578	9.12*	0.013	25200	25200	12.30**	0.006	0.11	0.11	1.73 ^{NS}	0.218	1.05	1.05	3.72*	0.083
X ₁₁	1	1663	1663	0.86 ^{NS}	0.375	51.28	51.28	0.03 ^{NS}	0.878	3.21×10 ⁻³	3.21×10 ⁻³	0.05 ^{NS}	0.824	0.47	0.47	1.66 ^{NS}	0.226
X ₂₂	1	1596	1596	0.83 ^{NS}	0.384	27.84	27.84	0.01 ^{NS}	0.910	0.67	0.67	10.85**	0.008	7.57×10 ⁻³	7.57×10 ⁻³	0.03 ^{NS}	0.873
X ₃₃	1	390.02	390.02	0.20 ^{NS}	0.662	451.84	451.84	0.22 ^{NS}	0.649	6.30×10 ⁻⁴	6.30×10 ⁻⁴	0.01 ^{NS}	0.922	0.33	0.33	1.18 ^{NS}	0.303
Residual	10	19267	1926.7			20484	2048.4			0.62	0.062			2.82	0.28		
Lack of Fit ^b	5	15864	3172.8	4.66 ^{NS}	0.058	12182	2436.5	1.47 ^{NS}	0.342	0.10	0.019	0.18 ^{NS}	0.958	0.14	0.03	0.05 ^{NS}	0.997
Pure Error	5	3402.8	680.57			8302	1660.4			0.52	0.1			2.68	0.54		
Correlation	19	1.38×10 ⁵	13189			1.43×10 ⁵				3.01	0.27			18.41	1.73		
Total																	

sum of squares; df, degree of freedom; MS, mean sum of squares; F, ratio of variances; p, probability

Subscript for X: 1 for Sodium hydroxide Concentration (X₁); 2 for Temperature (X₂); 3 for Scraper Speed (X₃)

*: Significant; ^b: Non-significant

NS: Not Significant, * Significant at 5 % level ($P \leq 0.05$), ** Significant at 1% level ($P \leq 0.01$)

Fig.2 Response surface and contour plots of chemical rinsing duration / (lye) time and total CIP time as influenced by level of. (a) concentration and temperature at 225 rpm scraper speed, (b) scraper speed and concentration at 70°C temperature, (c) scraper speed and temperature at 1.375 % sodium hydroxide concentration

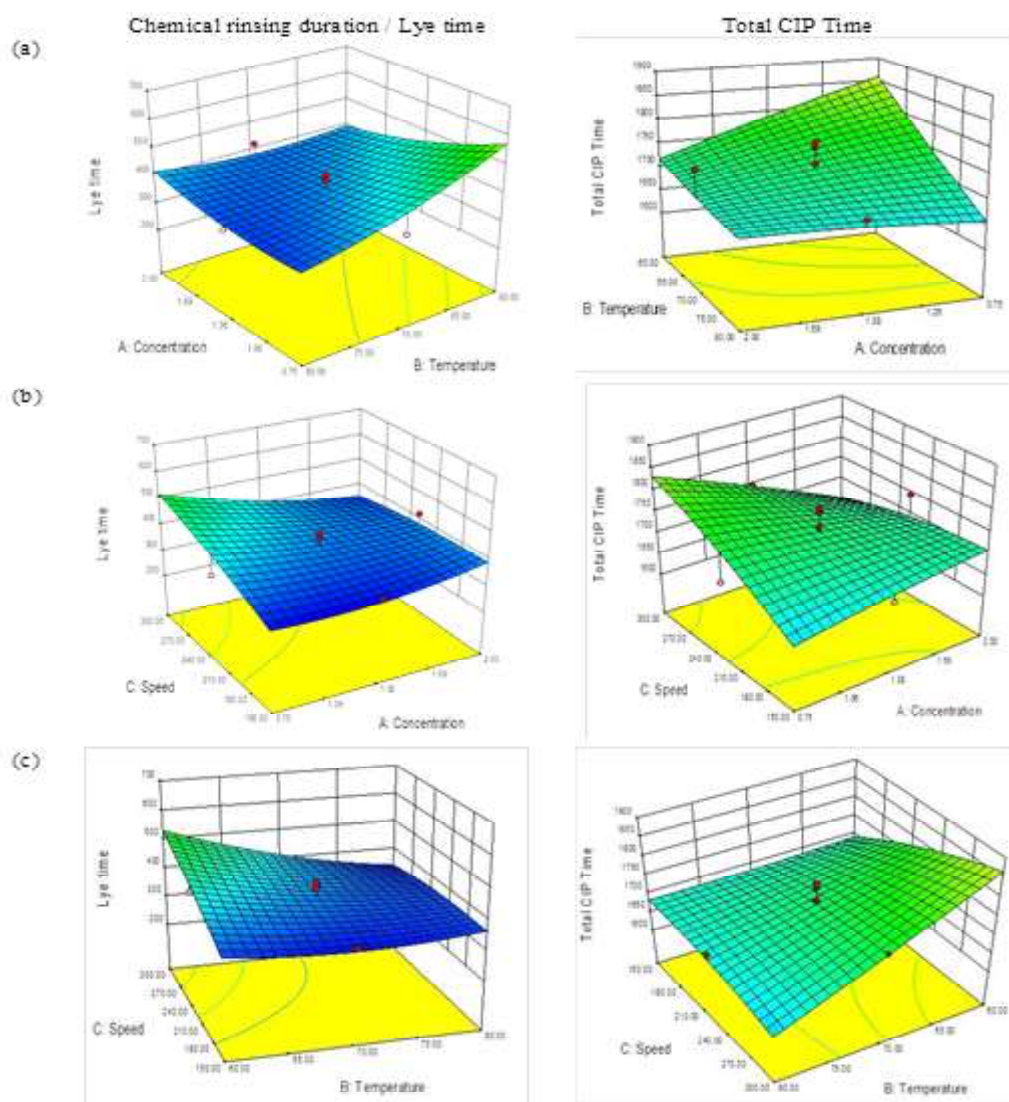


Table 3 Constraints, criteria for optimization, solution along with predicted and actual response values

Constraints	Goal	Lower limit	Upper limit	Importance	Solution	ARV	%P	T Value
X ₁	in range	0.750	2.000	3	0.850	-	-	-
X ₂	in range	60.00	80.00	3	72.70	-	-	-
X ₃	in range	150	300	3	150	-	-	-
Y ₁	Minimize	333	740	3	326.328	341.33±4.04	4.38	-2.87
Y ₂	Minimize	1549	2004	3	1648.7	1648.5±4.5	0.18	0.07
Y ₃	Target 0	0	1.15	5	0.1039	0	-	-
Y ₄	Target 0	0	3.5	5	0.1012	0	-	-

Factors (X): X₁, Sodium hydroxide Concentration (%); X₂, Temperature (°C); X₃, Scraper Speed (rpm)

Responses (Y): Y₁, Chemical rinsing duration (s); Y₂, Total CIP time (s); Y₃, TPC (cfu/cm²); Y₄, Coliform Count (cfu/cm²)

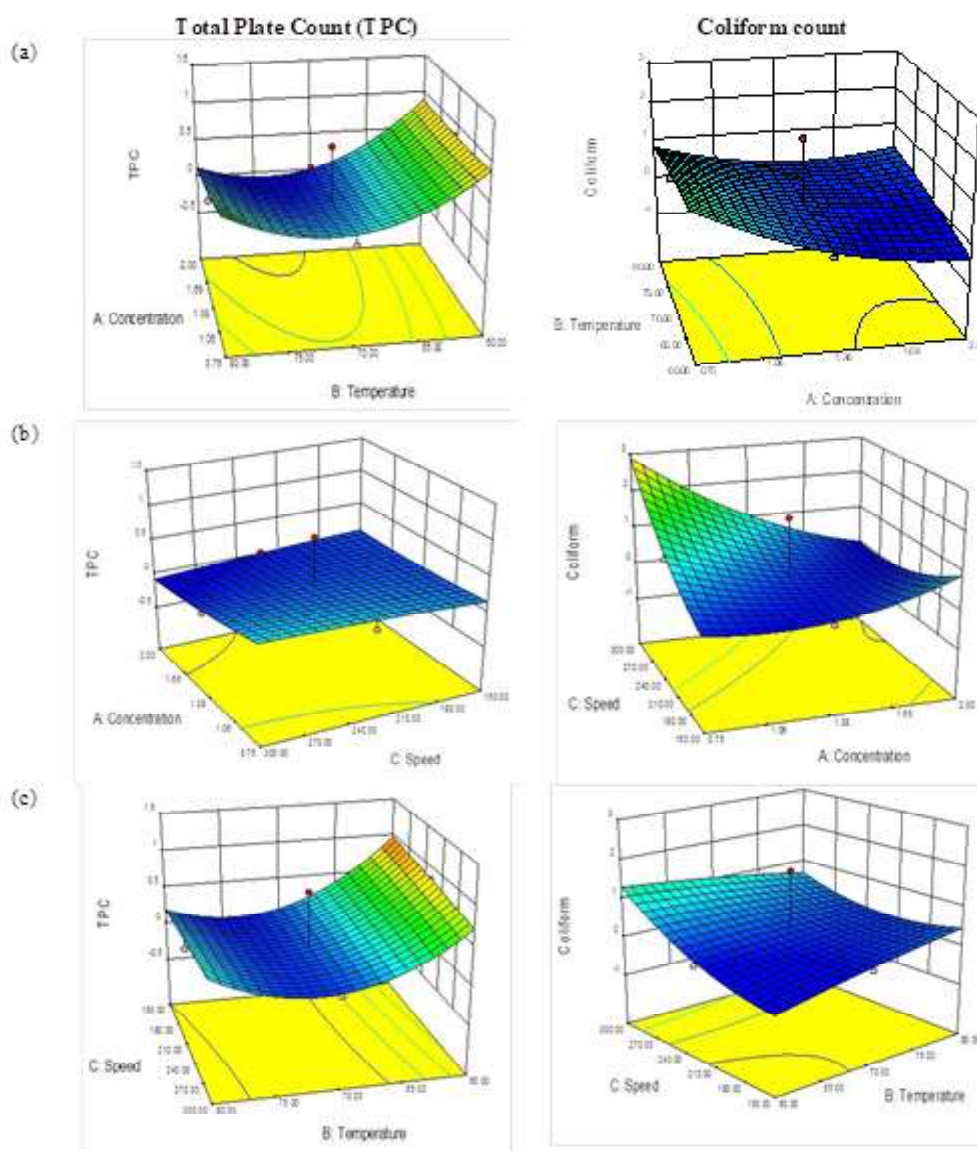
ARV Actual response values (Mean ± SD)

The predicted values and actual reported values for any response differed non-significantly (p<0.05)

scores in relation to concentration-temperature, speed-concentration and speed-temperature have been shown in Figure 3. The coefficient of determination ($R^2 = 0.80$) was in reasonable

agreement with adjusted R^2 of 0.61. At interaction level, all process variables were non-significant (Figure 3, Table 2).

Fig.3 Response surface and contour plots of total plate count (TPC) and coliform count as influenced by level of (a) concentration and temperature at 225 rpm scraper speed, (b) scraper speed and concentration at 70°C temperature, (c) scraper speed and temperature at 1.375 % sodium hydroxide concentration



Influence of process parameters on Coliform counts

The maximum coliform count observed was 3.5 cfu/cm², while the minimum was 0 cfu/cm² (Table 1). With increase in concentration at constant temperature and speed, coliform count decreased significantly ($p \leq 0.05$), which may be due to increased mortality of microbes as concentration increased. Under flow conditions, Lelievre et al. (2002a,b) observed that 0.5% concentration of sodium hydroxide was adequate for removal of majority of bacterial spores. With increase in scraper speed at constant temperature and concentration, coliform count increased significantly ($p \leq 0.05$), which may be due to separation of microbes from hot surface at higher scraper speed. However, CIP solution temperature as well as quadratic levels of concentration, temperature and scraper speed were having non-significant effect (Table 2). The response surface and contour plots for coliform

count scores in relation to concentration-temperature, speed-concentration and speed-temperature have been shown in Figure 3. The coefficient of determination ($R^2 = 0.85$) was in reasonable agreement with adjusted R^2 of 0.71. There was a significant decreasing effect on coliform count due to interaction effect of concentration and scraper speed ($p < 0.01$) as well as temperature and scraper speed ($p < 0.05$) (Figure 3, Table 2).

Optimization of process parameters

Table 3 represents the constraint criteria for optimization, solution along with predicted and actual values of responses. Using constraint criteria, the optimum process conditions were obtained as 0.85% sodium hydroxide concentration (X_1), 72.70°C temperature of CIP solution (X_2) and 150 RPM scraper speed (X_3). The experimental data was compared with predicted data by

conducting statistical analysis (student's t-test) and it was observed that the experimental values were not significantly different from the predicted values with respect to all responses at 5% level of significance. The %P (calculated using Equation(5)) in order to compare the precision of fit of the model and it was less than 5, for all responses suggesting that the experimental data were in good agreement with the predicted values. Thus, the response surface optimization model was adequate. The TPC and coliform counts value during validation was found as zero which is necessary for proper CIP.

In conventional cleaning process, human interactions are time consuming and risk prone etc. The fouling of heat exchangers occurs during production of dairy products with negative impact on operational costs and product quality. CIP system reduces the downtime of production due to less cleaning time for process equipment. During CIP, cleaning steps were considered to be efficiently completed when electrical conductivity (EC) reading matched with forward EC (chemical and water rinsing). Developed CIP system will save the resources e.g. labour requirement and time etc. as compared to manual cleaning. The response i.e. chemical rinsing duration and total CIP time may vary with the nature of soil (product made), size and type of process equipment.

Conclusion

An innovative CIP system was developed and its performance (time and microbial counts) was evaluated for proper cleaning of TS-TFSSHE. Optimized process parameters were 0.85% concentration of sodium-hydroxide, 72.7°C CIP solution temperature and 150 rpm scraper speed. Optimized predicted values were non-significant with the mean experimental values ($p > 0.05$). TPC and coliform count were also not detected at optimized solution. This system is mobile CIP system (wheel mounted), so it may be used for other processing equipments at other places as well.

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Conflicts of interest

None.

Ethical guidelines

Ethics approval was not required for this research.

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RESEARCH ARTICLE

Preparation of synbiotic fermented milk and evaluation of short-chain fatty acids production during storage study

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Abstract: The fermented milk product was formulated using *Lactobacillus cultures* i.e., V3 and M5 with addition of corn-starch. 8% sugar level was selected for the preparation of fermented milks based on sensory attributes and overall acceptability. Optimized fermented milk (T) along with control where corn starch was replaced with skim milk powder (C) were analyzed for their proximate composition, short chain fatty acids (SCFAs) production as well as changes in physico-chemical and sensory properties during storage at refrigerated temperature ($5\pm 2^\circ\text{C}$) up to 28 days. The pH of fermented milks C and T had significant ($P<0.05$) differences during 28 days of refrigerated storage and decreased from 4.35 to 3.85 and 3.93, respectively. Acidity (% LA) of fermented milks C and T significantly ($P<0.05$) increased to 1.22% from 0.78 and 0.76%, respectively during storage. Average viable counts (log CFU/ml) of fermented milk T was 9.57 log CFU/ml, which decreased to 7.36 log CFU/ml after 28 days, which had no significant differences with C. All three short chain fatty acids (SCFA), acetic acid, propionic acid and butyric acid content ($\mu\text{g/mL}$) of fermented milks (C and T) significantly ($P<0.05$) increased during storage from 1.27 to 5.37 and from 4.17 to 11.00 $\mu\text{g/mL}$, 2.00 to 2.40 and from 2.52 to 3.64 $\mu\text{g/mL}$, 2.25 and 4.72 from 2.11 and 3.18 $\mu\text{g/mL}$, respectively during storage. Treated fermented milks were acceptable up to 21 days under refrigeration temperature ($5\pm 2^\circ\text{C}$) and obtained higher sensory scores for all the attributes which decreased with elevated storage period.

Keywords: Fermented milk, Lactobacilli, Short-chain Fatty Acids, Synbiotics

Introduction

The term “functional food” was first used in 1984. A study on the relationship between Japan nutrition, sensory satisfaction, robustness and modulation of physiological systems to explain food products fortified with specific ingredients that have beneficial physiological effects. The purposes of functional foods are diverse: they improve the general conditions of the body such as prebiotics and probiotics, reduce the risk of certain diseases (for example, cholesterol-lowering products) and Can be used for Treatment of some diseases (Bigliardi and Galati 2013). The largest segment of the functional food market comprises of foods containing probiotics, prebiotics and synbiotics. According to FAO/WHO (2001), probiotics are defined as a “live microorganisms that when administered in adequate amounts confer a health benefit on the host”. A number of potential benefits arising from the use of probiotics have been demonstrated, including increased resistance to infectious diseases (Kumar et al. 2012), alleviation of lactose intolerance, prevention of gut diseases, diarrhoea, vaginal and urogenital infections; reduced allergy and respiratory infections; reduced serum cholesterol concentration; increased resistance to toxins produced during cancer chemotherapy and decreased risk of colon cancer (Stavropoulou and Bezirtzoglou 2020). The diet and the intestinal milieu interact in a complex way with the bacterial population in the gut. Given the nutritive composition and natural buffering capacity, fermented milk is considered best career of probiotics. Positive health effects of fermented foods and especially of those with probiotic microorganisms are reported in many recent publications (Hasan et al. 2014).

In 2008, prebiotics were defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as “a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” (Hill et al. 2014), a definition that is currently being further revised by ISAPP. Prebiotics are fermented by the gastrointestinal microbiota and contribute to healthy modulation of the gut (Di Bartolomeo

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et al. 2013). Synbiotics are a relatively new area that involve a combination of probiotic and prebiotic in one product; the prebiotic is intended to improve the survival/growth/performance of the probiotic or other beneficial bacteria in the colon, which in turn has beneficial health effects on the host (Pranckute et al. 2014). Functional foods are foods or dietary components that can provide a health benefit beyond basic nutrition. The use of the functional oligosaccharides in diet is a part of the management of dyslipidemia (Jaiswal and Sharma 2016). Optimal intake of the functional oligosaccharides reduces the risk of obesity, blood pressure and many other cardiovascular diseases. Fibers that lead to high amounts of SCFAs, lower the pH in the colon, which in turn affects the composition of the colonic microbiota and thereby the SCFA production.

SCFAs, especially acetic, propionic and butyric acid, are vital to maintain the normal function of intestine and human body. Acetate is absorbed and transported to the liver and peripheral tissues, less metabolized in the colon and acts as substrate for cholesterol synthesis and lipogenesis (Chakraborti 2015). Propionate is a primary precursor for gluconeogenesis and it reduces the synthesis of hepatic cholesterol (Cheng and Lai 2000). Butyrate is considered one of the most important colon metabolites, as it serves as the majorly preferred energy source for the colonocytes, has anti-inflammatory properties and regulates gene expression, differentiation, cellular proliferation and apoptosis in host cells, resulting in reduced risk of colon cancer (Hamer et al. 2008; Canani et al. 2011). Very few research works are carried out in combining the functional aspects of lactic acid bacterial fermentation of milk and generation of short chain fatty acids during its storage. Hence, the current study was planned mainly focusing on these three interesting SCFAs in fermented synbiotic product during storage study.

Materials and Methods

Preparation and Analysis of Fermented Milk

The study was planned with the use of best starter cultures *Lactobacillus helveticus* (V3) (MTCC 5463) (Prajapati et al. 2011) and *Lactobacillus fermentum* (M5) with the pre-chosen level of prebiotic (3% Corn-starch) (Makwana 2019) Standard procedure for probiotic lassi making with minor modification of the procedure adopted by Patidar and Prajapati (1998) was used. Complete flow diagram for preparation of fermented milk with added prebiotic is given in Fig. 1.

Optimization of sugar addition rate for preparation of fermented milk

The blend of MTCC 5463 (V3) *L. helveticus* and *L. fermentum* (M5) (1:1 by weight) was admixed with different rate of sugar (8, 9 and 10%) to suit to consumer taste. The samples of fermented milk admixed with different level of sugar were subjected to

sensory evaluation by trained panel using affective testing method.

Sensory evaluation

Seven panellists were engaged for their liking and preference evaluation, which were performed on different occasions. Liking scales were defined using 9-point hedonic scale for sourness, flavour, appearance, consistency and overall acceptability as described by Stone and Sidel (2004). Coded samples of fermented blend were given to the panellists. The results of sensory evaluations were reported as mean value with standard error of mean.

Evaluation of stability of fermented milk during refrigerated storage

The influences of storage period (4°C for 28 days) on biochemical and sensory properties for fermented milk were analysed as follows. The samples of both blends were removed prior to incubation for analysis. Samples of both the types of fermented product were removed from the refrigerated storage (4 °C) at 0, 7, 14, 21 and 28 day. The samples were analysed for changes in pH and acidity, viability of *Lactobacilli* cultures, SCFAs production and sensory attributes (i.e. flavour, colour and appearance, body and texture and overall acceptability score).

Fresh toned milk (TM) having minimum 3.0 % fat and 8.5 % SNF was selected for preparing fermented milk.

Estimation of SCFAs

Analysis of SCFAs in the curd was performed by slight modification of the method of Roopashri and Varadaraj (2014). Aliquot of 2 ml of homogenized fermented milk sample was taken in a 15 ml centrifuge tube, and 7 ml of 10 mM NaOH containing 0.1 mM crotonic acid was added. The mixture was kept in a shaker incubator for 6 h at 30 °C. One ml chloroform was then mixed with the sample to remove fat soluble substances. This mixture was centrifuged at 10,000 rpm for 15 min at 4 °C in a refrigerated centrifuge. The supernatant was isolated and filtered through 0.22 µm membrane filter. Using Hamilton syringe, 20 microlitre samples were injected in a Shimadzu HPLC model LC-20 (Shimadzu, Japan) to perform the HPLC analysis. An analytical column [C 18] was used for this purpose. 0.1 % (v/v) phosphoric acid isocratic mixture was used as the solvent for elution at 30 °C and at a flow rate of 0.6 ml/min, using an UV detector at 210 nm.

Determination of pH

The pH values of the blend during fermentation were monitored using a pH meter (Mettler Toledo, USA).

Determination of titratable acidity

The titratable acidity was estimated by the procedure described in (AOAC,1995).

Estimation of Protein Content

Protein content was determined by following macro-Kjeldahl method as described by AOAC (2006).

Estimation of Fat Content

The fat content was estimated by Mojonnier method (IS: SP-18, Part-X1,1981).

Estimation of Ash Content

Ash content of the product samples were determined by procedure described in BIS handbook (BIS, 1981).

Microbiological Evaluation

Lactobacilli Enumeration

MRS agar, 61.15 g was suspended in 1000 ml distilled water containing 1 mL of tween 80 and boiled to dissolve the medium completely. It was then filled in flasks and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. The pH of the medium was adjusted to 5.4 at the time of plating by using sterile glacial acetic acid. 1 mL of diluted sample (suitable dilution) was transferred in each of the Petridishes in triplicate, 10-15 mL of the melted agar (at 45°C) was then poured and the contents were mixed well by

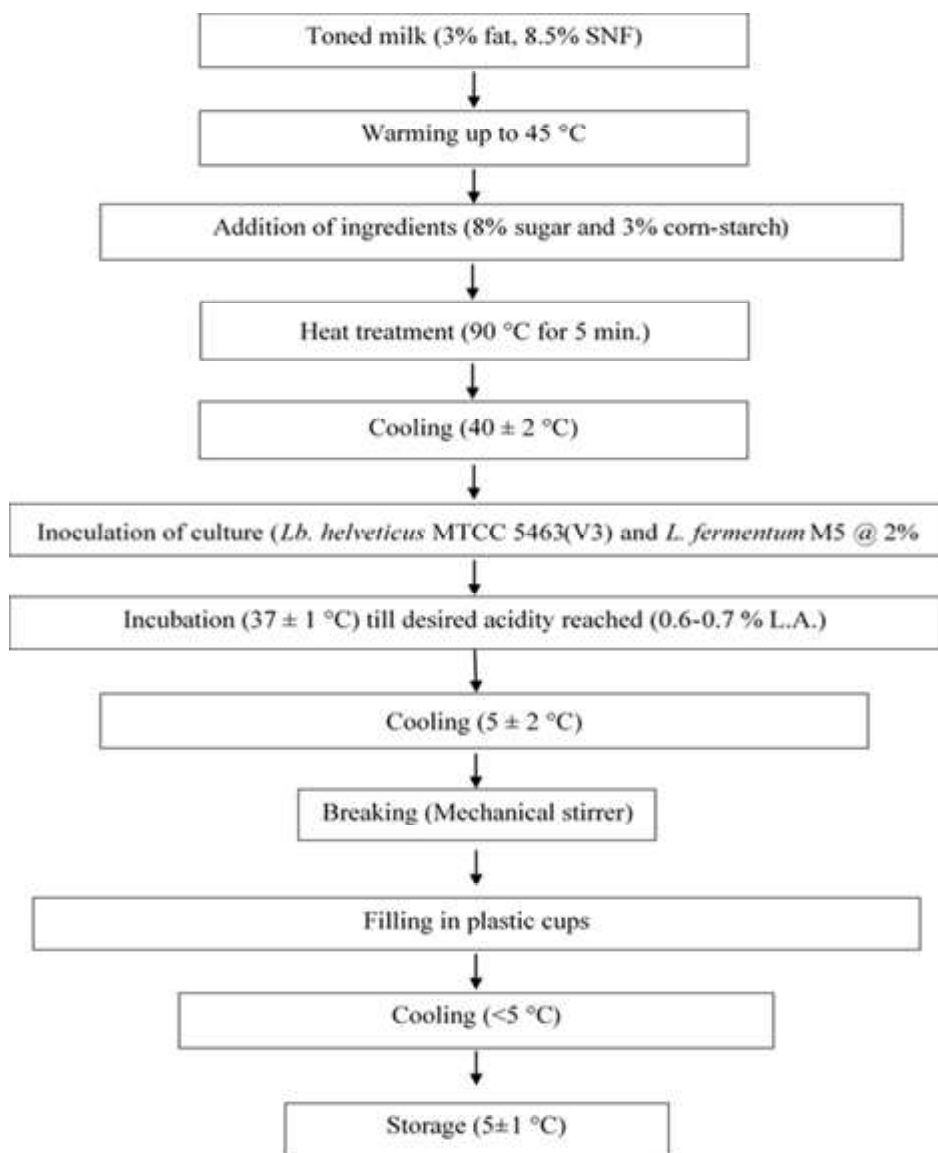


Fig.1 Flow chart for preparation of fermented milk

rotating in a horizontal position. The contents were allowed to solidify and a second of same agar (4-5 mL) was overlaid in each plate. The plates were then inverted and incubated at 37°C for 24-48 h and colonies formed on the medium were counted after incubation period.

Results and Discussion

Standardization of the sugar addition level for preparation of fermented milks

The selected LAB strains (V3 and M5 @ 2%) with 3% level of addition of corn-starch in toned milk medium were considered for optimization of rate of sugar addition. Varying amount of sugar (8%, 9% and 10%) were added. Sugar addition rate was optimized based on sensory evaluation of the blends. Different sensory properties viz. flavour, body, colour and appearance, sourness and overall acceptability were evaluated using affective testing method based on 9-point hedonic scale by seven expert panel members. The mean scores for sensory attributes of fermented milks as affected by addition of different level of sugar addition to fermented blend are presented in Table 1 and 2.

Mean scores for flavour, consistency, sourness, colour and appearance as well as overall acceptability as perceived by panellists were not significantly affected by the level of sugar.

Also, there was no significant difference observed within the different level of sugars as the highest mean score observed for flavour was for 8% sugar (8.68) followed by 9% (8.59) and 10% (8.51). It was significantly ($P<0.05$) higher as compared to control (6.94).

Assessment of biochemical characteristic and sensory attributes of fermented milk during refrigerated storage

The fermented milk (T), prepared with 8% sugar addition was studied for storage stability and other fermentation properties at refrigeration temperature along with the control product (C) for comparison replacing corn-starch (CS) with supplementation of SMP and fermented using the same method as given in Fig. 1. The both prepared fermented milks are shown in Fig. 2. They were analysed for changes in pH, viability of probiotic organisms and sensory attributes during refrigerated storage ($5\pm1^\circ\text{C}$) for 28 days. Different sensory properties viz. flavour, body, appearance, sourness and overall acceptability were evaluated using affective testing method based on 9-point hedonic scale by seven expert panel members.

Changes in overall acceptability of fermented milks

The mean score of overall acceptability for fermented milks is presented in Table 3. On average, the score for the control product (6.35) was significantly lower ($P<0.05$) than the developed product (7.88). However, the acceptability score significantly declined

Table 1: Sensory evaluation of fermented milks added with different levels of sugar

Treatments with sugar	Sensory Attributes				
	Flavor	Body	Sourness	Color & Appearance	Overall Acceptability
Control (0%)	6.94±0.5	8.55±0.1	8.65±0.1	8.73±0.08	7.41±0.4
8%	8.68±0.04	8.71±0.1	8.73±0.04	8.78±0.1	8.77±0.1
9%	8.59±0.04	8.68±0.09	8.7±0.1	8.75±0.1	8.64±0.1
10%	8.51±0.04	8.72±0.07	8.68±0.1	8.73±0.08	8.68±0.02
Sem	0.13	0.06	0.05	0.04	0.11
CD _(0.05)	0.40	NS	NS	NS	0.36
CV%	3.16	1.42	1.13	1.10	2.80

Values represent mean (7 panellists × 3 replication) ± SD

Table 2: Optimization of sugar addition rate for preparation of fermented milk

Treatments	Physicochemical Attributes		
	pH	Acidity (%L.A.)	<i>Lactobacillus</i> Count (log CFU/ml)
Control (0%)	4.15±0.01	1.09±0.01	9.48±0.08
8%	4.16±0.03	1.09±0.03	9.50±0.1
9%	4.06±0.05	1.06±0.01	9.49±0.09
10%	4.15±0.06	1.09±0.02	9.49±0.1
Sem	0.05	0.01	0.05
CD _(0.05)	NS	NS	NS
CV%	1.38	2.50	1.13

Values represent mean (7 panellists × 3 replication) ± SD

during the storage period ($P < 0.05$). Mean score 8.73 at 0 day declined significantly ($P < 0.05$) to 7.01 at 14 d for product T and subsequently declined during the storage. Similarly, for the product C, mean score 7.88 at 0 day declined significantly to 6.95 at day 14 and continually declined further during the storage study.

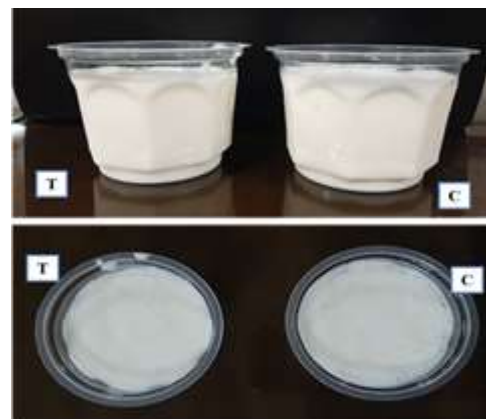
Changes in pH of fermented milks

The changes in pH of the fermented milk during the storage are presented in Table 4. During storage at 5° C, both types of fermented milks showed decrease ($P < 0.05$) in pH on 7th day followed by decrease ($P < 0.05$) up to 28 days of storage (Table 4). The average decline in pH was significantly higher on 7th day of storage. Subsequently further decline up to 21 days of storage was observed for both the milks. However, in the last week of storage study, mean pH value further declined significantly ($P < 0.05$) to 3.89.

Changes in viability of LAB

The changes in the total viable counts of *Lb. helveticus* MTCC 5463 + *Lb. fermentum* M5 are presented in Table 5. For both the fermented milks, the total viable counts of the products C and T significantly declined ($P < 0.05$) on 7th day, then after remained unaffected up to 21 days. However, significant loss in viability of starter bacteria ($P < 0.05$) were also observed on 28th day for both the milks.

Fig. 2 Prepared fermented milk (T) and Control (C)



Changes in titratable acidity of fermented milks

The changes of the acidity in both the types of fermented milks is presented in Table 6. In both the products, the acidity significantly increased ($P < 0.05$) on 7th day and 14th day, then after remained unaffected up to 28 days. However, the effect of treatments and the interaction effects of starter cultures during storage resulted in insignificant changes ($P > 0.05$) in the acidity of two different milks.

Overall, mean score value of all sensory properties remained above 6 (liking slightly) throughout the storage period up to 21 days indicating the product is acceptable. However, consumer to consumer variation, temperature of storage, packaging material and conditions, post production handling and other microbial

Table 3: Changes in overall acceptability of fermented milks during storage

Treatments	Storage period in days (P)					Mean (T)
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	7.88±0.28	7.59±0.37	6.95±0.08	5.87±0.27	5.51±0.06	6.35
T	8.73±0.21	8.2±0.24	7.01±0.2	6.58±0.15	5.98±0.11	7.88
Mean (P)	7.84	7.30	7.04	6.97	6.43	
Source		SEm	CD _(0.05)		CV%	
Period (P)		0.09	0.26			
Treatments (T)		0.05	0.17		3.14	
P*T		0.12	NS			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

Table 4: Changes in pH of fermented milks during storage

Treatments	Storage period in days (P)					Mean (T)
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	4.35±0.1	4.08±0.03	4.07±0.05	4.10±0.008	3.85±0.03	4.11
T	4.35±0.05	4.14±0.06	4.06±0.05	4.04±0.05	3.93±0.02	4.09
Mean (P)	4.35	4.11	4.07	4.07	3.89	
Source		SEm	CD _(0.05)		CV%	
Period (P)		0.02	0.07			
Treatments (T)		0.01	NS		1.33	
P*T		0.03	NS			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

contaminations etc. are the factors which needs to be taken in considerations during deciding the shelf life of the product.

Effect of LABs and Corn-starch on SCFAs production in Fermented Milks During Storage Study at 5±1 °C

In the present objective, estimation of SCFAs production for both the types of milks throughout the storage study was carried out at 0, 7, 14, 21 and 28 days and data are presented in Table 7, 8 and 9 for acetic acid, propionic acid and butyric acid production, respectively.

Changes in acetic acid (µg/ml) production in fermented milks during storage

The mean score of acetic acid production by fermented milks is presented in Table 7. Significant difference ($P<0.05$) was observed among both the products with regards to acetic acid production. In general, the acetic acid production score for control product (2.93) was significantly lower ($P<0.05$) than the product T (8.14). The acetic acid production scores also did significantly increase during the storage periods ($P<0.05$). For developed product T, acetic acid production was significantly increased from 4.17 on 0 day to 11.0 µg/ml on 28th day.

Changes in propionic acid (µg/ml) production in fermented milks during storage

The mean score of propionic acid production for fermented milks is presented in Table 8. Significant difference ($P<0.05$) was observed among both the fermented milks with regards to propionic acid production. In general, the acetic acid production score for control(C) product (2.26) was significantly lower ($P<0.05$) than T (2.94). The propionic acid production scores also did significantly increase during the storage period ($P<0.05$). Product T had non-significant increase in propionic acid production up to 14th day, thereafter, it significantly increased up to (3.64) on 28th day, while for control, the propionic acid production remains non-significant throughout the storage study.

Changes in butyric acid production in fermented milks during storage

The mean score of butyric acid production for fermented milks is presented in Table 9. Significant difference ($P<0.05$) was observed among the products with regards to butyric acid production. In general, the butyric acid production for the product C (2.21) was significantly lower ($P<0.05$) than the developed product T (3.77). The butyric acid production scores also did significantly increase during the storage period ($P<0.05$). The butyric acid production

Table 5: Changes in log count of starter bacteria in fermented milks during storage

Treatments	Log count (CFU/ml)					Mean (T)
	Storage period in days (P)					
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	9.53±0.02	8.69±0.2	8.53±0.5	8.29±0.1	7.26±0.1	8.46
T	9.57±0.01	8.76±0.09	8.66±0.1	8.36±0.2	7.36±0.2	8.54
Mean (P)	9.55	8.72	8.60	8.32	7.31	
Source		SEm	CD _(0.05)	CV%		
Period (P)		0.09	0.27			
Treatments (T)		0.05	NS	2.62		
P*T		0.13	NS			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

Table 6: Changes in titratable acidity of fermented milks during storage

Treatments	Acidity (% L.A.)					Mean (T)
	Storage period in days (P)					
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	0.78±0.03	1.07±0.07	1.15±0.09	1.21±0.01	1.22±0.03	1.09
T	0.76±0.03	1.09±0.06	1.19±0.03	1.21±0.01	1.22±0.05	1.10
Mean (P)	0.77	1.08	1.17	1.21	1.22	
Source		Sem	CD _(0.05)		CV%	
Period (P)		0.02	0.06			
Treatments (T)		0.01	NS		5.0	
P*T		0.03	NS			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

in product T on 0 day was 3.18 µg/ml, which increased significantly to 4.72 on 28th day, while for control it remained non-significant throughout the storage period.

Standardization of the sugar addition level for preparation of fermented milks

The addition of sugar did not have any significant effect on body, acidity, color and appearance. The score for color and

appearance ranged from 8.73 to 8.78 and for body, it ranged from 8.55 to 8.72. The overall acceptability of sugar added products were better than the control product. Hence, the product with the lowest possible sugar addition (8%), was prepared and analysed for storage study attributes. The physico-chemical attributes in terms of pH and acidity did not change significantly with different rates of addition of sugar. Average pH of the product was 4.1 and the acidity was 1.08% L.A. The *Lactobacilli* count in the product did not change with the addition of the sugar, and

Table 7: Changes in acetic acid (µg/ml) production in fermented milks during storage

Treatments	Acetic acid production (µg/ml) Storage period in days (P)					Mean (T)
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	1.27±0.1	1.75±0.2	2.79±0.2	3.44±0.1	5.37±0.1	2.93
T	4.17±0.1	6.44±0.2	8.7±0.2	10.4±0.2	11±0.1	8.14
Mean (P)	2.53	4.10	5.74	6.92	8.05	
Source		SEm	CD _(0.05)		CV%	
Period (P)		0.09	0.27			
Treatments (T)		0.05	0.17		4.1	
P*T		0.13	0.38			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

Table 8: Changes in propionic acid (µg/ml) production in fermented milks during storage

Treatments	Propionic acid (µg/ml) production Storage period in days (P)					Mean (T)
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	2±0.1	2.21±0.1	2.21±0.1	2.27±0.08	2.4±0.1	2.26
T	2.52±0.1	2.57±0.1	2.71±0.07	3.25±0.06	3.64±0.07	2.94
Mean (P)	2.26	2.39	2.49	2.84	3.02	
Source		SEm	CD _(0.05)		CV%	
Period (P)		0.04	0.13			
Treatments (T)		0.02	0.08		4.2	
P*T		0.05	0.18			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

Table 9: Changes in butyric acid (µg/ml) production in fermented milks during storage

Treatments	Butyric acid (µg/ml) production Storage period in days (P)					Mean (T)
	0 th day	7 th day	14 th day	21 st day	28 th day	
C	2.11±0.1	2.23±0.08	2.23±0.08	2.25±0.05	2.25±0.1	2.21
T	3.18±0.2	3.46±0.2	3.55±0.06	3.94±0.1	4.72±0.1	3.77
Mean (P)	2.64	2.84	2.89	3.10	3.48	
Source		SEm	CD _(0.05)		CV%	
Period (P)		0.06	0.17			
Treatments (T)		0.03	0.11		4.95	
P*T		0.08	0.25			

C= Control product Probiotic milk prepared by replacing CS with SMP

T = Developed product Probiotic milk prepared using CS

the average count was observed to be 9.49 log CFU/ml. A carbonated probiotic fermented milk with *Lactobacillus helveticus* MTCC 5463 and *Streptococcus thermophilus* MTCC 5460 was prepared and standardized with respect to carbon dioxide pressure, sugar and salt concentrations based on sensory, physico-chemical and microbial parameters and the product was acceptable on 28th day of storage under refrigeration condition (Shah and Prajapati, 2014).

Assessment of biochemical characteristic and sensory attributes of fermented milk during refrigerated storage

Changes in flavour of fermented milks

The mean score for flavour remained above 7 (moderate liking preference) at day 14, thereafter significantly ($P < 0.05$) decreased during storage. Usually, the lipolytic and proteolytic activity of LAB is generally responsible for causing off-flavours in fermented milk (Tamime and Robinson, 2007). Apart from these activities, in the present study, the flavour profile of corn-starch also contributes to overall flavour perception. El-Aidie et al. (2017) studied the sensory evaluation of cereal-based fermented milk product and reported that all products have good sensory acceptability.

Changes in body of fermented milks

The mean score for body (7.18) remained above 7 (moderate liking preference) on 14th day for the product T, thereafter significantly ($P < 0.05$) decreased during storage. Viscosity and consistency of food system is usually affected by sugar and other macromolecules through their interaction with the solution or solvent (Zapsalis and Beck 1985). The level of addition of sugar and corn starch may have influenced the higher viscosity of our fermented milk supplemented with CS. Mohammad (2004) found that seven different stabilizers (pectin, guar gum, carboxymethylcellulose (CMC), carrageenan, sodium alginate, corn starch and gelatin), storage time and total solids combined with the thickeners significantly affected the properties such as syneresis and texture of the yogurt samples. Textures (hand-felt and oral) of yogurts were significantly affected by starch concentrations and/or storage time. Williams et al. (2003) reported that the addition of starch has beneficial effects on yogurt properties.

Changes in sourness of fermented milks

The mean score for mouthfeel (7.48) remained above 7 (moderate liking preference) on 14th day for product T, thereafter significantly ($P < 0.05$) decreased during storage. It was observed by Supavititpatana et al. (2010) that the acidity of the yoghurt samples increased during the 28 days storage. This increase in acidity during storage agreed with the findings of for corn milk yoghurt. The increase in titratable acidity may be as a result of anaerobic microbial activities resulting in the formation of lactic and other organic acids. It has been reported that increase in

titratable acidity and the extent of increase was influenced by the type of lactic acid bacteria present (Sanni et al. 1999; Buckner et al. 2008). However, as titratable acidity increased, the pH decreased as a function of fermentation time (Walia et al. 2013).

Changes in appearance of fermented milks

The mean score for appearance (7.62) remained above 7 (moderate liking preference) up to 14th day, thereafter significantly ($P < 0.05$) decreased up to 28 days of storage. Similar to our observations, Yasni & Maulidya (2014) also found the color of corn yoghurt light yellow because of the yellowish pigment in sweet corn.

Changes in overall acceptability of fermented milks

The mean score for overall acceptability (7.01) remained above 7 (moderate liking preference) on 14th day. Alim et al. (2016) evaluated the sensory qualities based on different attributes for sweetened cow milk yoghurt (without homogenization) with incorporation of corn starch at different levels. They found that all the sensory scores of different samples were significantly different ($p < 0.01$) and the samples showed various degrees of acceptability. However, they concluded that the highest overall acceptability was found at 2.0% of incorporation level for corn-starch.

Changes in pH of fermented milks

The effect of treatments and the combined effects of starter bacteria and storage periods, however, did not observe to influence ($P > 0.05$) the changes in pH of fermented milks. Kpodo et al. (2014) reported that skimmed milk powder addition increases the concentration of lactose that could be degraded by the starter culture enzymes to produce lactic acid. This acid in turn increases the acidity and automatically reduces the pH. Onweluzo and Nwakalor (2009) reported similar relationship between pH and acidity. Ifediba & Ozoh (2018) reported that their product breadfruit-corn yoghurt contained corn protein (zein) that may lower the pH, the acidification effect of skim milk could bear more influence owing to faster utilization of lactose by the fermenting microorganisms, whereas, the commercial milk yoghurt used in their study equally contained corn starch as declared on the label, an indication that the zein protein may have equally contributed to the acidity. Supavititpatana et al. (2010) reported similar trend in pH reduction during 35 days of corn milk yoghurt and commercial milk yoghurt storage, with greater reduction found in corn milk yoghurt.

Changes in viability of LAB

Our results of loss of viability in prolonged refrigeration storage are in agreement with the previous reports (Dave and Shah 1998~ Akalin et al. 2007~ Roseburg et al. 2010). However, the interaction effects of starter cultures during storage resulted in non-significant changes ($P > 0.05$) in the total viable counts of starter bacteria. Imamoglu et al. (2017), reported that the amount of starter culture bacteria increased as storage time increased, which is typical for cultured yogurt (added with two types of starch: corn

and kudzu) products and was to be expected. **Changes in titratable acidity of fermented milks:** Walia et al. (2013) reported that fermentation time had a positive effect on acidity but a negative effect on pH in mango soy fortified yoghurt. In our study, acidity of the fermented milks samples increased during the 28 days storage. This increase in acidity during storage agreed with the findings of Supavititpatana et al. (2010) and Adeiye et al. (2013) for corn milk yoghurt and groundnut milk respectively. Similarly, Yasni & Maulidya (2014) found the observation of the total titrated acid of corn milk yoghurt for 4 weeks at refrigerator temperature showed that the lactic acid percentage of corn milk yoghurt continually increased. In the initial week (0 week) the lactic acid percentage was 0.99% and after 4-week observation it increased to $1.22\% \pm 0.01$.

Effect of LABs and Corn-starch on SCFAs Production in Fermented Milks During Storage Study at $5 \pm 1^\circ\text{C}$

Changes in acetic acid ($\mu\text{g/ml}$) production in fermented milks during storage

There was significant increase in acetic acid production during every week of storage in both the products. Further, the acetic acid production in our CS added product was almost two to three times higher than the control product prepared without CS. The higher amount of acetic acid production seen in this product was difficult to explain as there are many factors which are playing role. One of the factors is use of toned milk instead of RSM used in phase one and two of the study. Similarly, Adhikari et al. (2001), showed that the diversity of organic acids in fermented dairy foods is due to the activity of the added probiotic bacteria such as *Bifidobacterium* (*B. longum* B6 and ATCC15708). By using HPLC method, they showed that the concentrations of acetic and lactic acid increased during storage.

Changes in propionic acid ($\mu\text{g/ml}$) production in fermented milks during storage

The mean score for propionic acid production (2.84) after 21 days of storage significantly ($P < 0.05$) increased to (3.02) on 28th day of storage. Similarly, an increase in the amount of propionic acid during storage in plain yogurt was reported by Fernandez-Garcia and McGregor (1994). Adhikari et al. (2001) reported that no particular pattern was observed for the stirred yogurts. However, the propionic acid content increased by about 10 mg/100 g yogurt for the non-encapsulated *B. longum* B6 treatment and by about 20 mg/100 g yogurt for the encapsulated *B. longum* ATCC 15708 treatment.

Changes in butyric acid production in fermented milks during storage: The mean score for butyric acid production (2.89) significantly differ after 14 days of storage ($P < 0.05$) which significantly increased to (3.1) and (3.48) on day 21 and 28, respectively. Adhikari et al. (2001) reported that in fermented dairy foods the treatments containing non-encapsulated

bifidobacteria or encapsulated *B. longum* B6 showed comparable values and they were stable throughout the storage period. Yadav et al. (2007), studied the production of fatty acids and conjugated linoleic acid (CLA) in the ordinary and probiotic yogurt dahi (prepared with buffalo milk) containing *L. acidophilus* and *L. casei*, during fermentation and after 10 days storage at 5°C . They reported that an increased level of fatty acids especially butyric acid during fermentation and storage in the probiotic yogurt samples is mainly due the lipolysis of milkfat which was higher in the presence of probiotic bacteria.

Conclusion

The findings of present study suggested that fermented milk prepared with V3 and M5 cultures (T) in combined showed superior sensory and better storage stability with increased SCFAs production up to 28 days as compared to the control (C). Proximate compositions of developed product were 9.84 percent total solids, 2.48 percent fat; 2.9 percent protein, 3.62 percent carbohydrate and 0.84 percent ash. These two promising *Lactobacillus* cultures could be used for the development of fermented milks with health beneficial properties. The validation of the health claim needs to be conducted on human subjects.

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Estrus intensity scoring and conception rate in postpartum anestrus buffaloes using estrus induction protocols

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Abstract: The onset of cyclicity of oestrus cycle and initiation of ovulation during postpartum period in buffaloes constitute a major problem and results into long postpartum anestrus and delayed breeding with consequent serious economic losses in the milk production and efficient reproduction. Effective treatment of postpartum anestrus in buffaloes using estrus induction protocols can efficiently improve their milk productivity. The objective of this study was to compare the estrus intensity score and conception rate in postpartum anestrus buffaloes using estrus induction protocols. Twenty four postpartum buffaloes (5-10 years age) reared under field conditions with the history of not showing signs of estrus for 60 days or more postpartum were considered to be affected with anestrus. These buffaloes were examined per rectally twice at 10 days apart to confirm ovarian activity and genital status. These animals were randomly divided into 4 groups (03 treatment and 01 control group) and each group having 06 anestrus buffaloes. Group I, II and III were treated with CIDR plus Ovsynch, CIDR plus Cosynch and eCG plus Cosynch protocols, respectively, while the control group (Group IV) animals were not given any treatment. The mean weighted oestrus intensity score and conception rate for group I, II, III and IV were 71.33±3.04, 67.66±3.04, 78.33±5.85, 65.00±5.00 and 50.00, 33.33, 66.67 and 33.33 per cent, respectively. It can be concluded that addition of eCG to a GnRH and prostaglandin based protocol (eCG+Cosynch) substantially improved the estrus intensity and conception rates in postpartum anestrus buffaloes.

Keywords: Postpartum anestrus, estrus intensity score, conception rate, estrus induction protocols

Introduction

Buffalo is regarded as a shy / poor breeder with delayed onset of puberty, long postpartum ovarian quiescence, poor signs of estrus and long inter-calving intervals (Brar and Nanda, 2004). In Indian climatic conditions, postpartum acyclicity is the most common single cause of infertility in rural buffaloes (Iyer, 1978). Incidence of anestrus varies from herd to herd and under different managemental conditions. Silent ovulation (lack of overt signs of estrus) and unobserved estrus (poor estrus detection efficiency) can greatly increase the incidence of anestrus.

Although signs of estrus normally are less pronounced in buffaloes, the majority of farmers detect estrus based on visual observation. When presented for AI, clinicians often base their diagnosis of estrus in buffaloes on transrectal evaluation of uterine tone, palpation of an ovulatory sized follicle and cervical status and visualization of the ovulatory follicle and/or uterus. Singh and Kharche (1985) devised a score card system with 75 and 70 as maximum weight score, respectively, to categorise the buffaloes into intense, intermediate and weak on the basis of oestrus behaviour pattern and status of reproductive organ.

Estrus induction protocol using various hormones with fixed-time artificial insemination (FTAI) have been tried for the treatment of anestrus with variable success rates (Kumar et al. 2014).

The present investigation was planned to evaluate the oestrus intensity scoring of modified Ovsynch and modified Cosynch protocols in postpartum anestrus buffaloes.

Materials and Methods

Selection of animal

The present research work was carried out in different villages of Jabalpur (M.P.). Twenty four postpartum buffaloes (5-10 years age) reared under field conditions with the history of not showing signs of estrus for 60 days or more postpartum were considered

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to be affected with anestrus. These buffaloes were examined per rectally twice at 10 days apart to confirm ovarian activity and genital status. These animals were randomly divided into four groups *i.e.* three treatment group and one untreated control group (n=06 animals/group) and were dewormed. The animals of treatment groups were subjected to various synchronization protocols (Table 1) after 07 days of deworming as follows:

Application and removal of CIDR

The CIDR is made of a silicone rubber impregnated with progesterone and molded over nylon spine that is T- shaped. The wings of the CIDR fold upon themselves when placed in the applicator. The buffaloes were restrained in trevis and CIDR was inserted into the vagina. Extra nylon tail attached to the end of the CIDR was cut to prevent pulling out by neighboring animals. The CIDR was removed by pulling out the nylon tail that is exposed from the reproductive tract.

Estrus detection and breeding

Detection of oestrus was carried out twice daily (morning and evening) by visual observations. Buffaloes at induced oestrus were bred by artificial insemination (AI) on the fixed day of treatment protocols. The observations regarding induction and intensity of estrus after the end of treatment were recorded.

Estrus intensity score

Table 1: Treatment protocols for different groups

Groups (n=06 animals /group)	Treatment regimen	Schedule
I (Modified Ovsynch protocol/ CIDR + Ovsynch)	Inj. GnRH (Buserelin acetate 20µg, I/M) + CIDR Implant Inj. PGF _{2α} (Cloprostenol 500µg, I/M) + Removal of CIDR Implant Inj. GnRH (Buserelin acetate 10µg, I/M) FTAI	Day 0 Day 07 Day 09 Day 10 (24 hrs. after 2 nd GnRH injection)
II (Modified Cosynch/ Cosynch)	Inj. GnRH (20µg, I/M) + CIDR Implant Inj. PGF _{2α} (Cloprostenol 500 µg, I/M) + Removal of CIDR implant Inj. GnRH (Buserelin acetate 10µg, I/M) + FTAI	Day 0 Day 07 Day 09
III (Modified Cosynch eCG+Cosynch)	Inj. eCG (400 IU, I/M) Inj. GnRH (Buserelin acetate 20µg, I/M) Inj. PGF _{2α} (Cloprostenol 500 µg, I/M) Inj. GnRH (Buserelin acetate 10µg, I/M) + FTAI	Day -3 (3 days prior to day 0) Day 0 Day 07 Day 09
IV (Untreated Control)	No treatment	AI on estrus

It was numerically scored on the basis of a proper weightage given for each of the parameter comprising estrus changes in the external and internal genitalia and behavioural expression. The estrus intensity was classified into intense, moderate and weak grade. Animals failed to exhibit signs of estrus were considered as silent estrus. For this purpose, the score card device of Sirmour (1999) was used.

Statistical analysis

The data was analysed on R platform (R Core Team, 2018) using “dplyr” library. Data from different experiments were presented as Mean ±SE. The pair-wise comparison of means was carried out using Fisher’s multiple comparison test as per standard statistical method described by Snedecor and Cochran (1994). The difference at $p < 0.05$ was considered to be satisfactory significances. The results of oestrus induction were expressed in percentage.

Results and Discussion

Oestrus Induction Efficiency

In response to different treatment protocols, oestrus was observed in 100.00(06/06) per cent postpartum buffaloes in group I, II and III. In control group (group IV), 33.33 per cent of postpartum buffaloes with anestrus showed oestrus signs (Table 2).

Oestrus induction efficiency obtained in treatment group I (100 per cent) in present study was in corroboration with the findings of Mujawar et al. (2019) in postpartum Marathwadi buffaloes, Kundulkar et al. (2016) in postpartum buffaloes and Baruselli et

al. (2007) in anestrus buffaloes. However, oestrus induction efficiency obtained in present study were higher than that reported by Azawi et al. (2012) in northern Iraqi buffaloes, Vikash et al. (2014) in anestrus buffaloes of Haryana (95.74 per cent) and Ghallab and Nosier (2016) in Egyptian buffaloes (80 per cent).

Oestrus induction efficiency obtained in treatment group II (100.00%) in present study were similar to the findings of Patil et al. (2020) in postpartum anestrus buffaloes and Sah et al. (2019) in anestrus cows. It was higher in present study as compared to the study of Kumar et al. (2016) using cosynch protocols (81.30%) in postpartum anestrus buffaloes.

Oestrus induction rate of 100.00 per cent was observed in CIDR + Ovsynch group of buffaloes in present study might be attributed to prolonged exogenous progesterone priming from CIDR device with negative feed-back effect on hypothalamo-hypophyseal-gonadal axis favouring GnRH, FSH and LH storage. Its sudden withdrawal in circulatory concentration promotes the release of GnRH as the negative feedback of progesterone, thus stimulating FSH and LH secretion and folliculogenesis and subsequent resumption of ovarian cyclicity (Zaabel et al. 2018).

Oestrus induction efficiency obtained in treatment group III (100.00%) in present study were higher than that observed by Dhaka et al. (2019) in postpartum Murrah buffaloes (86.60 per cent).

Rathore et al. (2017) in their study reported lower oestrus response (90.00 and 88.00%) than present study by eCG + CIDR and Ovsynch+eCG in postpartum anestrus Nili-Ravi buffaloes.

Suboptimal functioning of the hypothalamus–pituitary–gonadal (HPG) axis and low FSH and LH peaks is related to postpartum anestrus in the buffalo cow under tropical and subtropical conditions. In another study of Murugavel et al. (2009), eCG subsequent to 13 day of progesterone treatment increased plasma concentrations of LH and FSH and enhanced the LH peak during estrus in non-cyclic buffalo heifers. Thus, administration of eCG probably helps complete the recovery of HPG axis function already stimulated by the progesterone treatment.

The variation in results could be due to the reproductive status or stage of oestrus cycle at the beginning of the protocol, in addition variations in nutrition, managerial practices, breed, parity, age and geographical condition.

In control group animals 33.33 per cent animals showed oestrus induction which can be attributed to utero-ovarian massage during palpation resulting in enhancement of blood supply to ovary and uterus, activation of intra-ovarian factors increases the availability of hormones and growth factors; stimulation of local oxytocin production by the ovaries which consequently influence local blood circulation and luteolysis, if CL is present (Mwaanga et al. 2010).

Oestrus intensity score

Group wise total oestrus intensity score in postpartum anestrus buffaloes of different treatment and control group is depicted in table 3.

Statistical analysis revealed non-significant variation ($p \geq 0.05$) was observed between the different groups for oestrus intensity.

Table 2: Estrus induction efficiency and conception rate to various hormone protocols in control and different treatment groups

Groups (N=06/Group)	Estrus induction efficiency (per cent)	Conception rate (per cent)
Group I (CIDR+Ovsynch)	100.00 (6)	50.00 (3)
Group II (CIDR+Cosynch)	100.00 (6)	33.33 (2)
Group III (eCG+Cosynch)	100.00 (6)	66.67 (4)
Group IV (Untreated control)	33.33 (2)	33.33 (2)

Figures in parenthesis indicate number of animals

Table 3: Group wise total oestrus intensity score in postpartum anestrus buffaloes of different treatment and control group

Group	Name of group	Oestrus intensity Score	
		Range	Mean±SE
I	CIDR+Ovsynch (06/06)	64-84	71.33±3.04
II	CIDR+Cosynch (06/06)	60-88	67.66±4.20
III	eCG+Cosynch (06/06)	58-92	78.33±5.85
IV	Control (02/06)	60-70	65.00±5.00
Overall (20/24)		58-92	70.58±2.89

Figures in parenthesis indicate no. of buffaloes

Table 4: Group wise oestrus intensity at induced/observed oestrus in postpartum anestrus buffaloes

Group	Name of group	Oestrus intensity Score (per cent)			Total
		Intense	Moderate	Weak	
I	CIDR+Ovsynch (n=06)	16.67 (01)	50.00 (03)	33.33 (02)	06
II	CIDR+Cosynch (n=06)	16.67 (01)	16.67 (01)	66.67 (04)	06
III	eCG+Cosynch (n=06)	66.67 (04)	00.00 (00)	33.33 (02)	06
IV	Control (n=02)	00.00 (00)	50.00 (01)	50.00 (01)	02
	Total	30.00 (06)	25.00 (05)	45.00 (09)	20

Figures in parenthesis indicate no. of buffaloes

Table 5: Oestrus intensity at induced oestrus in postpartum anestrus buffaloes

Character	Intense	Moderate	Weak
Range	80-92	68-74	58-64
Mean weighted score	86.66±1.97	70.80±1.02	62.22±0.77
Conception rate (%)	66.67	80.00	50.00

In group III administration of PMSG three days before first GnRH resulted in development of follicle which was luteinized and transformed in to CL by first GnRH which acted as source of progesterone (Dhaka et al. 2019). The amount of progesterone secreted by this CL was sufficient enough to induce heat symptoms and additional supplementation of progesterone to improve heat intensity is not required in eCG plus Cosynch protocol. However, in other treatment groups intra vaginal device (CIDR) performed the same function by providing exogenous progesterone. So, non-significant variation ($p > 0.05$) was observed between the different groups for oestrus intensity.

Per cent distribution of buffaloes exhibited intense, moderate and weak oestrus in each treatment group is presented in the table 4.

Oestrus intensity score of buffaloes in intense, moderate and weak oestrus intensities at induced/observed oestrus are depicted in table 5. Difference in the oestrus intensity score between treatment groups clearly indicates the influence of CIDR plus Ovsynch, CIDR plus Cosynch and eCG plus Cosynch protocol at induced oestrus with different oestrus intensities. The highest oestrus intensity was observed in eCG plus Cosynch compared to other groups might be due to the effect of exogenous eCG, which sensitizes the hypothalamus receptors for the estrogen. The FSH and LH like effects of eCG is implemented through its attachment to FSH and LH receptors in theca and granulosa cells (Murphy, 2012). Higher estradiol production by larger follicles in this group mainly responsible for behavioral signs of oestrus (Nayak et al. (2009).

Non-significant ($p \geq 0.05$) variation between oestrus intensity score at induced/observed oestrus. As far as the conception rate based on the intensity of oestrus response is concerned, the results of this study revealed that conception rate was higher in animals showing moderate heat signs (80.00%) than intense (66.67%) and weak heat sign (50.00%). Sirmour (1999) studied oestrus intensity in 34 crossbred heifers and reported that 26, 7 and 1 heifers were in intense, moderate and weak oestrus (76.47, 20.59 and 2.94 per cent), respectively and Ali et al. (2012) studied oestrus intensity in 18 anestrus buffaloes treated with Ovsynch, Heatsynch and CIDR protocol and reported that 09, 03 and 02 buffaloes show intense, moderate and weak oestrus (64.28, 21.43 and 14.28 per cent), respectively. The high conception rate in moderate and intense estrus intensity animals may be attributed to optimum level of estrogen which might have been sufficient enough to induce LH surge for ovulation.

Conclusion

It can be concluded that addition of eCG to a GnRH and prostaglandin based protocol (eCG+Cosynch) substantially improved the estrus intensity in postpartum anestrus buffaloes.

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Physical and morphometric characteristics of unidentified cattle breed of northern Karnataka region of India

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Abstract: A study was undertaken to characterise the non-descript cattle of North Karnataka region. Raichur and Yadgir districts were randomly selected and divided into two blocks each and five villages were selected in each block. The morphological characteristics of male and female cattle of 0-3 months, 3-6 months, 6-12 months, 1 to 3 years and >3 years age was recorded from totally 388 animals. The results indicated that these cattle were short in nature, docile in temperament with three colour variants viz., brown, black and white. These cattle are called Javari in their breeding tract. Horn of these cattle was directed upward, medium sized with horizontal ears. The average horn length, chest girth, body length and height at withers (cm) were 19.63 ± 0.49 , 142.81 ± 0.88 , 118.73 ± 0.81 and 105.89 ± 0.48 in adult cows respectively, whereas in adult bulls were 26.65 ± 0.61 , 173.23 ± 1.20 , 142.15 ± 1.08 and 118.05 ± 0.56 , respectively. These cattle were reared under semi intensive system, sent for grazing in the day time without any supplemental feeding and kept under Kutcha housing or in open space during night hours. Therefore it was evident from the current study, that these cattle were having unique characteristic features.

Keywords: Cattle, non-descript, North Karnataka, morphometry, biometry

Introduction

In India, there are 53 recognized native cattle breeds that are categorised as 3 groups: milch, draught, and dual purpose, based on their use in dairying or agriculture. The phenotypic, utility pattern, and adaptability of cattle populations grown and adapted in India's various agro-climatic settings and production systems are vastly different (Srivastava et al. 2019). The indigenous breeds are now prone to rapid genetic degradation and dilution mainly due to unplanned breeding and the entry of exotic germplasm through cross breeding also because of mechanization of farm operations (Groeneveld et al. 2010). Genetic diversity conservation is critical for any species' long-term survival, especially for the rapidly changing environmental conditions (Tesfa et al. 2017). The loss of these breeds is equal to lose of global insurance policy against food security issues in the upcoming years (Shah et al. 2016). Around 22 per cent of well-known livestock breeds have been extinct in the previous century, with another 27 per cent facing varied degrees of extinction (Rege and Tawah, 1999). As per the available reports, each week, nearly two breeds of poultry and cattle are lost (FAO, 2007). Therefore, the conservation of native animal resources is a viable strategy for reducing the extinction of these livestock breed diversity (Srivastava et al. 2019).

There is a chance that at least 30 to 50 new cattle breeds may be discovered in the next 20 years (Sharma and Niranjana, 2016). According to the 20th Livestock Census, India has 192.49 million cattle population, an increase of 0.8 per cent compared to the previous Census. There are 50.42 million exotic/crossbred cattle and 142.11 million indigenous/non-descript cattle. Karnataka is having rich indigenous genetic resources with six cattle breeds, one buffalo breed, two goat breeds, five sheep breeds and one poultry breed (NBAGR, 2023). The indigenous cattle population of Karnataka accounts for 1,22,88,358 but still much of the population is unidentified. With this background the current investigation was undertaken to characterise the cattle population of Raichur and Yadgir districts of Karnataka.

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Materials and Methods

Locale of the study

The current study was conducted purposively in Raichur and Yadgir districts of Karnataka because it is breeding tract of non-descript cattle called as Javari.

Yadgir and Raichur districts were selected and classified in to four blocks; two from each district were selected randomly. The non-descript cattle (Javari cattle) was grouped in to five categories based on the age. From each block, sixteen animals were selected in 0 to 3 and 3 to 6 months age group, twenty animals in 6 to 12 months and 1 to 3 years old, each group with equal number of males and females. Whereas, in adult (>3 years) 10 male animals and 15 female animals were selected. The total number of animals used for the study was 388, each block consisting of 97 animals. The study was conducted from May 2022 to January 2023.

Physical and morphometric data on males and females of different age group were recorded. In the selected blocks, sampling was done in 5 villages. For morphological characterization, care was taken to collect the data randomly, from animals belonging to true-to-type non-descript cattle, as identified on the basis of their external appearance and typical morphometric characteristics. Various physical characteristics *viz.* basic temperament, size of hump, dewlap, Navel flap, penis sheath flap, and coat colour, skin colour, muzzle colour, eye lid colour, hoof colour, tail switch colour, horn colour and shape, horn size, length, thickness and orientation, head- fore head and face, udder shape and size, teat shape, milk veins were observed.

Biometric characteristics

The different biometric characteristics *viz.*, face length and width, body length, ear length and width, chest girth, paunch/barrel girth, tail length, horn length and circumference, height at withers, distance between hip bones and pin bones were recorded from the Javari cattle herds with the help of scale and measuring tape. Data collected was classified according to age group and sex of the animal.

Body weight

The body weight was 0 to 3, 3 to 6, 6 to 12 months old animals' was measured using hanging weighing balance. For adult cattle, it was calculated by the following formula: *Agarwal's modified Shaffer's formula*

Body weight = chest girth (inch) × body length (inch) / Y

Where, Y is

9, if chest girth is less than 65 inches

8.5, if chest girth is between 65-80 inches

8, if chest girth is over 80 inches

Statistical analysis

The collected data were scored, compiled, tabulated suitable statistical methods with help of using Microsoft Excel, 2016. The data was analysed systematically commensurate with objectives of the study using statistical software SPSS 22.0 (SPSS version 22, SPSS Inc. Chicago, Illinois) as per procedure described by Snedecor and Cochran (1994).

Results and Discussion

Origin, Geographical distribution and native tract of the breed

Karnataka State is located between 11.50° and 18.50° North latitudes and 74° and 78.50° East longitudes. The North Eastern Dry Zone, spread over 1762604 ha, accounts for 9.26 per cent of the total geographical area (1,91,791 sq.km.) of Karnataka State. It is situated between 15° 57' and 17° 36' north latitude and 76° 6' and 77° 33' east longitude. This zone comprises 5 blocks (Afzalpur, Chittapur, Gulbarga, Jewargi, and Sedam) of Gulbarga district, 3 blocks (Shahapur, Shorapur and Yadgir) of Yadgir district and 3 blocks (Devdurga, Manvi and Raichur) of Raichur district. Annual rainfall is between 633.22 to 806.6 mm.

Agriculture is the main occupation of the surveyed blocks of Yadgir and Raichur districts. The main crops of the districts are Paddy, Groundnut, Chilli, Green gram and Cotton, including horticulture crops. Sheep husbandry is also a major occupation of farmers in these districts.

Population status of cattle

This zone indicates the predominance of rain dependent dry land agricultural area. Total livestock population in Yadgir and Raichur districts were 10,05,218 and 13,14,529, respectively. The cattle population in the Yadgir district is 2,33,336 and in Raichur district is 2,45,374.

Physical characteristics

The results of physical characteristics of Javari cattle have been presented in Table 1. It was found that majority of the adult bulls and cows were docile in nature in the study area. The animals were handled by women for milking purpose. The cows were showing fright reaction towards entry of strange in their vicinity. Many of the cows were left for grazing in peri-urban area, so, they are acclimatized to human interactions and traffics. The bulls were owned by small and marginal farmers, they were also handled by women's and children's at home. Mostly, the animals in peri-urban area were more docile when compared to the animals reared under semi-intensive system of rearing in villages.

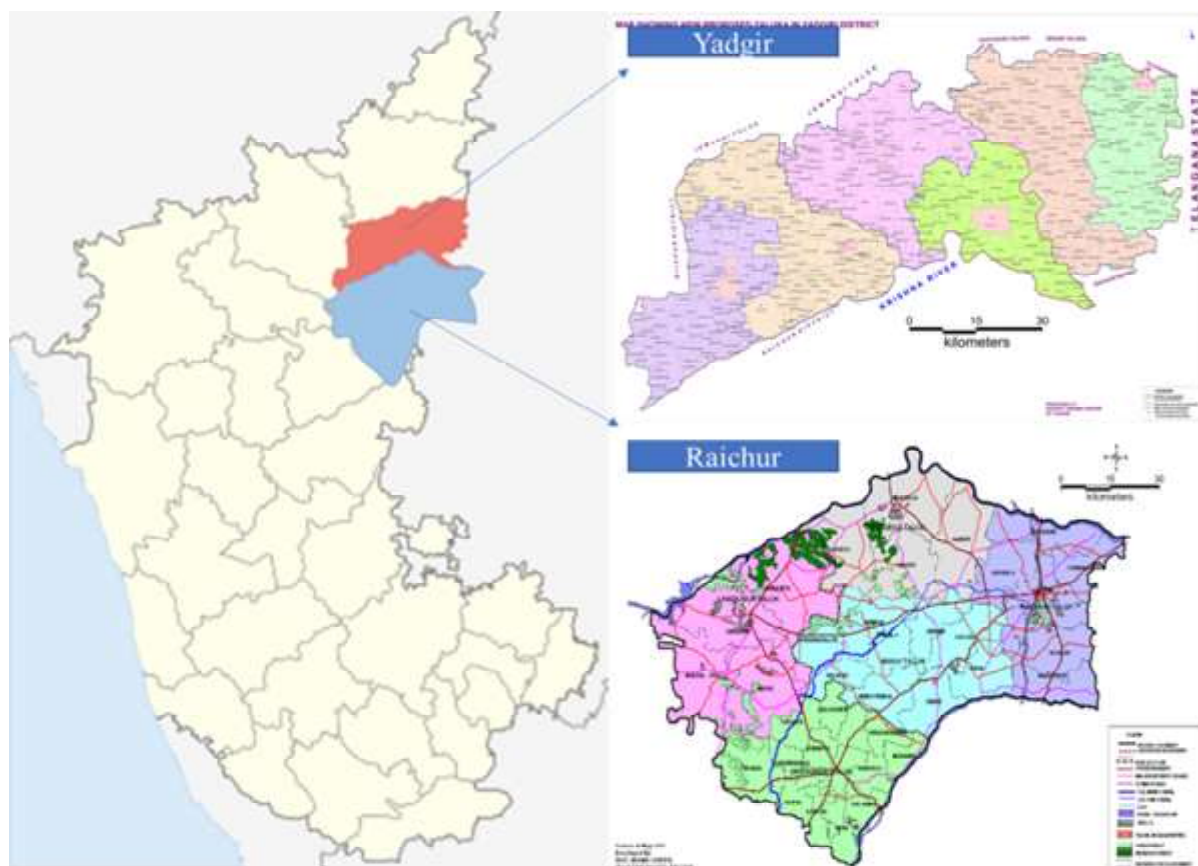


Fig. 1 Geographical maps of Yadgir and Raichur districts of North Karnataka

The predominant coat colour of the bulls and cows was found brown and white, black colour was rare. The predominant light coat colour might be due to hot climatic conditions of the region, which might have influenced the animals to adapt to such adverse climatic conditions which help them to protect against solar radiations. The close breed in the adjacent districts was Krishna valley, Karthickeyan et al. (2006) revealed that, the coat colour of the Krishna Valley bulls was found to be greyish white with dark colour shades on the fore- and hind limbs, cows were light grey in colour, but few brown coloured and black and white coloured were also noticed. But, the Deoni cattle were categorised into three strains on the basis of coat colour variation viz. Wannera (pure white along with black colour at the side parts of the face), Shevera (pure white body with irregular black patches) and Balankya (pure white along with black patches on the lower parts of the body) (Kuralkar et al. 2015). Similarly, short breed of Karnataka, the Malnad Gidda cattle were found in 5 different colours viz, black, brown, red, fawn and white, sometimes mixture of any two colours were also seen, but black colour was majorly present (Singh et al. 2008). In other Mysore type cattle, Singh et al. (2012) revealed that coat colours of Pulikulam cows were white or greyish white colour in most of animals and bulls were either black or blackish grey.

The white or light coat colour of the Javari cattle might help them to reduce the direct effect of heat stress by reflecting more incident solar radiation from the body (Katiyatiya et al.2017). The light coloured coats help to reflect 50 % to 60 % of direct solar radiation than the dark-coloured animal (McManus et al. 2009). Felius et al. (2011) mentioned that coat colour is the most obvious characteristic of cattle, at least for non-experts. Coat colour characteristics were also considered to indicate genetic purity and are relevant for the classifying a breed. Also, the coat colour is the important morphological adaptive trait, which imparts adaptive ability to livestock during heat stress exposure (Gaughan et al. 2019). The primary layer which protects the animals against solar radiation is coat colour. The coat colour of the breeds of tropical countries helps in reflecting the solar radiation; thereby protects the animals against the adverse climatic conditions (Fanta, 2017). Whereas, the breeds with a dark coat colour will absorb more solar radiation which increases their heat load. In a study, it was observed that the cow with lighter coloured coat showed lesser shade-seeking behaviour than the darker one (Tucker et al. 2008).

The muzzle colour of bulls was found black followed by white and brown colour whereas in females prominent was brown

followed by black colour. Similar results were found in Pulikulam cattle (Singh et al. 2012), tribal Kathani cattle (Kulkarni et al. 2013) and Konkan cattle (Singh et al. 2019). These results indicated that black muzzle colour was the most common in indigenous cattle. But, Pundir et al. (2007) noticed white, grey and black muzzle colour in Kenkatha, Kankrej and Gangatiri cattle.

The eyelid colour of the Javari cattle found was predominantly black in bulls and cows compared to brown colour. Similarly, Pundir et al. (2007) reported that Kenkatha and Red Sindhi cattle have black colour eyelids but, Kankrej cattle have grey coloured eyelids. Also, the eyelid colour of majority of the non-descript cattle of Konkan region was black (Khirari et al. 2014).

Table 1: Physical characteristics of unidentified cattle of North Karnataka

Parameters	Yadgir		Raichur		Overall	
	Cow (n=30)	Bullock (n=20)	Cow (n=30)	Bullock (n=20)	Cow (n=60)	Bullock (n=40)
Temperament						
Aggressive	7	2	5	3	12 (20%)	5 (12.50%)
Docile	23	18	25	17	48 (80%)	35 (87.50%)
Coat colour						
Brown	16	10	19	11	35 (58.33%)	21 (52.50%)
White	8	9	9	7	17 (28.33%)	16 (40.00%)
Black	6	1	2	2	8 (13.33%)	3 (7.50%)
Muzzle colour						
Brown	13	1	9	4	22 (36.66%)	4 (10%)
White	0	2	0	7	0	9 (22.50%)
Black	17	17	21	9	38 (72.33%)	27 (67.50%)
Eyelid colour						
Brown	1	0	0	1	1 (1.66%)	1 (2.50%)
Black	29	20	30	19	59 (98.33%)	39 (97.50%)
Hoof colour						
Yellow	3	0	0	1	3 (5%)	1 (2.50%)
Black	27	20	30	19	57 (95%)	39 (97.50%)
Tail switch						
Brown	17	12	10	14	27 (45%)	26 (65%)
White	1	0	1	0	2 (3.33%)	0
Black	12	8	19	6	31 (51.66%)	14 (35%)
Hump size						
Small	27	0	29	0	56 (93.33%)	56 (93.33%)
Medium	3	20	1	20	4 (6.67%)	40 (100%)
Large	0	0	0	0	0	0
Dewlap size						
Small	1	0	2	0	3 (5%)	0
Medium	29	20	28	20	57 (95%)	40 (100%)
Large	0	0	0	0	0	0
Penis sheath						
Small		1		0	1 (2.50%)	
Medium	-	19	-	20	39 (97.50%)	-
Large		0		0	0	
Navel sheath						
Small	30	3	30	0	60 (100%)	3 (7.50%)
Medium	0	17	0	20	0	37 (92.50%)
Large	0	0	0	0	0	0

Horn size						
Small	4	0	7	0	11 (18.33%)	0
Medium	26	20	23	20	49 (81.66%)	40 (100%)
Large	0	0	0	0	0	0
Horn colour						
White	8	5	7	2	15 (25%)	7 (17.50%)
Black	22	15	23	18	45 (75%)	33 (82.50%)
Horn shape						
Straight	30	20	30	20	60(100%)	40 (100%)
Horn orientation						
Upward - outward	30	20	30	20	60(100%)	40 (100%)
Head shape						
Straight	30	20	30	20	60(100%)	40 (100%)

Table 2: Udder characteristics of adult unidentified cows of North Karnataka

Parameters	Yadgir (N=30)	Raichur (N=30)	Overall (N=60)
Udder size			
Small	27	29	56 (93.33%)
Medium	3	01	4 (6.67%)
Udder shape			
Pendulous	1	0	1 (1.67%)
Pear	8	11	19 (31.67%)
Globular	21	19	40 (66.66%)
Teat size			
Small	27	25	52 (86.67%)
Medium	3	5	8 (13.33%)
Large	0	0	0
Teat shape			
Cylindrical	25	25	50 (83.33%)
Funnel	5	5	10 (16.67%)
Pear	0	0	0
Milk vein			
Prominent	0	0	0
Not prominent	30	30	15 (100%)

The hoof colour of Javari cattle found was black colour in most of the animals. Similar results were found in Assam cattle (Kayastha et al. 2011) and Kathani cattle (Kulkarni et al. 2013).

The tail switch colour of majority of the bulls was brown coloured followed by black. Whereas, in females, brown and black coloured tail switch was almost equally distributed and few animals had white coloured switch. But, the tail switch of the Krishna Valley cattle, Pulikulam cattle and Konkan cattle was mostly black in colour (Karthickeyan et al. 2006; Singh et al. 2012; Singh et al. 2019).

All the bulls in the study area had medium sized hump and dewlap whereas, majority of the cows had small sized hump and medium sized dewlap. It might be due to medium sized body of the animals and it can be classified as Mysore type cattle. In the present

study, it was observed that males had prominent humps compared to females. Similarly, majority of Konkan cows and bulls had small hump and followed by medium sized humps. In majority of bulls dewlap was large but, in case of cows, dewlap was mostly small sized (Singh et al. 2019). Similar results were found in Krishna Valley cattle (Karthickeyan et al. 2006), Pulikulam cattle (Singh et al. 2012) and Kathani cattle (Kulkarni et al. 2013).

Majority of the bulls had medium sized penis sheath and medium sized navel sheath whereas, all the cows had small sized navel flap. Similar findings were reported in Konkan cattle (Singh et al. 2019).

All the bulls studied had medium sized and straight horn but in case of females 81.66 % of the cows had medium sized horn. All animals had upward and outward orientation horns. But, 82.50 % of bulls and 75 % of cows had black coloured and 17.50 % of

bulls and 25 % of the cows had white coloured horn. All the animals had straight face and forehead with horizontal ears. But, in Krishna Valley cows Karthikeyan et al. (2006) found that the face of the breed is narrow and the forehead is wide and concave. The head of Krishna Valley is surmounted by short, slate coloured, curved horns usually emerging in an outward direction from the outer angles of the poll and slightly upwards and then inwards with a mild twist. Also, Singh et al. (2019) reported that among majority of the Konkan cattle the shape of horns was straight similar to Javari cattle of Yadgir and Raichur districts. Commonly, the horn orientation was found to be mostly outward, upward and backward. The horn colour was blackish, however creamy and mixed horn colour were also observed.

The horizontal orientation of ears indicates the alertness character of the animals. Most of the non-descript animals were low yielders and mainly used for draft purpose. These characters are of alert animals. Similarly, Joshi and Phillips (1953) recorded horizontal ears in Amritmahal cattle.

The body size of the cattle studied in all four blocks of Yadgir and Raichur districts of North Karnataka was small in comparison to other breeds in the vicinity viz. Khillar, Krishna Valley and

Deoni. The smaller body size of cattle breeds of tropical climatic conditions is beneficial for surviving in harsh environments (Sejian et al. 2018; Madhusoodan et al. 2019). The climatic conditions of the region are harsh and ambient temperature will be higher during summer which might be the reason for non-adaptability of cross bred cows in these districts, which indirectly influences the milk production of these districts. But, Javari cattle are **adopted** to these climatic conditions because of their physical and morphometric characteristics. A study showed that dwarf breeds of cattle use different heat tolerance mechanisms than standard cattle breeds, making them better adapted to hotter climates (Martin et al. 2018). Morphological characters of livestock are very important for the adaptation of animals during the stressful condition (Barendse, 2017) as they directly influence the heat exchange mechanisms such as cutaneous convection, radiation and evaporation between the animal and the surrounding environment (McManus et al. 2009). Morphological adaptation includes coat color, fur depth, hair type, and hair density, fat storage in hump or tail, skin color and body size (Khalifa, 2003).

The udder size of majority of the animals in the study area was small and majority of them had globular type of udder followed by pear shape and some of the animals had pendulous shaped

Table 3: Morphometric characteristics of different age groups of unidentified cattle of North Karnataka

Trait	Cow (N=60)	Bullock/Bulls (N=40)	1-3 Y Female (N=40)	1-3 Y Male (N=40)	6-12 m female (N=40)	6-12 m male (N=40)	Female calves (N=32)	Male calves (N=32)
Horn length	19.14±0.49	26.65±0.61	8.09±0.48	11.30±0.43	1.42±0.12	2.75±0.21	1.42±0.12	1.27±0.15
Horn Circumference	12.48±0.19	19.39±0.34	7.70±0.45	11.10±0.37	1.64±0.15	2.80±0.17	1.64±0.15	1.42±0.15
Face length	39.17±0.39	43.60±0.35	35.98±0.30	35.33±0.34	28.14±0.16	30.68±0.32	28.14±0.16	28.28±0.20
Face width	17.55±0.13	19.43±0.28	15.78±0.13	16.26±0.14	12.25±0.14	12.35±0.12	12.25±0.14	12.67±0.14
Ear length	18.34±0.24	20.20±0.19	17.15±0.20	16.86±0.16	14.47±0.27	15.86±0.17	14.47±0.27	14.66±0.25
Ear width	10.62±0.16	11.36±0.18	9.61±0.14	9.03±0.10	7.87±0.09	8.80±0.09	7.87±0.09	8.44±0.14
Chest girth	142.78±1.08	173.23±1.20	120.90±1.25	119.82±1.38	91.53±0.58	98.40±0.93	91.53±0.58	94.13±0.95
Paunch girth	152.99±1.72	180.43±1.26	129.31±1.20	124.20±1.47	94.96±0.75	101.40±1.07	94.96±0.75	96.06±1.07
Body Length	118.73±0.81	142.15±1.08	104.48±1.27	106.79±0.92	82.70±0.71	89.43±0.93	71.17±0.88	74.94±1.36
Body weight	244.12±4.81	419.45±7.98	152.05±5.24	156.68±4.81	87.69±2.24	90.15±1.95	72.06±1.09	75.81±2.06
Height at Withers	105.89±0.48	118.05±0.59	98.85±0.69	102.18±0.83	81.19±0.63	87.60±0.83	81.19±0.63	82.25±0.78
Height at rump	109.48±0.39	121.48±0.66	102.83±0.65	106.05±0.79	84.03±0.46	90.28±0.82	84.03±0.46	85.34±0.62
Top line	141.17±0.88	155.88±1.11	122.15±1.23	125.05±1.34	91.92±0.87	101.00±0.98	91.92±0.87	93.66±1.26
Distance bw Hip bones	30.38±0.31	34.12±0.53	24.84±0.36	24.38±0.39	18.20±0.31	18.92±0.25	18.20±0.31	17.71±0.34
Distance bw Pin bones	18.25±0.24	20.03±0.36	13.70±0.35	13.75±0.41	10.88±0.27	9.70±0.17	10.88±0.27	10.80±0.28
Tail length	94.38±0.94	107.40±1.24	80.52±0.95	83.20±1.16	57.31±0.55	63.97±1.10	57.31±0.55	56.22±1.05

udder as depicted in Table 2. Also, majority of the animals had small sized teats in Javari cattle of Yadgir and Raichur districts. The teat shape of many of the cows was cylindrical and some cows had funnel shaped teats. The smaller udder and teats might be due to their small body size and low milk yielding ability of the animals. But, in Krishnavally, the udder in the female is medium-sized with short teats and fore teats are longer than hind (Karthikeyan et al. 2006). The udder shape of tribal Kathani was observed as bowl followed by rounded, trough and pendulous in cows (Kulkarni et al. 2013). As the Javari cattle milk yield is lesser, the milk vein was not prominent in the cows studied.

Morphometric characteristics

The average biometric measurements of animals viz. horn length and horn circumference are presented in Table 3. The Deoni cattle had smaller horns compared to Javari cattle where, horns were medium, thick and emerge from the sides of the pole (Singh et al. 2002). Similarly, the Ponwar cattle were also having small to medium horns and curve inward with pointed tips (Gaur et al. 2015). Also, the horns of Pulikulam cattle of Tamil Nadu were generally wide-spread (in some cases tips were closer), long, thick at bottom and pointed at tips, orientation was outward, upward, backward and inward ending with pointed tips with varying sizes, smaller but thicker in bulls as compared to cows (Singh et al. 2012). But, in Khillar breed, it was observed that the average horn length in cows, bullocks and bulls were found to be longer. The curved horns were observed commonly in Khillar cattle (Adgale et al. 2017).

The results of face length, face width and ear length are depicted in Table 3. These results indicate that the Javari cattle had moderate/ medium sized face. Similarly, Face of Konkan cattle was moderate in length and width (Singh et al. 2019). But, the face length of Wannera, Shevera, Balankya strains of Deoni adult cows and bulls was found to be longer compared to the present findings (Kuralkar et al. 2015). The ears were short and horizontally placed in Javari cattle in all ages. Similar to our study, Singh et al. (2019) also recorded the similar average ear length of Konkan cattle in cows, bullocks, young males, young females and calves. Which indicates the proportionate confirmation of different bodily parts according to body size of the animals. Whereas, Singh et al. (2002) reported that the average ear length in Deoni cattle breed of 26.18 ± 0.52 cm. But, the average ear length observed in Krishna valley cattle breed was 15 ± 0.0 cm which is lesser than the reported results (Karthikeyan, 2006).

The results of average chest girth, paunch girth, body length, height at withers, height at rump and body weights of Javari cattle are presented in Table 3. It was found that the average chest girth Javari cattle is higher compared to of Konkan cattle (Singh et al. 2019). Chest girth reported in different breeds of cattle in India ranged from 121.60 ± 0.76 cm to 191.10 ± 0.41 cm (Pundir et al. 2007). The body lengths of Javari cattle are higher

compared to Malnad Gidda of Karnataka (Singh et al. 2008) and lesser than Konkan cattle (Singh et al. 2019). The height withers of Javari cattle is lesser compared to adult male and females of Deoni cattle (Singh et al. 2002). But higher than Malnad Gidda breed of Karnataka (Singh et al. 2008). Also, the Javari cattle are having higher height compared to Konkan cattle (Singh et al. 2019). The height at rump of Javari cattle is shorter as compared to other draught breeds of Karnataka. The height at rump was smaller than Khillar cattle (Jagdale, 2019). The body weight of Javari cattle was lesser compared to Khillar cattle (Jagdale, 2019).

The distance between hip bones and pin bones, and the length of tail with switch and without switch in Javari cattle is depicted in Table 3. The tail length of Javari cattle were higher compared to Konkan cattle (Singh et al. 2019).

Conclusions

The non-descript cattle in Yadgir and Raichur districts of North Karnataka have distinct set of morphological features, which are not found in other recognised cattle breeds in neighbouring regions. Animals adapted to harsh climatic conditions with low management inputs in terms of feeds, fodder, and health care. The cattle are mainly reared for draught, manure purpose and household milk consumption. The cattle have variation in coat colour (brown, black and white) and having white skin colour. The cattle have medium sized straight horns with outward orientation, black coloured hooves, horizontal ear orientation, poorly developed udder with small teats, low milk yielders. Further, it was observed during study that these cattle were reared under semi intensive system, sent for grazing in the day time without any supplemental feeding and kept under kutchra housing or in open space during night hours. Therefore, it was evident from the current experiment, that these cattle were having unique characteristic features and are different from the known breeds of Karnataka. Furthermore, their distinctive traits make them eligible for registration with the nodal agency as a new cattle breed.

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RESEARCH ARTICLE

Influence of calf-mother interaction on performance and behaviour of Murrah buffalo calves during heat stress

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Abstract: The primary objective of the research was to study how calf-mother contact and heat stress ameliorative measures would impact the behavior and growth of Murrah buffalo calves. The study was conducted from April to mid-September. A total of 21 calf-mother Murrah buffalo pairs were selected at the time of birth and categorized into three groups (n=7 pairs). In the first group (T0), the calves and mothers were separated at birth, but they were allowed limited interaction twice-daily for suckling during milking times. In the second group (T1), the calves had continuous unrestricted contact with their mothers and were free to suckle colostrum for up to 5 days. Afterward, the calf-mother pairs were housed in adjacent enclosures separated by a fenceline. The third group (T2) received the same provisions as the second group (T1), but in addition, the calf-mother pairs were provided with fans and foggers to alleviate heat stress. Statistical analysis involved comparing means using one-way ANOVA and univariate general linear models (GLM) both within and between the groups. Results showed that among the experimental calves, those in group T2 exhibited significantly higher ($p<0.05$) average daily gain (ADG) compared to those in groups T1 and T0 at weekly interval. Calves in group T2 spent significantly ($p<0.05$) more time resting, eating, and engaging in rumination compared to calves in groups T1 and T0. Both T2 and T1 calves demonstrated a significantly ($p<0.05$) lower frequency of abnormal behaviors than the T0 calves. The findings clearly demonstrates that provision of fenceline mother-calf contact along with fan-fogger system in the shed reduces the stress and improves the growth, behaviour and welfare of calves than restricted contact calves.

Keywords: Calf-mother contact, Murrah calves, fan and fogger, behaviour,

Introduction

The emotional connection between a mother and her young can be understood as a special, affectionate attachment that lasts a long time and survives temporary separations (Newberry and Swanson, 2008). This bond is marked by behaviors like grooming, providing nourishment, warmth, and protection, resting in close contact, and coordinating activities together. Staying close fulfills a social comforting role, offering a soothing effect to both mothers and their young. The calming and satisfying aspect of physical touch, sharing food, and grooming among bonded individuals is evident in the reduction of heart rate and the release of natural pain-relieving chemicals (Feh and de Mazieres, 1993). The process of bonding begins as progesterone levels decrease and estrogen levels rise, leading to an increase in oxytocin hormone. This bonding is fully established within five minutes after birth. Comparatively, when compared to complete separation, allowing contact between a mother and her offspring through a barrier reduces the behavioral reaction to weaning (Price et al. 2003). However, in organized and commercial dairy farming systems, this social bond is broken when calves are separated from their mothers shortly after birth. This results in lowered immunity, reduced productivity, and an increase in abnormal behaviors displayed by both mothers and their young (Kumar et al. 2017). Buffaloes possess a heightened sensitivity to heat stress due to their dark body color, fewer hairs, and a lower density of sweat glands. Their thicker skin reduces the potential for cutaneous evaporation, making them less capable of dissipating excess metabolic heat, rendering them susceptible to heat stress (Marai and Haebe, 2010). Significant environmental factors impacting buffaloes in India encompass air temperature, relative humidity (RH), solar radiation, and the temperature humidity index (THI) (Chaudhary et al. 2015). All these environmental elements contribute to heat stress in animals, when the combinations of environmental variables exceed the animal's thermoneutral zone (TNZ) (Buffington et al. 1981). Predictions suggest that global temperatures could rise by 1.8-4°C by the year 2100 (Field et al. 2015). The Intergovernmental Panel on Climate Change has also

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highlighted the heightened vulnerability of developing countries like India to extreme climatic events due to their reliance on climate-sensitive sectors like agriculture and its allied fields. Multiple studies indicate that abrupt temperature changes (increases in maximum temperatures) during summer are likely to exacerbate stress in buffaloes, detrimentally impacting their productive capabilities, resulting in substantial losses (Upadhyay et al. 2007).

Several techniques have been devised to mitigate heat stress in buffaloes, aiming to uphold their production performance. These methods primarily focus on enhancing the dissipation of heat and lowering skin temperature, ultimately ensuring the well-being of the buffaloes. Providing shade to the animals alone can decrease over 30% of the total emitted heat. Within sheltered environments, strategies such as utilizing air movement (fans), wetting the animals, facilitating air cooling through evaporation, and using shade to minimize direct solar radiation are employed to augment heat dissipation, proving effective in heat loss. Approaches such as employing ceiling fans, spray cooling, misters, foggers, and combinations thereof, such as sprinkler and fan cooling systems, are extensively adopted to alleviate heat stress in buffaloes (Yadav et al. 2016; Kumar et al. 2018). Wallowing, a natural behavior of buffaloes, also serves as a method to counteract heat stress. Among these methods, time-controlled evaporative cooling has demonstrated favorable outcomes in reducing the Temperature-Humidity Index (THI), thereby safeguarding the production and reproductive functions of buffaloes (Sinha et al. 2017; Ahmad et al. 2017).

Considering the effects of fenceline calf contact and heat stress ameliorative measures the present study was undertaken to investigate its effect on growth and behaviour of the calves. The aim of the study was to alleviate stress of buffalo calves that they bear during summer season which usually leads to loss of farmers.

Materials and Methods

Location of experiment and Climatic condition

The study was carried out at the Livestock Research Centre (LRC), ICAR-NDRI, Karnal. This center is positioned at coordinates 29° 42' 20" N Latitude and 76° 58' 52.5" E Longitude, at an elevation of 247 meters above sea level. During the summer, the highest recorded temperatures range from 42 to 46 °C, while in winter, they range from 2 to 5 °C, with a daily fluctuation of 16–22°C. The typical annual rainfall in this area amounts to approximately 650 mm.

Experimental animals

The research was undertaken during the summer months spanning from April to mid-September. For this study, a total of 21 pregnant Murrah buffaloes in advanced stages of pregnancy were chosen from the institute's buffalo herd. These buffaloes

were relocated to the maternity section 15 days before their expected calving date. Once calving was successful, the pairs of mothers and their calves were split into three groups, each having 7 pairs. The groups were divided according to the parity levels (3.43 ± 0.43). In the first group (T0), the buffalo mothers were allowed to nurse their calves with colostrum and then milked twice daily. After milking, they were separated from their calves and kept in a shelter without any additional measures to protect them from heat stress. In the second group (T1), the buffalo mothers were in direct contact for first five days and were allowed free choice colostrum suckling and afterwards were housed in proximity to their calves through a fence line. They were permitted to nurse their calves twice a day, following the morning and evening milking sessions, similar to the first group. These buffaloes were also housed in a shelter without specific measures to mitigate heat stress. In the third group (T2), all conditions were similar to T1 group but these buffaloes were provided with time controlled fogging and fanning system to protect them from the effects of heat stress.

Housing and feeding of experimental animals

Housing

About 15 days prior to the expected calving date, pregnant animals in advanced stages of pregnancy were moved to maternity pens specifically designed for calving. These pens consisted of both open and covered areas placed next to each other. Each pen had a total floor space of 12 m², with the floor being made of concrete. To prevent injuries, rubber mattresses were placed on the floor. After 5 days from the time of calving, the buffaloes were transferred to experimental shed. These sheds also had adjacent open and covered sections. In the experimental shelter used by the groups FCC and FCC-HSP, a barrier made of galvanized iron pipes (1 inch in diameter) was set up along the entire length of the shelter, including both the covered and open areas. This barrier was at a height of 5 feet from the ground and was equipped with wire mesh (1x1 centimeter) to allow for visual, auditory, olfactory, and limited tactile interaction. In the FCC-HSP group's shelter, additionally foggers and fans were installed in covered area. The foggers and fans were automated and operated on a time-based schedule. Cross foggers with four outlets were set at a 90° angle. Positioned at a height of 8 feet within the covered space, these foggers emitted fine droplets (85 Micron) @ 22 liters per hour. Two wall-mounted fans (36 inches in diameter) were placed on one side of the shelter, spaced 6 meters apart and blowing air at a rate of 10000-11000 cubic feet per minute (cfm) with a maximum throw distance of 9 meters. Cross foggers were dispersing fog in intervals of 45 seconds, followed by fanning in every 5 minutes from 11:00 AM and 4:00 PM.

Feeding

The calf's diet was adjusted in line with the ICAR-2013 standard guidelines for the nutrition of growing buffalo calves. Calves were offered buffalo milk twice daily amounting to 10% of their

body weight, until they reached 3 to 4 months of age. Starting from their second week of life, they were provided with chopped maize green fodder without any restrictions, along with clean water and a salt lick block. The calves were given calf starter from second week onwards @ of 1% of their body weight, and they had unrestricted access to chopped green fodders. The calf starter consisted of maize (33%), wheat bran (21%), gram (10%), groundnut cake (33%), mineral mixture (2%), and common salt (1%). This included a formulation comprising 21% digestible crude protein, energy content of 2632 kcal/g, and 70% of total digestible nutrients.

Recording of climatic variables and temperature humidity index (THI)

To analyze the Temperature Humidity Index (THI) of every shed, a Zeal (made in UK) dry-wet bulb thermometer was employed to measure the dry and wet bulb temperatures. This measurement was done daily between 2:30 PM and 3:00 PM throughout the experimental duration. The THI was computed using the NRC (1971) formula.

$$\text{THI} = 0.72 (\text{Tdb} + \text{Twb}) + 40.6$$

Where, Tdb = dry bulb temperature (°C)

Twb = wet bulb temperature (°C)

Statistical analysis

Comparisons of calf body weight, ADG, and various behaviors were conducted using IBM SPSS version 28.0.1.1 software, employing a one-way analysis of variance (ANOVA) and univariate general linear models (GLM). The model incorporated treatment and time as fixed factors, along with their interaction.

Differences were considered statistically significant when $p < .05$. Results are presented as LS means $\pm$ SE.

Results and Discussion

Environmental parameters

Data on fortnightly average maximum temperature is presented in the table 1. The heat stress ameliorative measuring provided by time controlled fogging coupled with fanning were able to reduce the maximum temperature inside the shed housing T2 buffaloes to the extent of 3.51 °C (the reduction in overall mean between T0 and T2 sheds). Data on fortnightly average afternoon dry bulb (Db) and wet bulb (Wb) temperature are presented in the table 2. Fortnight average THI data is presented in the table 3. The overall difference in THI between the T1 and T2 shed was 3.34. The environmental variables like maximum temperature and THI are the best indicators of environmental heat stress on animals (Armstrong 1994).

Body weight and average daily gain (ADG) of calves

The average body weight of calves at birth among the three treatment groups had no significant differences (Table 4). The significant ($p < 0.05$) difference in calves' body weight was seen from 3rd week onwards and continued till the 12th week of study. At third week the body weight of T2 calves was higher ($p < 0.05$) than T0 calves. At the end of 12 weeks of age the average body weight was significantly ($p < 0.05$) different among the three treatment groups, with T2 calves having higher average body weight followed by T1 and T0 calves. The data on average ADG recorded at weekly intervals in calves is presented in the table 5. The ADG was higher ($p < 0.05$) in T2 and T1 group as compared to T0 group right from first week onwards till fourth week. After fourth week no significant difference was seen in ADG between the groups till the tenth week. The significant ($p < 0.05$) differences in ADG among the three groups of calves were again seen in 11th

Table 1: Fortnightly mean maximum and minimum temperature (°C) inside the sheds

Fortnight	Maximum temperature (°C)		
	T0 shed	T1 shed	T2 shed
1	36.14 ^a ±0.63	36.15 ^a ±0.61	32.83 ^b ±0.46
2	36.24 ^a ±1.08	36.26 ^a ±1.95	33.04 ^b ±0.93
3	36.60 ^a ±0.70	36.67 ^a ±0.75	32.1 ^b ±0.72
4	35.69 ^a ±0.99	35.62 ^a ±0.91	31.76 ^b ±0.81
5	36.47 ^a ±0.82	36.37 ^a ±0.80	32.37 ^b ±0.73
6	36.40 ^a ±0.64	36.48 ^a ±0.60	32.1 ^b ±0.64
7	36.57 ^a ±1.06	36.55 ^a ±1.06	32.07 ^b ±1.06
8	31.92 ^a ±0.71	31.90 ^a ±0.72	28.17 ^b ±0.56
9	33.17 ^a ±0.37	33.13 ^a ±0.31	30.37 ^b ±0.37
10	32.88 ^a ±0.66	32.87 ^a ±0.60	30.08 ^b ±0.69
11	30.81 ^a ±0.49	30.82 ^a ±0.48	29.31 ^b ±0.42
Overall mean	34.81 ^a ±0.26	34.78 ^a ±0.23	34.81 ^a ±0.26

Data are presented as LS means $\pm$ SE. a, b indicate differences between the mean values of different groups. Differences at all points for each parameter were considered at $P < 0.05$.

and 12th week of study. The overall average ADG was significantly higher ($p < 0.05$) in T2 (0.560 ± 0.02 kg) group as compared to T1 (0.506 ± 0.02 kg) and T0 (0.473 ± 0.01 kg) groups.

The overall higher average body weight of calves in T1 and T2 group compared to T0 group might be due to direct contact with mother during first five days after birth and free choice colostrum sucking and thereafter fenceline contact with mothers after 5 days which might have helped them achieve greater body weight due to lesser stress. Similar findings were reported by Hassan et al. (2019) and Chaudhary et al. (2022). While the higher body weight achieved in T2 group compared to T1 group might be due to cumulative effect of fenceline calf contact and heat stress

amelioration provided the shed of T2 group. The initial higher ADG during first 4 weeks after birth may be due to consequence of free choice colostrum suckling during first five days after birth in T1 and T2 group of calves. The superiority of the fenceline group calves on ADG in the current study may be attributable to free choice colostrum suckling during the first five days after birth along with a lower level of stress caused by the social stimulation offered by mother contact to the calves through the provision of fenceline contact. Our findings are consistent with those of Hassan et al. (2019), Chaudhary et al. (2022) for buffalo calves and Price et al. (2003) and Kisac et al. (2011) for cattle calves, who found that fenceline mother-reared calves saw greater

Table 2: Fortnightly mean afternoon dry bulb (Db) and wet bulb (Wb) temperature ($^{\circ}\text{C}$) inside experimental sheds

Fortnight	Db temperature ($^{\circ}\text{C}$)			Wb temperature ($^{\circ}\text{C}$)		
	T0 shed	T1 shed	T2 shed	T0 shed	T1 shed	T2 shed
1	35.71 ^b ±0.61	35.51 ^b ±0.6	32.91 ^a ±0.61	20.92 ^b ±0.47	20.72 ^b ±0.54	18.12 ^a ±0.47
2	35.12 ^b ±1.15	34.92 ^b ±1.17	32.32 ^a ±1.15	21.36 ^b ±0.43	21.16 ^b ±0.50	18.56 ^a ±0.43
3	34.53 ^b ±1.14	34.73 ^b ±1.16	30.13 ^a ±1.14	24.13 ^b ±0.36	23.93 ^b ±0.43	20.93 ^a ±0.36
4	34.85 ^b ±1.12	35.05 ^b ±1.14	30.45 ^a ±1.12	25.06 ^b ±0.41	24.86 ^b ±0.48	21.86 ^a ±0.41
5	35.67 ^b ±0.85	35.47 ^b ±0.87	31.87 ^a ±0.85	26.47 ^b ±0.35	26.67 ^b ±0.42	23.27 ^a ±0.35
6	34.43 ^b ±1.06	34.23 ^b ±1.08	31.53 ^a ±1.06	26.24 ^b ±0.22	26.44 ^b ±0.29	24.94 ^a ±0.22
7	34.47 ^b ±1.36	34.27 ^b ±1.38	32.17 ^a ±1.36	27.60 ^b ±0.44	27.20 ^b ±0.51	26.10 ^a ±0.44
8	30.53 ^b ±0.66	30.33 ^b ±0.68	28.43 ^a ±0.66	28.21 ^b ±0.29	27.81 ^b ±0.36	26.91 ^a ±0.29
9	31.70 ^b ±0.49	31.90 ^b ±0.51	30.20 ^a ±0.49	28.17 ^b ±0.24	28.37 ^b ±0.31	27.77 ^a ±0.24
10	31.73 ^b ±0.71	31.93 ^b ±0.73	29.83 ^a ±0.71	28.33 ^b ±0.25	28.53 ^b ±0.32	27.23 ^a ±0.25
11	30.20 ^b ±0.50	30.00 ^b ±0.52	27.80 ^a ±0.5	27.69 ^b ±0.31	27.49 ^b ±0.38	25.29 ^a ±0.31
Overall mean	33.52 ^b ±0.28	33.46 ^b ±0.30	30.67 ^a ±0.30	25.86 ^b ±0.20	25.77 ^b ±0.27	23.75 ^a ±0.27

Data are presented as LS means $\pm$ SE. a, b indicate differences between the mean values of different groups.

Differences at all points for each parameter were considered at $P < 0.05$.

*Db = Dry Bulb ; Wb = Wet bulb

Table 3: Fortnightly mean THI inside different experimental sheds

Fortnight	THI (Afternoon)			Difference between THI in T1 and T2 sheds
	T0 shed	T1 shed	T2 shed	
1	81.37 ^{bWXY} ±0.63	81.15 ^{bWXY} ±0.68	77.27 ^{aYZ} ±0.63	-3.88
2	81.27 ^{bWX} ±1.10	81.05 ^{bWXY} ±1.15	77.16 ^{aYZ} ±1.10	-3.89
3	82.83 ^{bXYZ} ±1.04	82.90 ^{bYZ} ±1.09	78.30 ^{aZ} ±1.04	-4.60
4	83.74 ^{bXYZ} ±1.00	83.81 ^{bYZ} ±1.05	79.20 ^{aZ} ±0.99	-4.61
5	85.34 ^{bZ} ±0.77	85.34 ^{bZ} ±0.82	80.37 ^{aZ} ±0.77	-4.97
6	84.28 ^{bXYZ} ±0.90	84.28 ^{bYZ} ±0.95	81.26 ^{aZ} ±0.9	-3.02
7	85.29 ^{bYZ} ±1.26	84.93 ^{bYZ} ±1.31	82.55 ^{aZ} ±1.26	-2.38
8	82.89 ^{bXYZ} ±0.65	82.53 ^{bYZ} ±0.70	80.44 ^{aZ} ±0.65	-2.09
9	83.71 ^{bXYZ} ±0.47	84.00 ^{bXYZ} ±0.52	82.34 ^{aZ} ±0.47	-1.66
10	83.84 ^{bXYZ} ±0.68	84.13 ^{bYZ} ±0.73	81.68 ^{aZ} ±0.68	-2.45
11	82.29 ^{bWXYZ} ±0.52	82.07 ^{bXYZ} ±0.57	78.83 ^{aZ} ±0.52	-3.24
Overall mean	83.35 ^b ±0.26	83.29 ^b ±0.31	79.95 ^a ±0.29	-3.34

Data are presented as LS means $\pm$ SE. a, b indicate differences between the mean values of different groups.

Where, W, X, Y, Z indicate differences in the same group at different time intervals. Differences at all points for each parameter were considered at $P < 0.05$.

*THI= Temperature humidity index

average daily gains than the calves that had restricted contact with their mothers. While the higher ADG in T2 group confirms that summer protective measures help in better growth of the calves when deployed in conjunction with fenceline contact. Similar results of higher ADG under summer ameliorative measures were reported by Kamal et al. (2014) in cattle and Barman et al. (2017) in buffalo calves.

Resting behaviour of calves

The mean time spent resting was significantly ($p < 0.05$) different among the three groups, with T2 having longest resting time followed by T1 and T0 calves throughout the experimental period (Table 6). The mean time spent on resting showed declining trend from first of age till 12th week of age in all the three groups of

calves. The fenceline contact calves (T1 and T2) rested for a longer period of time than calves that had just restricted contact. These findings are consistent with those of Price et al. (2003) and Haley et al. (2006), who found that when mother and calf were in close proximity they rested more. The findings of Jensen (2004), Calvo-Lorenzo et al. (2016) and Chaudhary et al. (2022), who reported that calves spend 16–19 hours per day lying down between the ages of 21 and 70 days, also support for the findings of the current study. Higher resting time in T2 group compared to T1 and T0 group may be due to cumulative effect of fenceline mother contact and heat stress ameliorative measures provided in T2 shed. Higher resting time reported in T2 group calves in our study is in agreement with the finding of Chikkagoudara et al. (2022).

Table 4: Body weight (kg) of different treatment groups of calves recorded at weekly intervals

Week after birth	Body weight (kg)		
	T0	T1	T2
At birth	29.00±0.93	28.90±0.77	28.44±0.94
1	32.75±0.72	34.33±0.68	34.01±1.05
2	36.63±0.57	39.45±0.98	39.89±1.38
3	39.93 ^a ±0.83	44.53 ^{ab} ±1.28	45.14 ^b ±1.78
4	43.47 ^a ±0.85	48.89 ^{ab} ±1.41	50.00 ^b ±2.22
5	47.13 ^a ±0.78	52.33 ^b ±1.30	52.97 ^b ±2.02
6	50.18 ^a ±0.77	54.84 ^b ±1.13	55.54 ^b ±1.88
7	53.40 ^a ±0.87	57.30 ^b ±0.96	58.74 ^b ±1.56
8	56.15 ^a ±0.80	59.64 ^b ±0.98	61.76 ^b ±1.32
9	59.52 ^a ±0.81	62.06 ^{ab} ±0.90	64.91 ^b ±1.19
10	62.35 ^a ±0.82	64.89 ^a ±0.81	68.07 ^b ±1.11
11	65.75 ^a ±0.78	68.24 ^a ±0.80	71.64 ^b ±1.03
12	68.73 ^a ±0.72	71.43 ^b ±0.72	75.46 ^c ±0.89

Data are presented as LS means ± SE. a, b and c indicates differences between the mean values of different groups. Differences at all points for each parameter were considered at $P < 0.05$.

Table 5: Average daily gain (kg/d) of different groups of calves recorded at weekly intervals

Week after birth	Average daily gain (kg/day)		
	T0	T1	T2
1	0.536 ^a ±0.06	0.776 ^b ±0.03	0.796 ^b ±0.05
2	0.555 ^a ±0.05	0.732 ^{ab} ±0.06	0.839 ^b ±0.06
3	0.471 ^a ±0.07	0.726 ^b ±0.05	0.751 ^b ±0.06
4	0.505 ^a ±0.03	0.622 ^b ±0.04	0.694 ^b ±0.07
5	0.524±0.03	0.492±0.04	0.424±0.04
6	0.436±0.03	0.359±0.05	0.367±0.04
7	0.460±0.02	0.351±0.04	0.457±0.06
8	0.393±0.03	0.335±0.04	0.431±0.05
9	0.481±0.05	0.345±0.04	0.451±0.05
10	0.405±0.03	0.404±0.03	0.451±0.03
11	0.486 ^a ±0.02	0.480 ^b ±0.04	0.510 ^b ±0.03
12	0.426 ^a ±0.02	0.455 ^a ±0.02	0.545 ^b ±0.03
Overall	0.473 ^a ±0.01	0.506 ^a ±0.02	0.560 ^b ±0.02

Data are presented as LS means ± SE. a, b indicate differences between the mean values of different groups. Differences at all points for each parameter were considered at $P < 0.05$.

Standing behaviour of calves

The data on average daily time spent on standing by the calves is presented in the table 7. The time spent on standing by calves on day 6, 10, 15, 75 and 120 was significantly ($p < 0.05$) lowest in T2 followed by T1 and T0 group calves. While on rest of the recorded days, the time spent on standing was significantly ($p < 0.05$) shorter in T2 and T1 calves than T0 calves. The overall mean time spent on standing among the three groups of calves was significantly ($p < 0.05$) lower in T2 group of calves followed by T1 and T0 group of calves. The mean time spent on standing showed rising trend from first week of age till 12th week of age in all the three groups of calves.

The lower standing time in T2 and T1 group might be due to their mothers' continued olfactory, auditory, and visual contact with the calves. This suggested that fenceline interaction helped the calves cope with their new environment and minimize the separation anxiety. Fenceline housed calves (T1 and T2) spent less time standing than calves that were allowed restricted contact (T0). Our findings are consistent with the findings of Loberg et al. (2008), Enriquez et al. (2010) and Chaudhary et al. (2022). The lowest standing time recorded in T2 group calves then T1 calves might be due to synergistic effect of fenceline mother contact and heat stress amelioration provide in the shed. These findings are in agreement with Chikkagoudara et al. (2022).

Table 6: Average time spent on resting (min/d) by the calves during summer season

Day after birth	Resting time (min/day)		
	T0	T1	T2
6	1066.20 ^a ±4.20	1190.20 ^b ±6.51	1244.70 ^c ±3.89
10	1054.40 ^a ±4.02	1159.24 ^b ±5.21	1220.14 ^c ±5.26
15	1031.42 ^a ±4.11	1101.45 ^b ±3.52	1161.00 ^c ±5.82
30	993.39 ^a ±5.94	1057.13 ^b ±2.14	1095.87 ^c ±4.07
45	984.61 ^a ±7.38	1020.07 ^b ±4.63	973.69 ^c ±5.98
60	938.50 ^a ±7.48	971.52 ^b ±3.26	958.31 ^c ±5.56
75	903.85 ^a ±4.72	929.75 ^b ±5.21	961.69 ^c ±4.36
90	849.93 ^a ±4.62	932.21 ^b ±6.88	939.59 ^c ±4.99
120	831.74 ^a ±4.60	876.25 ^b ±5.11	899.66 ^c ±3.61
Overall	961.56 ^a ±11.26 (16.30 hrs)	1026.42 ^b ±14.18 (17.11 hrs)	1050.52 ^c ±17.08 (17.51 hrs)

Data are presented as LS means ± SEM. a, b, c indicate differences between the mean values of different groups. Differences at all points for each parameter were considered at $p < 0.05$.

Table 7: Average time spent on standing (min/d) by the calves

Day after birth	Standing time (min/day)		
	T0	T1	T2
6	373.80 ^c ±4.20	249.81 ^b ±6.51	195.30 ^a ±3.89
10	385.60 ^c ±4.02	280.76 ^b ±5.21	219.86 ^a ±5.26
15	408.58 ^c ±4.11	338.55 ^b ±3.52	279.00 ^a ±5.82
30	446.61 ^c ±5.94	382.87 ^b ±2.14	344.13 ^a ±4.07
45	455.39 ^b ±7.38	419.93 ^a ±4.63	466.32 ^a ±5.98
60	501.50 ^b ±7.48	468.48 ^a ±3.26	481.69 ^a ±5.56
75	536.15 ^c ±4.72	510.25 ^b ±5.21	478.31 ^a ±4.36
90	590.07 ^b ±4.62	507.79 ^a ±6.88	500.41 ^a ±4.99
120	608.26 ^c ±4.60	563.76 ^b ±5.11	540.34 ^a ±3.61
Overall	478.44 ^c ±11.26 (7.97 hrs)	413.58 ^b ±14.18 (6.90 hrs)	389.48 ^a ±17.08 (6.50 hrs)

Data are presented as LS means ± SEM. a, b, c indicate differences between the mean values of different groups. Differences at all points for each parameter were considered at $P < 0.05$.

Eating behaviour of calves

The average daily time spent eating by the calves is presented in the table 8. The significant ($p < 0.05$) difference in eating time among the three groups of calves were seen on day 30 and 120, with T2 calves having highest eating time followed by T1 and T0 group of calves. On the remaining recorded days T2 and T1 groups of calves had significantly ($p < 0.05$) higher eating time than T0 group of calves. When compared to the T0 calves, the

T1 and T2 calves began nibbling the meal significantly ($p < 0.05$) sooner. The mean time spent on eating showed an increasing trend from first week of age till 12th week of age in all the three groups of calves.

It was seen that fenceline calves (T1 and T2) spent more time eating than restricted contact calves (T0). This might be due to the free choice suckling in T2 and T1 group calves during the first five days after birth. It has already been reported that



Fig 1: Cross-sucking behaviour in T0 group of calves

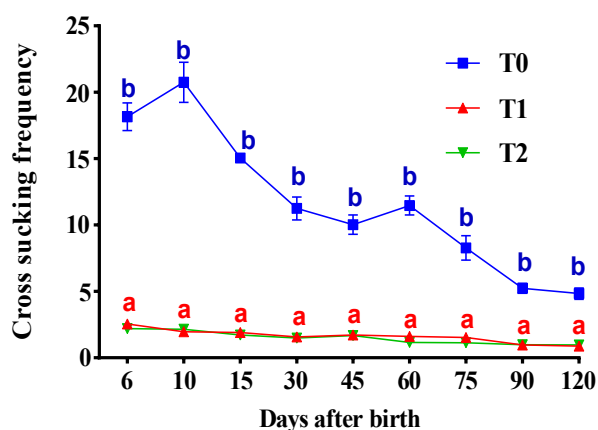


Fig 2: Mean frequency of cross-sucking by the calves

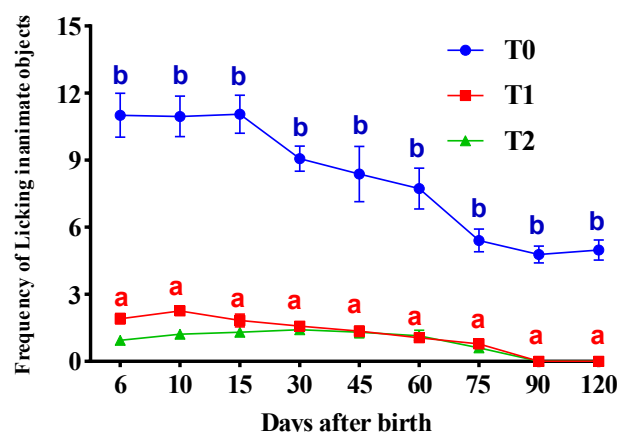


Fig 3: Mean frequency of licking inanimate objects by the calves

Table 8: Average time spent on eating (min/day) by the calves during summer season

Day after birth	Eating time (min/day)		
	T0	T1	T2
30	1.10 ^a ±0.16	14.42 ^b ±1.01	19.46 ^c ±1.06
45	15.85 ^a ±0.93	48.18 ^b ±0.86	50.01 ^b ±1.47
60	28.65 ^a ±1.01	68.80 ^b ±1.28	75.37 ^b ±3.51
75	70.99 ^a ±2.18	108.92 ^b ±4.30	113.80 ^b ±2.57
90	122.62 ^a ±2.94	188.68 ^b ±2.22	188.49 ^b ±3.12
120	196.96 ^a ±3.02	216.09 ^b ±3.11	231.48 ^c ±3.17

Data are presented as LS means $\pm$ SEM. a, b, c indicate differences between the mean values of different groups. Differences at all points for each parameter were considered at $P < 0.05$.

consuming more colostrum has significant impacts on GIT development and alters GI hormones and digestive enzymes (Parashar, 2021). Additionally, this might be due to transition from free-choice colostrum suckling to limited suckling and larger body weight, which led to earlier solid feed nibbling and longer feeding times in fenceline group calves (T1 and T2). These results are in accordance with those reported by Price et al. (2003), Haley (2006), Loberg et al. (2008) and Parashar (2021) who found that calves that had fenceline contact with their mothers ate for longer periods of time than calves those did not. Due to restricted suckling and restricted contact with mothers soon after birth might have lead to reduced eating time in restricted contact calves (T0). The highest eating time in T2 group might be due to the both free choice suckling followed by fenceline calf contact and the heat stress ameliorative measures provided in the T2 shed. The findings of higher eating time due heat stress amelioration are in agreement with that of Chikkagoudara et al. (2022).

Rumination behaviour of calves

The time spent by calves ruminating is presented in the table 9. The significant ($p<0.05$) difference in rumination time among the three groups of calves were seen on day 30 and 120, with T2 group calves having highest rumination time followed by T1 and T0 group of calves. On the remaining recorded days T2 and T1 groups of calves had significantly ($p<0.05$) higher rumination time than the T0 group of calves. The mean time spent on

rumination showed an increasing trend from recorded day till 12th week of age in all the three groups of calves.

In the current study, newborn calves did not engage in rumination until 15 to 20 days after birth. Higher feed intake and feeding duration may have contributed longer rumination times in fenceline calves (T1 and T2) from day 30 to 120 in our study. Similar findings were reported by Enriquez et al. (2010) and Chaudhary et al. (2022), who noted that fenceline-housed calves spent more time ruminating than the calves that were separated. Highest rumination time was seen in T2 in which heat stress amelioration was provided. The findings are in lines with those of Chikkagoudara et al. (2022) who reported similar findings; higher ruminating time in heat stress ameliorated group calves.

Abnormal behaviour

Cross sucking frequency in calves

The overall average and average frequency on all recorded days of cross sucking was significantly ($p<0.05$) higher in T0 group of calves than the T1 and T2 groups of calves (fig 2). More instances of cross sucking were observed in T0 group (Fig 1). There was declining trend in cross sucking frequency with the age in all the three groups (fig. 1). The frequency of overall mean $\pm$ SE of cross sucking were 11.67 ± 0.75 , 1.63 ± 0.09 and 1.49 ± 0.08 in T0, T1 and T2 group of calves respectively.

Fig 4: Calves licking inanimate objects



Table 9: Average time spent on rumination (min/day) by the calves during summer season

Day after birth	Rumination time (min/day)		
	T0	T1	T2
30	2.41 ^a $\pm$ 0.23	20.43 ^b $\pm$ 1.17	25.83 ^c $\pm$ 1.53
45	25.79 ^a $\pm$ 1.17	54.42 ^b $\pm$ 1.81	59.42 ^b $\pm$ 2.25
60	33.54 ^a $\pm$ 2.17	79.43 ^b $\pm$ 1.11	89.55 ^c $\pm$ 1.54
75	81.83 ^a $\pm$ 2.03	145.17 ^b $\pm$ 2.20	150.40 ^b $\pm$ 1.65
90	167.22 ^a $\pm$ 2.27	227.84 ^b $\pm$ 2.91	232.25 ^b $\pm$ 2.04
120	214.57 ^a $\pm$ 3.28	258.14 ^b $\pm$ 1.11	268.38 ^b $\pm$ 5.99

Data are presented as LS means $\pm$ SEM. a, b, c indicate differences between the mean values of different groups. Differences at all points for each parameter were considered at $P < 0.05$.

The calves in T2 and T1 group exhibited negligible cross-sucking behaviour, which may be due to the fact that the calves in T2 and T1 group had complete mother contact for first five days followed by fenceline contact after five day. The calves in these groups (T1 and T2) were having more visual, tactile and auditory contact with their mothers. This may have provided an opportunity to calves to concentrate more of its attention upon its mother and dietary habits, which may have resulted in negligible oral abnormal behaviour. The lower levels of behavioral discomfort shown in these calves may be another reason why there is less cross-sucking in T2 and T1 group of calves. Our findings are in agreement with the findings of Stookey et al. (1997), Price et al. (2003), Johnsen et al. (2015), Pérez-Torres et al. (2016), Chaudhary et al. (2022) and Ingle (2022) who used fenceline weaning in calves which supports our findings of fenceline contact between mother and young causes less behavioral distress than the sudden separation of calves from their mothers after birth.

Although restricted contact calves were given access to natural suckling and mother touch, even then they showed higher cross-sucking behaviour throughout the study, which might have been caused by their mothers' limited contact periods. The restricted contact calves (T0) in our study had shorter periods of contact, which increased their desire for natural suckling and increased the amount of non-nutritive suckling. The findings of the current study are in consistent with Enriquez et al. (2010) who reported that calves responded more behaviorally to temporary separation and came to the conclusion that these limited contacts cause significantly more psychological stress than the contact between mother and calves along the fence.

Frequency of licking inanimate objects in calves

The data on the average frequency of licking inanimate objects is presented in the fig 3. The overall average and average frequency on all recorded days of licking inanimate objects was significantly ($P < 0.05$) higher in T0 group of compared to T1 and T2 groups of calves. There was declining trend in frequency licking inanimate objects with the age in all the three groups (Fig. 3). The frequency of overall mean $\pm$ SE of licking inanimate objects were 8.15 ± 0.42 , 1.20 ± 0.12 and 0.88 ± 0.09 in T0, T1 and T2 group of calves respectively.

Direct mother contact and free will colostrum suckling during early life followed by fene-line contact in T1 and T2 group may have reduced the frequency of licking inanimate objects. The maternal contact and free choice colostrum suckling allows calves spent the majority of their time sleeping and resting because their nutritional social needs had been met. Dam's presence might have calmed the calves, resulting in less licking of the items in the calf pen. Similar finding was reported by Krohn et al. (1999) and Veissier et al. (2013), who found that calves who stay with their mother during the first four days of life exhibit less non-nutritive oral behaviour following weaning. Our findings are also

consistent with those of Haley et al. (2006) and Jung & Lidfors (2001), who reported that extending the length of milk consumption has been indicated to minimize non-nutritive licking and sucking in mother-fed calves. When newborn calves suckle the udder, it was observed that young one's to focus their actions more on milk or solid food consumption or on the mother. Natural suckling and fenceline interaction with the calves appear to benefit the calves by reducing non-nutritive oral behaviour. Similar findings of lower frequency of licking inanimate objects by the calves in fenceline mother contact have been reported by Choudhary et al. (2022) in buffalo calves and Ingle (2022) in Sahiwal calves.

Conclusion

Behaviour plays a critical role in evaluating the animal's comfort level, which in turn affects its production. The extended mother-calf contact and reduced stress levels associated with fenceline contact played a crucial role in promoting better growth and reducing abnormal behaviors. Notably, the lowest occurrence of cross-sucking and licking inanimate objects in fenceline groups indicates that these calves experienced less behavioral distress and displayed more natural behaviors. The findings of this research underscore the importance of early and continuous mother-calf contact, as well as the positive effects of heat stress amelioration, in promoting the growth, comfort, and behavioral well-being of calves. These insights can inform management practices aimed at optimizing calf rearing conditions for improved animal welfare and productivity.

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RESEARCH ARTICLE

Effect of non-genetic factors on production performance of Mehsana buffalo at organized farm

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Abstract: The present investigation included the data of lactation records of 301 Mehsana buffaloes, spread over 30 years (1991-2020), collected from the records maintained at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, District Banaskantha, Gujarat; to know the effects of non-genetic factors on the production performance of Mehsana buffaloes. The data about different performance traits was collected from the records of the farm and analysed to know the effects of non-genetic factors like- period of calving, season of calving, parity and age at first calving group on the performance traits. The overall LSM of 305-Days Milk Yield (DMY), Total Milk Yield (TMY) and Lactation length (LL) was observed 1714.75 ± 27.30 L (N=1171), 1765.55 ± 28.61 L (N=1186) and 273.28 ± 3.66 days (N=1164), respectively. The effects of period of calving ($P \leq 0.05$), season of calving ($P \leq 0.01$) and parity ($P \leq 0.01$) had significant effects on 305-DMY, but TMY was affected by period of calving, season of calving and AFC group ($P \leq 0.01$). Similarly, LL was significantly ($P \leq 0.05$) influenced by the period of calving and the AFC group, but the season of calving and parity had a highly significant ($P \leq 0.01$) effect on LL of Mehsana buffaloes. Therefore, based on these observations it was concluded that significant effect of non-genetic factors plays an important role in improving the production performance of the Mehsana buffalo herd, therefore emphasis must be given on nutrition, management and healthcare practices to improve the performance of the indigenous breed on the farm.

Keywords: Production performance, Non-genetic factors, Total milk yield, AFC Group

Introduction

India is a leading milk-producing country across the world. Gujarat possesses a rich biodiversity of buffalo population, and inhabits four well-established buffalo breeds viz., Mehsana, Surti, Jaffarabadi and Banni. As per 19th Livestock Census, the total number of buffalo in the country is 108.7 million. The buffalo constitute around 21.23% of the total livestock population of India (Annual Report, 2016-2017). Mehsana is one of the best dairy breed of buffalo and is considered to be regular breeder. Although the breed has contributed significantly in the milk production and had greater role in 'Operation flood' programme initiated to augment milk production in India, but the genetic potential of this breed has not been utilised to the fullest. Looking towards the contribution of buffalo, which has occupied an important place in the agricultural economy of India, because of their adaptability to harsh climatic conditions, tolerance to tropical diseases and have the ability to convert the poor quality roughages into the milk, meat and draught power, where important buffalo breeds originated from Gujarat and its total population is 10.5 million while total Mehsana buffalo population is 2.67 million. Among these breeds, Mehsana is well known for its characteristics like: higher milk production and "persistent milking and regular breeding".

It is important to remember that income from dairy enterprises largely depends upon the efficiency of production performance of the dairy herd. At the same time, it is highly desirable to record the major economic attributes such as lactation yield, lactation length, etc of the animals kept for milk production. non-genetic factors. Therefore, precise and accurate knowledge of different economic parameters is important to plan appropriate selection, breeding, feeding and marketing strategies for the improvement of the herd. Looking above facts, improvement in the production characteristics of indigenous breeds has become essential for keeping dairy enterprises economically viable, while improvement in the performance of indigenous breeds can be achieved by implementing appropriate management and breeding strategies. The non-genetic factors have a great role in determining the

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production traits. The season or period of calving determines what animal will get to feed when the animal will be lactating like availability of fodder, temperature, humidity etc. The present investigation has been done to study the effect of non-genetic factors on the production performance of Mehsana buffalo.

Material and Methods

The relevant data on the present investigation was collected from the history cum pedigree sheets maintained at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat during the period from 1991-2020. Geographically, Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar is located in North Gujarat. The climate of the livestock research station is semi-arid. The animals of all age groups are reared under similar climatic conditions. Management practices followed on the farm were uniform for the herd. All animals were housed under a loose housing system with adequate sheds for shelter against sun, rain and extreme winter. All animals at the farm were stall-fed with dry roughages, green fodder and concentrate in proper proportion. The animals having lactation length of less than 100 days, incomplete lactation due to sale or death during lactation, abortion and stillbirth etc. were considered as abnormal lactation and not included in the study. The data pertaining to Mehsana buffaloes was maintained over a period of 30 years from 1991-2020. The data was grouped into 6 periods with a duration of 5 years viz. P1: 1991-1995, P2: 1996-2000, P3: 2001-2005, P4: 2006-2010, P5: 2011-2015 and P6: 2016-2020. Each year was delineated into 3 seasons each with a duration of 4 months viz. S1: Nov-Feb (winter), S2: Mar-Jun (summer) and S3: July-Oct (Rainy). The records on parity was collected from history sheets of individual animals having 1st to 5th parities. The age at first calving group were classified based on age at first calving of Mehsana buffaloes as A-1: <1151 days, A-2: 1151-1541 days and A-3: >1541 days. The traits included in the study were 305-Days Milk Yield, Total Milk Yield and Lactation Length. The period of calving, season of calving, parity and age at first calving group were considered as fixed effects for all production traits. The least squares analysis of variance for unequal sub-class numbers (Harvey, 1990) considering six periods, three seasons, five parities and three age at first calving groups was used to analyze the data on various production and reproduction traits using the following statistical model. The least squares of variance analysis was done using LSML software package

$$Y_{ijklm} = \mu + A_i + B_j + C_k + D_l + e_{ijklm}$$

Where,

Y_{ijklm} = mth record of buffalo calved in ith period, jth season, kth parity and lth age at first calving group

μ = Population mean

A_i = Fixed effect of ith period of calving where i = 1, 2, 3, 4, 5 and 6

B_j = Fixed effect of jth season of calving where j = 1, 2 and 3

C_k = Fixed effect of kth parity where k = 1, 2, 3, 4 and 5 & above

D_l = Fixed effect of lth age at first calving group where l = 1, 2 and 3

e_{ijklm} = Random error assumed to be normally and independently distributed with zero mean and constant variance (NID, 0, σ^2).

The difference of means between any two subclasses of period, season, parity and age at first calving group was tested for significance using Duncan's Multiple Range Test (DMRT) as modified by Kramer (1957).

Results and Discussion

The present investigation was conducted at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, District-Banaskantha, Gujarat to evaluate the production performance of Mehsana buffaloes maintained from 1991-2020. The climatic conditions of the region are semi-arid, where summer is dry and hot and the temperature goes up to 40°C, while winter is not stressful as the average temperature remains from 10°C to 30°C with relative humidity 69% and 48% in morning and afternoon, respectively. The data about 305 DMY, TMV and LL was evaluated from the records maintained at the farm and analyzed to know the effects of non-genetic factors like period, season, parity and AFC group studied and results are depicted in Table: 1, 2, 3 and 4.

305-Days Milk Yield (305-DMY)

The LSM for 305 DMY of Mehsana buffaloes having 1171 lactation records was calculated as 1714.75±27.30 lit (Table 1). The values for 305-DMY were in close agreement with Gupta et al. (2012) as 1760.69 ± 42.25 kg and Thiruvankadan et al. (2014) as 1804.9 ± 14.7 kg in Murrah buffaloes. The present values are contrasted with the findings of Bharat et al. (2004) for Mehsana buffaloes and Rathod et al. (2018) in Surti buffalo. However, comparatively higher estimates of 305 DMY than the present findings were reported by Pawar et al. (2012), Jamuna et al. (2015) and Jakhar et al. (2016). Lactation milk yield of dairy animal's upto 305-days of lactation is the criterion most commonly used for the selection of dairy animals and study the performance of such trait is of paramount importance for carrying out selection. However, 305 days milk yield of Mehsana buffaloes is 1988 kg (AGRI-IS, NBAGR).

The average LSM for 305 DMY of Mehsana buffaloes during different periods of calving viz. P1, P2, P3, P4, P5 and P6 were 1600.51 ± 40.07 lit, 1669.89 ± 46.00 lit, 1707.00 ± 46.48 lit, 1707.49 ± 43.29 lit, 1761.88 ± 51.95 lit and 1841.75 ± 60.24 lit, respectively (Table 1). Further, it was revealed that 305 DMY was lowest in P1 (1600.51 ± 40.07 lit) and highest in P6 (1841.75 ± 60.24 lit). In the present study, it was found that the period of calving had a significant ($P \leq 0.05$) effect on 305 DMY. These findings are in close agreement with Galsar et al. (2016a) in Mehsana buffaloes and Verma et al. (2017) in Murrah buffaloes. Moreover, Thiruvankadan et al. (2010), Chaudhary (2015) and Chaudhari (2016) reported highly significant effect ($P \leq 0.01$). The significant effect of period of calving indicated that this improvement in the performance of the herd might be due to better management, grading-up practices followed on the farm and better health coverage of the animals.

The performance of Mehsana buffaloes calving during winter, summer and rainy season were 1803.72 ± 35.62 lit, 1724.84 ± 54.20 lit and 1615.70 ± 28.95 lit, respectively (Table 2). However, the 305 DMY of Mehsana buffaloes calving during winter and summer season was highest and it differed significantly from that of rainy season calvers. The season of calving had a highly significant ($P < 0.01$) effect on 305 DMY in this investigation. However, similar effects were reported by Thiruvankadan et al. (2014) and Chaudhary (2015) in Murrah buffaloes; Chaudhari (2016) in Mehsana buffaloes observed highly significant ($P \leq 0.01$) effect of the season of calving on 305 DMY, which were in line with the present findings of Mehsana buffaloes. The decrease in milk yield might be because of high environmental temperature and humidity during summer and rainy season, respectively. Season can affect milk production in two ways: by including deficiency of fodder and by the extreme temperatures that may suppress production at the peak of lactation curve (Afzal et al. 2007; Hernandez-Castellano et al. 2019). Whereas, it is in contrast with the findings of Verma et al. (2017).

The least squares mean of 305 DMY were observed under different parities viz. L1, L2, L3, L4 and e" L5 viz: 1604.31 ± 37.26 lit, 1733.25 ± 44.48 lit, 1753.34 ± 48.32 lit, 1767.27 ± 55.27 lit and 1715.60 ± 37.45 lit, respectively (Table 3). The effect of parity was highly significant ($P \leq 0.01$) on 305 DMY in the present findings of Mehsana buffaloes. It was revealed that 305 DMY increased from 1st to 4th parity and thereafter it started declining. It is in close agreement with the findings of Thiruvankadan et al. (2014) and Chaudhary (2015) in Murrah buffaloes. Bharat et al. (2004) and Verma et al. (2017), observed that significant ($P \leq 0.05$) effect of parity on 305 DMY.

The LSM of 305 DMY were observed under different AFC groups viz. A1, A2 and A3 as 1641.90 ± 41.08 lit, 1735.42 ± 26.08 lit and 1766.94 ± 55.39 lit, respectively (Table 4). However, results shows that AFC group did not have any affect on 305 DMY in the present study, but the lowest 305 DMY was observed in A1 (1641.90 ± 41.08 lit) and the highest 305 DMY was observed in A3 (1766.94 ± 55.39 lit). These findings are in close agreement with Thiruvankadan et al. (2010), Jamuna et al. (2015), Jakhar et al. (2016) and Verma et al. (2017). Chaudhary (2015) observed a significant ($P \leq 0.05$) effect of age at first calving group on 305 DMY in Murrah and Nili-ravi buffaloes. The age at first calving is also an important trait for bringing improvement in milk production. Also, AFC considerably affects the productive life of an animal and the number of calves produced during her life time. From the economic point of view, reduction in AFC is desirable for reducing the rearing cost of heifer and milk production cost.

Total Milk Yield (TMY)

It is the total amount of milk produced by an animal during a particular lactation period. Milk yield is an important polygenic trait of dairy animals and it is directly related to the genetic potential of the breed and management practices of the herd.

Table 1: Effect of period of calving on 305-DMY, TMY and LL of Mehsana buffaloes

Sr. No.	Factors	305-days milk yield (lit)	Total milk yield (lit)	Lactation length (days)
1.	Population Mean ($\mu \pm$ S.E.)	1714.75 ± 27.30 (1171)	1765.55 ± 28.61 (1186)	273.28 ± 3.66 (1164)
2.	Period of calving	*	**	*
	P1	1600.51 ± 40.07 (239) ^c	1660.20 ± 42.58 (239) ^c	287.41 ± 5.53 (234) ^a
	P2	1669.89 ± 46.00 (218) ^{bc}	1711.44 ± 48.68 (219) ^{bc}	272.04 ± 6.38 (205) ^{bc}
	P3	1707.00 ± 46.48 (222) ^b	1741.95 ± 49.27 (222) ^{bc}	264.82 ± 6.34 (222) ^c
	P4	1707.49 ± 43.29 (251) ^b	1743.19 ± 45.60 (256) ^{bc}	271.06 ± 5.93 (252) ^{bc}
	P5	1761.88 ± 51.95 (140) ^{ab}	1794.13 ± 53.84 (147) ^{ab}	263.18 ± 6.93 (144) ^c
	P6	1841.75 ± 60.24 (101) ^a	1942.39 ± 62.97 (103) ^a	281.15 ± 7.99 (107) ^{ab}

Note: Figures in parenthesis indicates number of observation/records., ** $P < 0.01$ highly significant; * $P < 0.05$ significant; NS= Non-significant; S.E.= Standard Error; N= Subclass means with different superscripts are significantly different from each other

The average TMY of Mehsana buffaloes based on 1186 observations was 1765.55 ± 28.61 lit (Table 1), which was lowest (1660.20 ± 42.58 lit) during the period from 1991 to 95 and highest (1942.39 ± 62.97 lit) during the period from 2016 to 2020. This shows that there is an increase of 282.19 lit of milk per lactation over the period of 30 years. This improvement might be due to better management practices and a proper breeding plan of the farm. The present findings are within the range of the estimates reported by Thiruvankadan et al. (2010) as 1686.2 ± 44.4 kg and Thiruvankadan et al. (2014) as 1855.6 ± 16.1 kg in Murrah buffaloes; Galsar et al. (2016a) as 1851.98 ± 19.73 lit in Mehsana buffaloes. The present estimates of TMY were much higher than those reported by Bharat et al. (2004), Charlini and Sinniah (2015) and Rathod et al. (2018). The present findings are contradictory to the findings of Chaudhary (2016) and Sathwara et al. (2020) for Mehsana buffaloes.

The average LSM of TMY of Mehsana buffaloes during different periods of calving viz. P1, P2, P3, P4, P5 and P6 were 1660.20 ± 42.58 lit, 1711.44 ± 48.68 lit, 1741.95 ± 49.27 lit, 1743.19 ± 45.60 lit, 1794.13 ± 53.84 lit and 1942.39 ± 62.97 lit, respectively (Table 1). The TMY was lowest in P1 (1660.20 ± 42.58 lit) and highest in P6 (1942.39 ± 62.97 lit). The period of calving had highly significant ($P \leq 0.01$) effect on TMY in the present study and it is in close agreement with Chaudhary (2015) and Galsar et al. (2016a) for Nili-ravi and Mehsana buffaloes, respectively. Similarly, significant ($P \leq 0.05$) effects of the period of calving on TMY were reported by Bharat et al. (2004), Jakhar et al. (2016) and Verma et al. (2017). The significant effect of the period of calving indicated that there may be differences in feeding practices, proper breeding plan, management practices and climatic conditions during different periods.

The LSM of TMY of Mehsana buffaloes during winter, summer and rainy season were 1856.57 ± 37.30 lit, 1783.25 ± 57.27 lit and 1656.82 ± 30.29 lit, respectively (Table 2). The TMY was highest during winter season and lowest during rainy season. The effect of season of calving on TMY was observed highly significant ($P \leq 0.01$) in the present study and it is in accordance with

Chaudhary (2015) in Murrah buffaloes. Thiruvankadan et al. (2014) and Verma et al. (2017) in Murrah buffaloes reported that significant ($P \leq 0.05$) effect of season of calving on TMY. In contrast to present results the findings of Chaudhary (2015) and Jakhar et al. (2016) reported that season of calving had non-significant effect on TMY. The buffaloes calved during winter season give their better performances than the buffaloes calved in rainy season might be due to the fact that winter season is followed by rainy season in which there is abundant availability of fodders in the subsequent winter and summer seasons.

The performance of Mehsana buffaloes under different parities viz. L1, L2, L3, L4 and e" L5 and found 1683.79 ± 39.10 lit, 1783.75 ± 47.04 lit, 1793.95 ± 51.04 lit, 1816.84 ± 58.20 lit and 1749.41 ± 39.19 lit, respectively (Table 3). However, TMY increased from 1st to 4th parity, but decline thereafter. In the present findings of Mehsana buffaloes found that parity had a non-significant effect on TMY and it is in accordance with Bharat et al. (2004) in Surti buffaloes. Contradictory to present findings, Jakhar et al. (2016) and Verma et al. (2017) reported a significant ($P \leq 0.05$) effect of parity which is not in accordance with present findings; Thiruvankadan et al. (2014) and Galsar et al. (2016a) reported highly significant ($P \leq 0.01$) effect of parity on TMY.

The age at first calving group had a highly significant ($P < 0.01$) effect on the TMY of Mehsana buffaloes. The TMY was lowest (1671.88 ± 43.42 lit) in the age at first calving group 1 (A1), followed by A2 (1782.73 ± 27.61 lit) and A3 (1842.04 ± 57.48 lit) (Table 4). Thiruvankadan et al. (2010) and Charlini and Sinniah (2015) reported that non-significant effect of age at first calving group on TMY. Although AFC group has significant effect on TMY, but with the increase in AFC, there was substantial increase in TMY also.

Lactation Length

Lactation length is one of the important trait which affects the lactation milk yield. However, there are various factors such as

Table 2: Effect of season of calving on 305-DMY, TMY and LL of Mehsana buffaloes

Sr.No.	Factors	305-days milk yield (lit)	Total milk yield (lit)	Lactation length (days)
1.	Population Mean ($\mu \pm$ S.E.)	1714.75 ± 27.30 (1171)	1765.55 ± 28.61 (1186)	273.28 ± 3.66 (1164)
2.	Season of calving	**	**	**
	S1	1803.72 ± 35.62 (322) ^a	1856.57 ± 37.30 (329) ^a	278.25 ± 4.83 (326) ^a
	S2	1724.84 ± 54.20 (124) ^{ab}	1783.25 ± 57.27 (125) ^a	278.97 ± 7.30 (127) ^a
	S3	1615.70 ± 28.95 (725) ^b	1656.82 ± 30.29 (732) ^b	262.61 ± 3.93 (711) ^b

Note: Figures in parenthesis indicates number of observation/records., ** $P < 0.01$ highly significant; * $P < 0.05$ significant; NS= Non-significant; S.E.= Standard Error; N= Subclass means with different superscripts are significantly different from each other

Table 3: Effect of parity on 305-DMY, TMY and LL of Mehsana buffaloes

Sr. No.	Factors	305-days milk yield (Kg)	Total milk yield (Kg)	Lactation length (days)
1.	Population Mean ($\mu \pm$ S.E.)	1714.75 $\pm$ 27.30 (1171)	1765.55 $\pm$ 28.61 (1186)	273.28 $\pm$ 3.66 (1164)
2.	Parity	**	NS	**
	L1	1604.31 $\pm$ 37.26 (336) ^b	1683.79 $\pm$ 39.10 (340)	296.37 $\pm$ 5.05 (338) ^a
	L2	1733.25 $\pm$ 44.48 (216) ^a	1783.75 $\pm$ 47.04 (217)	272.57 $\pm$ 6.06 (213) ^b
	L3	1753.34 $\pm$ 48.32 (173) ^a	1793.95 $\pm$ 51.04 (174)	263.77 $\pm$ 6.54 (175) ^b
	L-4	1767.27 $\pm$ 55.27 (128) ^a	1816.84 $\pm$ 58.20 (130)	271.58 $\pm$ 7.53 (128) ^b
	$\geq$ L-5	1715.60 $\pm$ 37.45 (318) ^a	1749.41 $\pm$ 39.19 (325)	262.09 $\pm$ 5.13 (310) ^c

Note: Figures in parenthesis indicates number of observation/records., **P<0.01 highly significant; *P<0.05 significant; NS= Non-significant; S.E.= Standard Error; N= Subclass means with different superscripts are significantly different from each other

Table 4: Effect of AFC group on 305-DMY, TMY and LL of Mehsana buffaloes

Sr. No.	Factors	305-days milk yield (Kg)	Total milk yield (Kg)	Lactation length (days)
1.	Population Mean ($\mu \pm$ S.E.)	1714.75 $\pm$ 27.30 (1171)	1765.55 $\pm$ 28.61 (1186)	273.28 $\pm$ 3.66 (1164)
2.	AFC group	NS	**	*
	A1	1641.90 $\pm$ 41.08 (217)	1671.88 $\pm$ 43.42 (220) ^b	262.47 $\pm$ 5.63 (215) ^b
	A2	1735.42 $\pm$ 26.08 (834)	1782.73 $\pm$ 27.61 (840) ^a	270.27 $\pm$ 3.55 (825) ^b
	A3	1766.94 $\pm$ 55.39 (120)	1842.04 $\pm$ 57.48 (126) ^a	287.09 $\pm$ 7.42 (124) ^a

Note: Figures in parenthesis indicates number of observation/records., **P<0.01 highly significant; *P<0.05 significant; NS= Non-significant; S.E.= Standard Error; N= Subclass means with different superscripts are significantly different from each other

availability of feed and fodder, managerial practices and seasonal variation which affects the lactation length.

The LSM for LL based on 1164 number of observation was found 273.28 $\pm$ 3.66 days, which was highest (296.37 $\pm$ 5.05 days) in 1st parity and lowest (262.09 $\pm$ 5.13 days) in 5th parity (Table 1). This shows that as the parity advanced, the duration of LL decreased (34.28 days) over 30 years. This improvement might be due to better management practices adopted at the farm. These findings are in close association with the reports of Bharat et al. (2004) as 294.44 $\pm$ 6.66 days in Surti buffaloes, Jamuna et al. (2015) as 286.08 $\pm$ 2.23 days in Murrah buffaloes, Galsar et al. (2016a) as 298.84 $\pm$ 4.33 days and Galsar et al. (2016b) as 281.17 $\pm$ 2.58 days in Mehsana buffaloes. The results are contrast with the findings of Gupta et al. (2012), Chaudhari (2016), Prajapati et al. (2018), Patel et al. (2019) and Bhatt (2019) reported comparatively higher LL. Moreover, Rathod et al. (2018) reported lower LL than that of present study. The variations observed in LL by research workers may be due to differences in feeding and management practices of buffaloes.

The LSM of lactation length of Mehsana buffaloes during different periods of calving viz. P1, P2, P3, P4, P5 and P6 were

287.41 $\pm$ 5.53 days, 272.04 $\pm$ 6.38 days, 264.82 $\pm$ 6.34 days, 271.06 $\pm$ 5.93 days, 263.18 $\pm$ 6.93 days and 281.15 $\pm$ 7.99 days, respectively (Table 1). It was observed that period of calving had significant (P<0.05) effect on lactation length of Mehsana buffaloes. The highest LL was observed during P1 (287.41 $\pm$ 5.53 days) and lowest during P5 (263.18 $\pm$ 6.93 days). The present findings was in accordance with those of Charlini and Sinniah (2015), Galsar et al. (2016b), Prajapati et al. (2018) and Bhatt (2019) in Mehsana buffaloes. The present findings are contrast with the findings of Jamuna et al. (2015) and Jakhar et al. (2016).

The average LL of Mehsana buffaloes calving during winter or summer season have longer LL viz: 278.25 $\pm$ 4.83 days and 278.97 $\pm$ 7.30 days, respectively than rainy season calvers, which have shorter LL i.e. about 262.61 $\pm$ 3.93 days (Table 2). The effects of season of calving of LL had highly significant (P $\leq$ 0.01) on Mehsana buffaloes. The season or period of calving determines what animal will get to feed when the animal will be lactating like availability of fodder, temperature, humidity etc. The present findings are in close agreement with Bharat et al. (2004) in Mehsana and Surti buffaloes. The present findings are contrast with the findings of Eldawy et al. (2021), Galsar et al. (2016a) and Galsar et al. (2016b) in Mehsana buffaloes.

The LSM of LL observed under different parities viz. L1, L2, L3, L4 and e"5 were 296.37 ± 5.05 days, 272.57 ± 6.06 days, 263.77 ± 6.54 days, 271.58 ± 7.53 days and 262.09 ± 5.13 days, respectively (Table 3). The effects of parity on LL was highly significant ($P \leq 0.01$) in this study on Mehsana buffaloes. Parity may have a role in lactation length as sex of calf, use of different sire etc. The mean comparison of LL was significantly highest in 1st parity and lowest in e"5th parity. The present findings are in accordance with the findings of Thiruvankadan et al. (2014), Chaudhary (2015) and Galsar et al. (2016a). Jakhar et al. (2016) and Galsar et al. (2016b). In present findings are contrast with the findings of Bharat et al. (2004) and Jamuna et al. (2015).

The analysis of variance (Table 4) revealed that AFC group had significant ($P \leq 0.05$) effect on lactation length of Mehsana buffaloes. The present findings is in accordance with Jamuna et al. (2015) in Murrah buffaloes and Prajapati et al. (2018) in Mehsana buffaloes. On the other hand, Chaudhary (2015) in Nili-ravi buffaloes and Bhatt (2019) in Mehsana buffaloes reported non-significant effect of age at first calving group on LL, which is reverse with the present findings of Mehsana buffaloes. The LL in A1 was lowest as 262.47 ± 5.63 days and highest in A3 as 287.09 ± 7.42 days.

Conclusion

On the basis of results obtained in the study, we can conclude that the milk production performance of Mehsana buffaloes has improved significantly over a period of 30 years (1991 to 2020) maintained under organized herd. Improvement of these traits might be due to better, feeding, breeding, health care and proper culling strategies followed on the farm. However, the season of calving, period of calving and parity have significant effects on the performance of the herd, hence such non-genetic factors must be taken into consideration in future, for further improvement of the herd

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Conflict of interests

The authors declare that there are no conflicts of interest.

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Economic analysis of costs and returns of milk production in Andhra Pradesh

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Abstract: The present study was conducted in the Chittoor and East Godavari districts in the state of Andhra Pradesh to estimate the costs and returns of milk production to know the status of milk profitability among the farmer-producer households. The milk productivity in the study area was higher in crossbreed cows than the buffalo and indigenous cattle. The overall gross cost of milk production estimated at ₹ 264.40 per animal per day, which was higher in the crossbreed cows than the Buffaloes and Indigenous cattle. The variable costs account for the major proportion of the cost of milk production (88.87%) than the fixed cost. Within variable costs, feed and fodder expenses represent the largest portion, accounting for 71.89 percent of milk production costs, exceeding labour, veterinary as well as miscellaneous costs. The average milk production per animal was 7.23 litres per day, was highest in the crossbreed cows with 10.37 litres per day than in buffalo (6.12 litres per day) and indigenous cattle (5.21 litres per day). The overall net returns per animal were highest in crossbreed cows with ₹ 83.14 per day than the buffalo (₹ 51.66 per day) and the indigenous cattle (15.66 per day). The cost of milk production and net returns per litre was highest in the buffalo with ₹ 44.89 per litre and ₹ 8.37 per litre than the crossbreed cows and the indigenous cattle. Even though the overall returns from milk production were positive among all

the farmer-producer households in the study area rearing of crossbreed cows and buffalo was found to be profitable when compared with the indigenous cattle.

Keywords: Milk production; Economic analysis, Labour; Feed; Indigenous cattle

Introduction

Dairying is essential to transforming Indian farmers' lifestyles. Farmers living in drought-prone regions where agriculture is only practiced for a brief period, depend heavily on livestock, making it their primary source of income. Both livestock and dairy employ more than one-third of the country's population (DAHD, 2021). Dairy plays a crucial role in the income generation and employment of the rural population, which provides employment for more than 8 crore farmers directly and solely contributes around 5 percent of the National Economy (Economic Survey, 2021-22). It also observed that dairying helped people overcome poverty and enhance rural welfare (Yadav et al. 2017).

The economics of milk production plays a crucial role in dairy farming. It indicates the profitability of the enterprise. A farmer producer can run the enterprise successfully when they get a sufficient amount of income from that, otherwise, they may incur losses, which affects the farmer as well as the family and also the income of the country. If the farmer makes a huge investment in the dairy enterprise from the borrowings from the banks, if he is not able to pay debt properly, he will become a wilful defaulter. So, the economics of the enterprise gives the idea to the farmer producer about profit or loss and also helps the public agencies in making the policy decisions in order to ensure the minimum price to the producer. So, cost and returns are important in estimating the profitability of the dairy enterprise. It also suggests the producer where to increase and decrease in order to run the farm sound. Costs incurred in dairy farm includes fixed (buildings, machinery, etc.) and variable cost (feed and fodder, labour charges, etc.). The returns are from the sale of the milk and milk products. To run the farm successfully the returns must be more than the costs, otherwise farmers suffer from losses. The variable costs had a keen role in the production of milk. Feed and fodder costs are most crucial in the production of milk as they influence the

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returns. Milk production depends on feed and fodder which increases production and productivity. So, the proper maintenance of feeding throughout the year is important. It is estimated that green fodder and dry fodder had shortages of 11.24 percent and 23.4 percent in the country (IGFRI, 2021). To overcome the problem of shortage, a proper fodder plan should be prepared and implemented throughout the year as it will enhance the productivity of the animal. The price of the milk is the most important incentive for the farmers to increase milk production (Chand et al. 2017) and also helps in decision-making in the allocation of resources (Dwivedi and Naik, 2017). Other factors like quality of breed, artificial insemination, and veterinary care also play a key role in milk production even though they cost very little in the total costs.

Andhra Pradesh state stands fifth in the country in milk production with 14.7 million metric tons (MT) of milk production (contributing 7 percent to the total country's milk production). Very few studies were conducted on the costs and returns of milk in the state. So, there is a need to study investment patterns, feed costs, and other costs along with the price of the milk for developing the proper policies in the study in order to enhance the welfare of the farmers.

Materials and methods

Study area

The study was conducted in Andhra Pradesh, focusing on Chittoor and East Godavari districts due to their high milk production. A multistage random sampling method was employed to select 80 farmer-producer households from four villages, with 20 households from each village.

Data collection and methodology

The primary data was collected from the farmer-producer households through the pre-structured interview schedules on different aspects of dairy farming during 2023. The 80 farmer-producer households were post-stratified into small (1-3 milch animals), medium (4-5 milch animals), and large farmers (6-14 milch animals) based on milch animals by using the cumulative square root frequency method. The data collected from the farmer respondents were subjected to tabular analysis. The methodology used for the study was presented in detail as follows:

1. Cost of milk production: The costs of milk production include the fixed and variable expenditures incurred by farmers on different items

A. Fixed costs: It consists of the rental value of the land, depreciation on fixed assets like buildings, machinery, chaff cutter, etc. The capital cost recovery cost method was used for accounting for the assets.

Capital Recovery Cost (CRC) of civil structures, machinery, and equipment

$$R = Z \left[\frac{(1+r)^n r}{(1+r)^n - 1} \right]$$

R = Capital recovery cost, Z = Initial value of the capital asset, r = Current interest rate, n = Useful life of the assets/animals

The formula for CRC estimation of milch animals is the same as above but the important aspect is ascertaining 'n' i.e., the useful life of the animal or its productive life. As per the subject matter specialists at the field level, the useful productive life of milch animals, defined in terms of age (year) and order of lactation, viz., the average number of calving per animal is: a) Local cow: 10 years with 6 calving's; b) Crossbred cow: 8 years with 5 calving's and c) Buffalo: 10 years with 6 calving's.

B. Variable costs: These include feed and fodder costs, labour costs, and veterinary and miscellaneous costs.

Feed and fodder cost: Feed and fodder costs include dry fodder, green fodder, and concentrates. If the feed and fodder are purchased, the cost is worked as the product quantity of feed fed to the animal and the purchase price of feed. In the case of feed and fodder produced at the home, the prices are farm harvest prices.

Labour cost: These costs considers both family and hired labour, with hired labour costs based on work type and wages, while family labour costs align with prevailing permanent farm labour rates. Various labour types (adult male, female, and child) were used, later standardized to male equivalent units using Patel et al.'s 1980 wage rate recommendations.

Veterinary and miscellaneous costs: The veterinary expenses encompass artificial insemination, natural service, vaccinations, medications, and other costs. On the other hand, miscellaneous expenditures cover fixed asset repairs, petroleum, oil, lubricants, insurance premiums, and other charges.

There are different types and ages of animals in the herd (indigenous cattle, crossbreed cows, and buffaloes). These different categories of animals are converted into homogenous units for the easy estimation of costs and returns. For the present study, the standard animal units developed by Sirohi et al. (2015) were used. As the study region falls in the southern region, SAUs for the southern region are presented in Table 1.

2. Returns from milk production: To calculate the gross returns from the animals the milk production and animal dung returns are taken. The sale value of animals is not taken into consideration while calculating the returns from milk production.

3. Other costs and return concepts:

- Gross cost = Total fixed cost + total variable cost
- Net cost = Gross cost – value of dung

Net cost per animal

- Cost of milk production (¹ / litre) =

Average milk yield of animal

- Gross returns = Quantity of milk × price of milk
- Net returns = Gross returns – net cost

Net returns per animal per day

- Net returns per litre of milk =

Average milk yield of animal

Results and Discussion

Costs and returns of milk production for Indigenous cow

The cost and returns of milk production of indigenous cattle across different herd size categories in farmer-producer households. Table-2 shows that the overall gross cost of milk production was ₹ 203.04 with 86.59 percent variable cost and 13.14 percent fixed cost. Similar results were found in Sunil et al. 2017, Kumar et al. 2015 and Mohapatra et al. 2021. Among the variables, the total feed and fodder account for 68.34 percent of gross cost followed by labour cost (16.86 percent) and miscellaneous cost (1.39 per cent). In the feed and fodder costs,

green fodder cost, ₹ 75.35 was more than the concentrates, ¹ 44.90 and dry fodder ¹ 18.51.

Inter-category analysis shows that the feed fodder cost was highest in the large herd size category (₹ 147.50) followed by medium (₹ 138.79) and small (¹ 130.00). while in the case of labour cost, it was highest for large herd size (₹ 35.16) followed by medium (₹ 33.13) and small herd size (₹ 34.38). In the case of miscellaneous charges, it was highest for large herd size (₹3.16) followed by medium (₹ 2.83) and small herd size (₹ 2.46).

The overall net cost of production was obtained by subtracting the dung value from the gross cost. The overall net cost of indigenous cattle has been estimated of ₹ 200.75, which was highest in large herd size ₹ 216.02, followed by medium ₹ 200.40 and small herd size ₹ 185.83. The overall average price of the milk was ₹ 41.55, was highest in the case of large herd size (₹ 42.00) followed by medium (₹ 41.64) and small herd size (₹ 41.00). The overall average production of milk per day was found to be of 5.21 litres per day, was highest in case of large herd size category 5.58 litres per day followed by medium herd size, 5.21 litres per day and small, 4.82 litres/day.

The gross returns were highest in the case of large herd size (¹ 234.50 per animal per day) followed by medium (₹ 217.12 per animal per day) and small (₹ 197.62 per animal per day) with an average of ₹ 216.41 per animal per day. It was found that the average net returns per day were ₹ 15.66 per day per animal, which was highest in large herd size (₹ 18.48 per day per animal) followed by medium (₹ 16.72 per day per animal) and small (₹ 11.79 per day per animal).

The overall cost of milk production per litre was ₹ 38.56, which was highest in large herd size (₹ 38.69), followed by medium (₹ 38.43 per litre) and small herd size (₹ 38.55 per litre). The overall net returns per litre were ₹ 2.99, which was highest in large herd size (₹ 3.31) followed by medium (₹ 3.21) and small herd size (₹ 2.45). The table revealed that with an increase in milk production, the net returns were also increasing. While in the case of indigenous cattle, milk yield was low when compared with other crossbreed cows and buffalo. The net returns were found positive

Table 1: Standard animal units for the Southern region

Animals	Indigenous cattle	Crossbreed cows	Buffalo
Adult male	0.97	1.12	1.04
Adult female	1.00	1.62	1.24
Young stock male (<1 year)	0.22	0.24	0.24
Young stock female (<1 year)	0.27	0.30	0.28
Young stock male (≥ 1 year)	0.54	0.63	0.60
Young stock female (≥ 1 year)	0.47	0.52	0.51
Heifer	0.82	0.86	0.77

among all the categories of farmers. similar findings were also obtained by other studies in Madhya Pradesh by Chand et al.2017 and Agrawal and Raju, 2021. Some studies also showed that the net returns from the indigenous cattle were negative (Singh et al. 2012, Sunil et al.2017 and Jaiswal and Singh, 2015).

Cost and returns of milk production of crossbreed cows

The cost and returns of milk production of crossbreed cows across different herd size categories in the study area are shown in Table-3. The overall average gross cost of milk production was ₹ 313.05, which accounts for 91.27 per cent variable cost and 8.73 per cent fixed cost similar results were observed in the study of Kumar et al. 2022.

The Feed and fodder cost accounts for 77.33 per cent of the total cost of milk production followed by labour costs at of 13.53 per cent and miscellaneous costs of 1.00 per cent. Among the feed and fodder cost, green fodder accounts for about 35.28 per cent of the total cost of production, followed by concentrate cost,

30.99 per cent and dry fodder cost, 10.79 per cent. Inter-category analysis revealed that feed and fodder cost was highest in the large herd size category¹ 258.22, followed by medium,¹ 245.90 and small, ₹ 216.55. while in the case of green fodder, it was highest in large herd size (₹ 118.53) followed by medium (₹ 115.64) and large (₹ 97.15) with an overall average of ₹ 110.45. In the case of dry fodder, it was highest for large (₹ 36.96) followed by medium (₹ 34.63) and small (₹ 28.92). while in concentrate cost also follows a similar pattern *i.e.*, large (₹ 102.69), medium (₹ 95.63) and small herd size (₹ 90.48). The large herd size (₹ 44.11) had the highest labour charges followed by medium (¹ 41.92) and small (₹ 41.07). while in the case of miscellaneous cost, it was highest in large (₹ 3.22) followed by medium (₹ 3.16) and large (₹ 3.00) with an average of ₹ 3.13.

The overall average net cost was ₹ 310.64, which was highest in large herd size ₹ 334.18, followed by medium ₹ 317.98 and small ₹ 279.77. The overall average sale price of milk was ₹ 37.91, with large herd size ₹ 38.50, medium ₹ 38.48 and small ₹ 36.75. The overall production of milk production per day was highest in

Table 2: Costs and returns of milk production of Indigenous cattle in farmer producer households (₹/ animal/ day)

Cost and Returns components	Small	Medium	Large	Overall
Green fodder	70.00 (37.09)	75.71 (37.37)	80.33 (36.89)	75.35 (37.11)
Dry fodder	18.00 (9.54)	18.29 (9.03)	19.25 (8.84)	18.51 (9.12)
Concentrate	42.00 (22.25)	44.79 (22.10)	47.92 (22.00)	44.90 (22.11)
Feed and fodder cost	130.00 (68.88)	138.79 (68.50)	147.50 (67.72)	138.76 (68.34)
labour cost	34.38 (18.21)	33.13 (16.35)	35.16 (16.35)	34.22 (16.86)
Veterinary & Miscellaneous cost	2.46 (1.30)	2.83 (1.40)	3.16 (1.40)	2.82 (1.39)
Total Variable Cost	166.84 (88.40)	174.75 (86.25)	185.83 (85.32)	175.81 (86.59)
Total Fixed Cost (TFC)	21.89 (11.60)	27.85 (13.75)	31.97 (14.68)	27.24 (13.41)
Gross Cost (TFC+TVC)	188.73 (100.00)	202.60 (100.00)	217.79 (100.00)	203.04 (100.00)
Value of Dung	2.90	2.20	1.77	2.29
Net Cost	185.83	200.40	216.02	200.75
Price of milk (₹/litre)	41.00	41.64	42.00	41.55
Milk production (litre/animal/day)	4.82	5.21	5.58	5.21
Gross Return	197.62	217.12	234.50	216.41
Net Returns	11.79	16.72	18.48	15.66
Cost of milk production (₹/litre)	38.55	38.43	38.69	38.56
Net Returns (₹/litre)	2.45	3.21	3.31	2.99

Note: Figures in the parenthesis indicate the percentage of the gross cost of milk production

large herd size (11.11 litres per animal per day) followed by medium (10.53 litres per animal per day) and small (9.48 litres per animal per day) with an average of 10.37 litres per animal per day. The gross returns were highest in large herd size (₹ 427.78), followed by medium (₹ 405.00) and small (₹ 348.57) with an average of ₹ 393.78. The net returns per animal were highest in large herd size (₹ 93.60) followed by medium (₹ 87.03) and small (₹ 68.79), with an average of ₹ 83.14 per animal per day. The overall average cost of milk production per litre was ₹ 29.93, was highest in medium herd size (₹ 30.21) than in large (₹ 30.08) and small (₹ 29.50). The net returns per litre were highest in large (₹ 8.42), medium (₹ 8.27) and small (₹ 7.25) with an average of ₹ 7.98 per litre. The overall returns found similar to that of studies conducted by Sunil et

al. 2017 in Karnataka and Agarwal and Raju, 2021 in Madhya Pradesh. The increased returns per litre in case of large herd size are due to the increase in milk production per animal.

Costs and returns of milk production for Buffalo

The costs and returns of milk production of buffalo across different herd size categories was shown in Table-4. The fixed and variable costs account for about 12.16 per cent and 87.84 per cent of the total cost of milk production. Similar findings were reported in Banekol et al. 2023. Among the variable cost, feed and fodder cost account for 69.00 per cent of the total cost of milk production, labour cost, 17.63 per cent and miscellaneous

Table 3: Costs and returns of milk production for crossbred cows in farmer-producer households (₹ / animal/ day)

Cost and Returns components	Herd size category			Overall
	Small	Medium	Large	
Green fodder	97.15 (34.44)	115.64 (36.10)	118.58 (35.22)	110.45 (35.28)
Dry fodder	28.92 (10.34)	34.63 (10.89)	36.96 (11.06)	33.50 (10.79)
Concentrate	90.48 (32.34)	95.63 (30.08)	102.69 (30.73)	96.27 (30.99)
Feed and fodder cost	216.55 (77.40)	245.90 (77.33)	258.22 (77.27)	240.23 (77.33)
labour cost	41.07 (14.56)	41.92 (13.08)	44.11 (13.10)	42.37 (13.53)
Veterinary and miscellaneous cost	3.00 (1.07)	3.16 (0.99)	3.22 (0.96)	3.13 (1.00)
Total Variable Cost	260.63 (92.39)	290.97 (90.83)	305.56 (90.76)	285.72 (91.27)
Total Fixed Cost (TFC)	21.48 (7.61)	29.38 (9.17)	31.12 (9.24)	27.33 (8.73)
Gross Cost (TFC+TVC)	282.11 (100.00)	320.35 (100.00)	336.68 (100.00)	313.05 (100.00)
Value of Dung	2.33	2.38	2.50	2.40
Net Cost	279.77	317.98	334.18	310.64
Price of milk (₹/litre)	36.75	38.48	38.50	37.91
Milk production (litre/animal/day)	9.48	10.53	11.11	10.37
Gross Return	348.57	405.00	427.78	393.78
Net Returns	68.79	87.03	93.60	83.14
Cost of milk production (₹/litre)	29.50	30.21	30.08	29.93
Net Returns (₹/litre)	7.25	8.27	8.42	7.98

Note: Figures in the parenthesis indicate the percentage of the gross cost of milk production

costs, 1.21 per cent of the total cost of production. The overall feed and fodder cost was highest in green fodder with ¹ 98.56, which was highest in large herd size (₹ 111.90), then in medium (₹ 92.74) and small (₹ 91.05). After greenfodder, concentrates had a high amount ₹ 72.01, in which large herd size (₹ 75.79) shares higher share followed by medium (₹ 74.19) and small (₹ 66.03). In the case of dry fodder, it follows a similar pattern of concentrates and green fodder *i.e.*, large (₹ 24.14), medium (₹ 19.76) and small (₹ 18.05) with an average of ₹ 20.65. After feed and fodder costs, the labour cost, ₹ 48.84 was the highest variable cost than the miscellaneous cost, ₹ 3.36. In the case of labour charges, a large herd size (₹ 51.31) had a higher cost than medium (₹ 49.92) and small (₹ 45.30).

The overall average net cost per animal was ₹ 274.57, which was highest in large, ₹ 302.57, medium, ₹ 268.58 and small, ₹ 252.54. The average price per litre of milk was ₹ 53.26, which was highest in large (₹ 54.88), medium (₹ 52.92) and small (₹ 51.98). the average milk production per animal was highest in large, 6.65 litres per animal per day followed by medium, 6.12 litres per animal per day and small, 5.58 litres per animal per day, with an average of 6.12 litres per animal per day. The overall average gross returns per animal were ₹ 326.22, was highest in large herd size (₹ 364.69), followed by medium (₹ 323.93) and small (₹ 290.05). The net

Table 4: Costs and returns of milk production for buffalo in farmer-producer households (₹ / animal/ day)

Cost/Returns components	Herd size category			Overall
	Small	Medium	Large	
Green fodder	91.05 (35.64)	92.74 (34.23)	111.90 (36.69)	98.56 (35.57)
Dry fodder	18.05 (7.07)	19.76 (7.29)	24.14 (7.92)	20.65 (7.45)
Concentrate	66.03 (25.85)	74.19 (27.38)	75.79 (24.85)	72.01 (25.98)
Feed and fodder cost	175.13 (68.56)	186.69 (68.90)	211.82 (69.46)	191.22 (69.00)
labour cost	45.30 (17.73)	49.92 (18.42)	51.31 (16.82)	48.84 (17.63)
Veterinary and miscellaneous cost	3.28 (1.28)	3.33 (1.23)	3.47 (1.14)	3.36 (1.21)
Total Variable Cost	223.71 (87.57)	239.94 (88.55)	266.60 (87.42)	243.42 (87.84)
Total Fixed Cost (TFC)	31.75 (12.43)	31.02 (11.45)	38.36 (12.58)	33.71 (12.16)
Gross Cost (TFC+TVC)	255.46 (100.00)	270.96 (100.00)	304.96 (100.00)	277.13 (100.00)
Value of Dung	2.92	2.38	2.39	2.56
Net Cost	252.54	268.58	302.57	274.57
Price of milk (₹/litre)	51.98	52.92	54.88	53.26
milk production (litre/animal/day)	5.58	6.12	6.65	6.12
Gross Return	290.05	323.93	364.69	326.22
Net Returns	37.50	55.35	62.12	51.66
Cost of milk production (₹/litre)	45.26	43.88	45.53	44.89
Net Returns (₹/litre)	6.72	9.04	9.35	8.37

Note: Figures in the parenthesis indicate the percentage of the gross cost of milk production

Table 5: Cost and returns of milk production in farmer-producer households (¹ / animal/ day)

Cost and Returns components	Indigenous cattle	Crossbred cows	Buffalo	Overall
Green fodder	75.35 (37.11)	110.45 (35.28)	98.56 (35.57)	94.79 (35.85)
Dry fodder	18.51 (9.12)	33.50 (10.70)	20.65 (7.45)	24.22 (9.16)
Concentrate	44.90 (22.11)	96.27 (30.75)	72.01 (25.98)	71.06 (26.87)
Feed and fodder cost	138.76 (68.34)	240.23 (76.74)	191.22 (69.00)	190.07 (71.89)
labour cost	34.22 (16.86)	42.37 (13.53)	48.84 (17.63)	41.81 (15.81)
Veterinary and miscellaneous cost	2.82 (1.39)	3.13 (1.00)	3.36 (1.21)	3.10 (1.17)
Total Variable Cost	175.81 (86.59)	285.72 (91.27)	243.42 (87.84)	234.98 (88.87)
Total Fixed Cost (TFC)	27.24 (13.41)	27.33 (8.73)	33.71 (12.16)	29.42 (11.13)
Gross Cost (TFC+TVC)	203.04 (100.00)	313.05 (100.00)	277.13 (100.00)	264.40 (100.00)
Value of Dung	2.29	2.40	2.56	2.42
Net Cost	200.75	310.64	274.57	261.99
Price of milk (₹/litre)	41.55	37.91	53.26	44.24
Milk production (litre/animal/day)	5.21	10.37	6.12	7.23
Gross Return	216.41	393.78	326.22	312.14
Net Returns	15.66	83.14	51.66	50.15
Cost of milk production (₹/litre)	38.56	29.93	44.89	37.79
Net Returns (₹/litre)	2.99	7.98	8.37	6.45

Note: Figures in the parenthesis indicate the percentage of the gross cost of milk product

returns per animal per day were highest in large (₹ 62.12) than in medium (₹ 55.35) and small (₹ 37.50) with an average of ₹ 51.66 per animal per day. The overall cost of milk production per litre was highest in large (₹ 45.53), followed by small (₹ 45.26) and medium (₹ 43.88) with an average of ₹ 44.89. The net returns per litre were highest in large (₹ 9.35), medium (₹ 9.04) and small (₹ 6.72) with an average of ₹ 8.37. The overall similar findings were found in the studies conducted by Sunil et al.2017 and Agrwal and Raju, 2021.

Cost and returns of milk production

The cost and returns of milk production across different animals of farmer-producer households was shown in Table-5. The overall fixed and variable costs across different animals were 11.13 per cent and 88.87 per cent of the total cost of milk production.

Among the total variable costs, feed and fodder costs account for 71.89 per cent of the total cost of production, followed by labour, 15.81 per cent and miscellaneous costs, 1.17 per cent. In the case of feed and fodder costs, crossbreed cows, ₹ 240.23 had the highest cost followed by buffalo, ₹ 191.22) and indigenous cattle, ₹ 138.76. In the case of feed and fodder, green fodder (35.85 per cent) shared a large amount than followed by concentrates (26.87 per cent) and dry fodder (9.61 per cent) of the total cost of production. while the green fodder cost was highest in crossbreed cows, ₹ 110.45, followed by buffalo ₹ 98.56 and indigenous cattle ₹ 75.35. Similar pattern is followed for concentrates and dry fodder *i.e.*, crossbreed (₹ 96.27, ₹ 33.50), buffalo (₹ 72.01, ₹ 20.65) and indigenous cattle (₹ 44.90, ₹ 18.51). while in the case of labour costs, it was highest for crossbreed cows, ₹ 42.37, followed by buffalo, ₹ 48.84 and indigenous cattle,

₹ 34.22 with an average of ₹ 41.81. In the case of miscellaneous costs also follow the same as that of labour charges *i.e.*, crossbreed cows (₹ 3.13), buffalo (₹ 3.36) and indigenous cattle (₹ 2.82) with an average of ₹ 3.10.

The overall net cost was ₹ 261.99, was highest in crossbreed cows (₹ 310.64) followed by buffalo (₹ 247.57) and indigenous cattle (₹ 200.75). The average price of the milk in the study area was ₹ 44.24, it was highest in buffalo (₹ 53.26) followed by indigenous cattle (₹ 41.55) and crossbreed cows (₹ 37.91). The highest price of buffalo milk was due to the high-fat content of both the indigenous cattle and crossbreed cows. The overall milk production per animal per day was 7.23 litres, with crossbreed cows (10.37 litres per day), buffalo (6.12 litres per day) and indigenous cattle (5.21 litres per day). The gross returns were highest in crossbreed cows (₹ 393.78) followed by buffalo (₹ 326.22) and indigenous cattle (₹ 216.41) with an average of ₹ 312.14. the lower returns in the case of buffalo and indigenous cattle are due to the lower productivity of the animal in the study area. The net returns were highest in crossbreed cows (₹ 83.14 per animal) followed by buffalo (₹ 51.66 per animal) and indigenous cattle (₹ 15.66 per animal) with an average of ₹ 50.15 per animal. The cost of production of milk per litre was highest in buffalo (₹ 44.89), followed by indigenous cattle (₹ 38.56) and crossbreed cows (₹ 29.93) with an average of ₹ 37.79 per litre. The net returns per litre were highest in buffalo (₹ 8.37), followed by crossbreed cows (₹ 7.98) and indigenous cattle (₹ 2.99) with an average of ₹ 6.45. The results are in line as found in the study by Dwivedi et al. 2024. The overall maintenance cost per litre as well as net returns per litre was highest in the case of buffalo than in crossbreed cows and indigenous cattle.

Conclusion

The study on the costs and returns on milk production concludes that the milk productivity of crossbreed cow was higher than the buffalo and indigenous cattle in the study area. The overall returns from the crossbreed cow were found more than the buffaloes and indigenous cattle. The feed and fodder costs were playing the major role in the total milk production and indirectly affects the profitability of the dairy. The dairy was main source and also improved the welfare of the farmers. Even though net returns per litre was higher in the buffaloes but the overall returns were low due to low milk productivity. The basic infrastructure like the improvement of hospitals, cattle sheds and subsidies for concentrates and mineral mixtures is to be provided for the betterment of the dairy industry in the state. Providing village-level training to dairy farmers on scientific management practices enhances milk production, meeting the demand of both domestic and global consumers. The overall study indicates that the rearing of dairy animals by the farmers was not only profitable but also an efficient source of income for the dairy producers.

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Elucidating the associations of polymorphism of growth hormone gene with milk production traits in Jamunapari goats of India

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Abstract: Growth hormone (GH) gene can be utilized as a major gene because its polymorphisms have been associated to milk traits in various livestock species. The aim of this present study was to investigate the polymorphism in the exon 1, 3 and 5 of gGH (goat GH) gene and to evaluate the possible associations with milk traits in Jamunapari goat, maintained at the ICAR-Central Institute for Research on Goats, Makhdoom, Uttar Pradesh, India. In this study, 100 primiparous lactating goats were randomly chosen, and their productive performances like 90-days, 140-days and total milk yield as well as milk compositional traits like fat %, SNF%, casein%, protein%, lactose% and ash % of three consecutive lactations were recorded. The exon 1, 3 and 5 of the gGH gene was PCR amplified and the resulting products were analyzed by Single-strand conformation polymorphism (SSCP). Two conformational patterns (A and B) for each the GH exon 3 and 5 were detected but no polymorphism existed in exon 1 of growth hormone gene. The frequency of the patterns varied from 41.4 to 58.6% in these two fragments. Association studies of SSCPs patterns at GH gene with milk production and milk composition traits in Jamunapari breed showed that only polymorphic patterns at exon 3 were positively associated with milk production traits, i.e., with 90-days milk yield, 140-days milk yield and total milk yield of animals but there was no significant

effect on milk composition traits. Animals with pattern A/A for exon 3 were significantly superior milk producers ($P < 0.01$) than animals having B/B pattern for 90-days, 140-days and total milk yield of animals, whereas GH conformation patterns (A and B) for exon 5 didn't have any impact on milk compositions. Further, in case of exon 5, the milk yields and milk compositions of animals didn't vary significantly between animals having different GH conformation patterns (A and B). These findings may be used for preserving genetic diversity of the population and may be useful for marker assisted selection in order to improve milk production of this breed.

Keywords: Growth hormone gene, Milk traits, SSCP polymorphism, Jamunapari Goat

Milk production is a physiological function that is under the control of several genes. Genotyping animals for all the genes encoding a polygenic traits seems impractical and so it appears more realistic to focus on only a few genes having effects that account for a significant genetic variation in milk production traits. The growth hormone (GH), an anabolic hormone synthesized and secreted by the anterior pituitary somatotroph cells, plays an important role in regulation of postnatal growth and metabolism in mammals and is directly involved in animal processes such as lactation, protein, lipid and carbohydrate metabolism, tissue growth, and fertility in dairy animals (Seevagan et al. 2015; Agaoglu et al. 2019). The wide physiological activities of growth hormone gene make it an important candidate gene worth investigating for its role in growth and milk production traits of animals. Several authors demonstrated the associations of GH gene polymorphisms with growth (Singh et al. 2015; Pandya et al. 2021; Rashijane et al. 2022), milk yield (Moneva et al. 2020) and milk compositions (Dettori et al. 2009; Dettori et al. 2013) in different goat breeds.

Single-strand conformation polymorphism (SSCP) is a powerful method for identifying sequence variation in amplified DNA. The SSCP analysis of genes, whose product is associated with production traits, could be a valuable alternative approach for the establishment of allelic variants useful as markers to aid selection. Therefore, growth hormone gene is a potential target for studies of genetic sequence variation in connection with

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production performance of animals. This prompted us to investigate Single-strand conformation polymorphisms (SSCP) in each of the three exons of the goat GH gene and to establish the possible relationships of polymorphism patterns with milk production (lactation and milk compositional traits) traits in Jamunapari goats.

The present study was carried out on Jamunapari goats belonging to a farm located at ICAR- Central Institute for Research on Goats, Makhdoom, Uttar Pradesh, India. A total of 100 primiparous lactating goats were randomly chosen, and the lactation and milk compositional traits of these selected animals were recorded for three consecutive lactations for a period of 3 years. Milk yields at 90-days, 140-days and total lactational yield were recorded for each lactation and fresh milk samples for all animals under study were collected at 15 days intervals from kidding to 90 days of lactation. Milk samples were analyzed to estimate the fat, solid-not-fat (SNF), casein, protein, lactose and ash content of milk (Prajapati et al. 2017). Blood sample (8–10 µl) was collected from each animal by jugular vein puncture in vacuum tubes treated with 15% ethylene di-amine tetra acetic acid (EDTA) as an anticoagulant and stored at 4°C till further processing. Genomic DNA was isolated from whole blood using phenol-chloroform method (Sambrook et al. 1989) with minor modifications.

Based on the published nucleotide sequence of exon 1, 3 and 5 of goat growth hormone (Marques et al. 2003 and Malveiro et al. 2001a,b), the three exons of gGH gene were amplified by PCR using the following primer pairs shown in Table 1.

PCR reactions were performed using advanced primus 96 thermocycler using 200 mM each of dATP, dTTP, dGTP and dCTP; 50mM KCl, 10mM Tris-HCl (pH 9.0, 0.1% Triton X -100, 1.5 mM magnesium chloride; 0.75 unit of Taq DNA polymerase; 0.5 µM of

each primers and 50–100 ng of genomic DNA in the final volume of 25 µl). The amplification began with denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56–61°C for 30s extension at 72°C for 30 s and final extension at 72°C for 5 min. The amplified product of each fragment was analyzed by electrophoresis on 2% agarose gel (5V/cm) using ethidium bromide staining.

PCR-Single strand conformation polymorphism (PCR-SSCP), a method for mutation detection, relies on the fact that denatured DNA molecules migrate across non-denaturing polyacrylamide gel according to their size and their sequence. For SSCP analysis, 6 µl of the each PCR product is mixed with 8 µl of denaturing loading buffer (0.05% xylene cyanol and 0.05% bromophenol blue, 5.5mM EDTA, pH 8.0, in formamide), denatured at 95°C for 5 min, and snap-chilled on ice for 2 min. Samples were then loaded onto a polyacrylamide gel containing 0.5x TBE (0.045M Tris-borate, 0.001M EDTA, pH 8.0). Acrylamide concentration was 9.2%, glycerol concentration was 1%, *bis* ratio (29.1), running temperature 10°C, and TBE concentration was 0.5x in using Bio-rad Seq GT Electrophoresis Systems. After the electrophoresis run, gels were silver stained after fixing for 5 min and stained for 20 min. The PCR-SSCP gels were scored based on differential conformation and movement patterns of each of the single strand of amplified DNA (Orita et al. 1989).

To study the associations of SSCP patterns of growth hormone gene with milk production and milk composition traits, least-squares analysis of fitting constant (Harvey, 1990) was conducted with the following model:

$$Y_{ijklmn} = \mu + Y_j + F_k + P_l + S_m + e_{ijklmn}$$

Table 1: Primer sequences along with fragment length of exon regions of growth hormone gene

Exons	Primer sequences	Fragment length and localization(bp)	References
1	5'-CAGAGACCAATTCCAGGATC-3' 5'-TAATGGAGGGGATTTTGTG-3'	112 (360-471)	Marques et al. 2003
3	5'-GTGTGTTCTCCCCCAGGAG-3' 5'-CTCGGTCCTAGGTGGCCACT-3'	157 (1063-1219)	Marques et al. 2003
5	5'-AAAGGACAGTGGGCACTGGA-3' 5'-CCCTTGGCAGGAGCTGGAAG-3'	289 (1854-2142)	Malveiro et al. 2001a,b

Table 2: The band patterns and their frequencies at different fragments of growth hormone gene of Jamunapari goat

Exons	No. of pattern	Band Patterns	Pattern Frequencies (%)
gGH-1	1	A	100
gGH-3	2	A	41.4
		B	58.6
gGH-5	2	A	45.5
		B	54.5

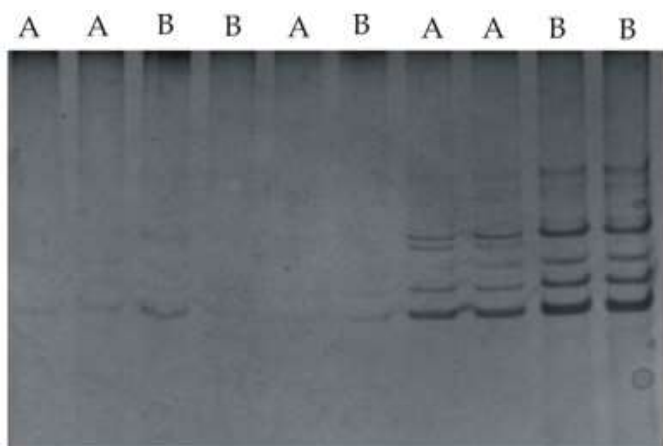


Fig. 1 PCR-SSCP of DNA region spanning from exon 3 of gGH gene in Jamunapari goat

Where, Y_{ijklmn} is the record for the n^{th} animal, μ is overall mean, Y_j is the effect of j^{th} year of kidding ($j=1,2,3$), F_k is the effect of the k^{th} season of kidding ($k=1, 2$), P_l is the effect of the l^{th} parity of dam ($l=1, 2, 3, 4, 5, 6$ or more), S_m is the effect of the m^{th} SSCP patterns ($m=1, 2$) and e_{ijklmn} is the residual error element with standard assumptions.

In this study, SSCP analysis of exons 1, 3 and 5 of the gGH gene of Jamunapari goats was performed on the fragments amplified by PCR using the primers described in Table 1, which showed the expected lengths. Polymorphism of gGH gene in Jamunapari goats showed two conformation patterns (A and B) for each of the GH exon 3 and 5 (Fig. 1 and 2), whereas no polymorphism was found in exon 1 (Fig. 3). The frequencies of each pattern are depicted in the Table 2. The SSCP pattern of growth hormone gene of Jamunapari goat in this study revealed polymorphism only in exon 3 and 5 regions. SSCP patterns analysis of exon 1, 3 and 5 of growth hormone gene of Algarvia goat was also observed by Malveiro et al. (2001a, b) and they reported that two conformational patterns existed in exon 1, four in exon 3 and five in exon 5 as compared to the present study. Marques et al. (2003) reported the existence of 6, 10, 5 SSCP pattern in exons 3, 4 and 5, respectively in Serrana goat. Vyas et al. (2008) also observed that high level of polymorphism existed at exons 3, 4 and 5 of the growth hormone gene of Jamunapari goats by SSCP analysis. In their study, they reported the presence of 7, 5 and 4 conformational patterns in exons 3, 5 and 4 of gGH gene of Jamunapari goats. Singh et al. (2018) observed the genetic polymorphism for growth hormone gene at 1 exon in Jamunapari kids by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), which was contrary to the present study. PCR-SSCP patterns of exons 4 and 5 of growth hormone gene of Jakhrana goats of India revealed the presence of 3 conformation patterns (viz., A, B and C) as reported by Gupta et al. (2009). In another study on polymorphism at the goat GH (gGH) gene, Mousavizadeh et al. (2009) reported the presence of nine conformational patterns in exon 4 of the gGH gene in SSCP-PCR

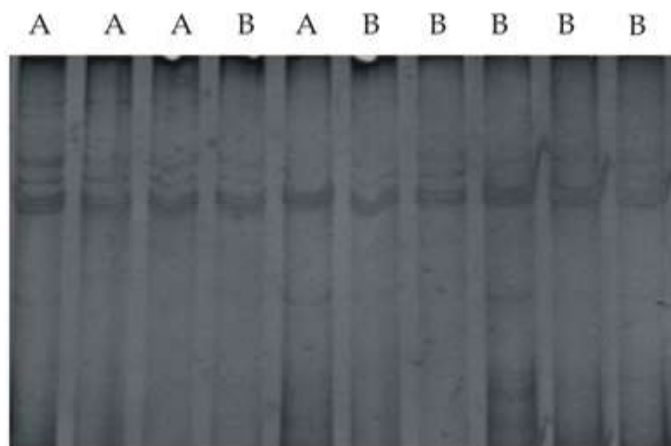


Fig. 2 PCR-SSCP of DNA region spanning from exon 5 of gGH gene in Jamunapari goat

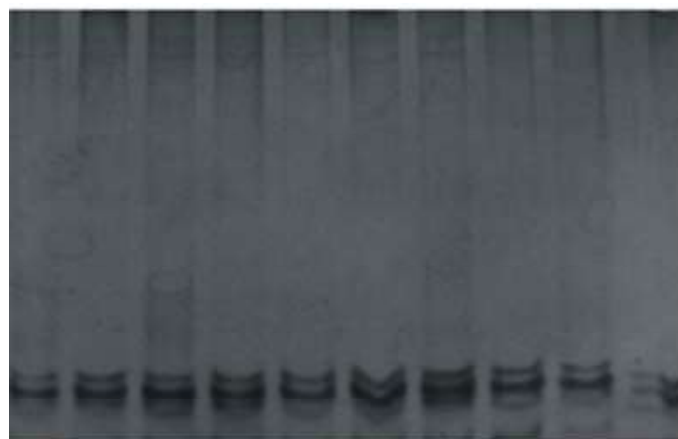


Fig. 3 PCR-SSCP of DNA region spanning from exon 1 of gGH gene in Jamunapari goat

analysis in Iranian Talli goats. The exon 3 of the gGH gene in Sarda goats was analysed after SSCP-PCR analysis by Dettori et al. (2009) and they detected six conformational patterns at the exon3 region of goat hormone gene. Wickramaratne et al. (2010) observed 3 conformational patterns (A, B and C) at each of exon 3 and 5 regions of growth hormone gene in Osmanabadi and Sangamneri goats. Further, genetic variations in the goat growth hormone (gGH gene) were investigated by single strand conformation polymorphism (SSCP) analysis in Sirohi goats by Kumar et al. (2011) and the fragments consisting of exon 1, exon 4 and exon 5 revealed 6 variants.

The least-squares means along with standard errors associated with milk production and milk composition parameters for SSCP patterns at exon 3 and 5 of GH gene in Jamunapari goats have been depicted in Table 3. Different environmental factors associated with milk production and milk constituents traits of Jamunapari goats were found non-significant ($P>0.05$) for this set of data. The results also showed that SSCP patterns at exon

Table 3: Least-squares means ($\pm$ SE) for lactation traits and milk compositional traits of Jamunapari goat associated with patterns of exon3 and exon 5

Traits	Patterns			
	Exon 3 A/A	B/B	Exon 5 A/A	B/B
Milk Production traits				
90-days MY	74.03 ^a $\pm$ 3.03 (41)	65.05 ^b $\pm$ 2.98 (58)	68.18 $\pm$ 3.28 (45)	69.73 $\pm$ 3.07 (54)
140-days MY	102.87 ^a $\pm$ 4.52 (36)	89.72 ^b $\pm$ 4.15 (52)	95.52 $\pm$ 4.72 (39)	95.76 $\pm$ 4.19 (49)
Total MY	109.46 $\pm$ 5.24 (41)	96.75 $\pm$ 4.73 (58)	101.53 $\pm$ 5.18 (45)	103.08 $\pm$ 4.84 (54)
Milk compositional traits				
Fat %	2.81 $\pm$ 0.07 (41)	2.89 $\pm$ 0.06 (58)	2.82 $\pm$ 0.07 (45)	2.88 $\pm$ 0.06 (54)
SNF %	9.26 $\pm$ 0.07 (41)	9.21 $\pm$ 0.06 (58)	9.31 $\pm$ 0.07 (45)	9.16 $\pm$ 0.06 (54)
Casein %	2.93 $\pm$ 0.03 (41)	2.91 $\pm$ 0.02 (58)	2.93 $\pm$ 0.02 (45)	2.92 $\pm$ 0.02 (54)
Protein %	3.28 $\pm$ 0.03 (41)	3.23 $\pm$ 0.03 (58)	3.26 $\pm$ 0.03 (45)	3.24 $\pm$ 0.03 (54)
Lactose %	5.00 $\pm$ 0.07 (41)	4.90 $\pm$ 0.06 (58)	5.01 $\pm$ 0.06 (45)	4.89 $\pm$ 0.06 (54)
Ash %	0.79 $\pm$ 0.01 (41)	0.78 $\pm$ 0.01 (58)	0.79 $\pm$ 0.01 (45)	0.78 $\pm$ 0.01 (54)

3 had only significant effect ($P < 0.01$) on all milk yield traits. Animals with pattern A/A for exons 3 were significantly superior milk producers ($P < 0.01$) for 90-days, 140-days and total milk yield of animals than animals having B/B pattern, whereas the milk yields and milk compositions of animals of different GH conformation patterns (A and B) for exon 5 didn't varied significantly between each other. Associations were established in the Portuguese Algarvia goat breed between gGH SSCP polymorphic patterns in exon 4 and 5 and milk production (Malveiro et al. 2001), later confirmed by Marques et al. (2003) for the Sarda goat. SSCP polymorphic patterns in exon 3 were also associated ($P < 0.01$) with milk yield, fat and protein percentages, and with lactose content ($P < 0.05$) in Sarda goats (Dettori et al. 2009). Further, Dettori et al. (2013) also reported that polymorphic patterns at exon 1 and exon 4 were positively associated with milk production, and with both fat and protein content in Sarda goat. The findings of the present study are preliminary based on small samples, and it should be confirmed on a larger sample size. The gGH gene polymorphisms may be used for marker assisted selection in Jamunapari goat, also taking into account short and long-term effects on population structure and rates of inbreeding, in order to improve dairy production along with preserving genetic diversity of the population.

In this study, Single-strand conformation polymorphism (SSCP) analysis of exons 1, 3 and 5 of the growth hormone gene of Jamunapari goats revealed that two conformation patterns (A and B) for each of the GH exon 3 and 5 but no polymorphism existed in exon 1 of growth hormone gene. Association studies between SSCPs patterns at GH gene with milk production and milk composition traits in this breed showed that only polymorphic patterns at exon 3 were positively associated with milk production traits of animals. As SSCP polymorphism at the exon 3 of the gGH exert a positive influence in milk production of Jamunapari goats in this study, so there is a possibility of exploring this approach for the search of genetic markers located at this

region. The SSCP polymorphic variation makes it a potential candidate for the establishment of the association with quantitative traits. If specific haplotypes can be defined at this candidate gene that could be associated with milk production traits as well as milk composition traits, it would be a valuable genetic resource for improvement of this caprine breed.

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INSTITUTIONAL FORM Sustaining Membership (1 Year) Benefactor Membership (8 Year)	12,500 70,000	Nil Nil	2,250 12,600	14,750 82,600	Nil Nil	14,750 82,600	APR-MAR APR-MAR	

Contents

ISSN 0019-5146 (Print)

ISSN 2454-2172 (Online)

RESEARCH ARTICLES

Physicochemical quality of cow milk collected from different sources in Adewa, Central Zone of Tigray, Ethiopia

Etsemeskel Tadele and Brhane Gebreananya

Comparison of acid casein-based Mozzarella cheese analogue with natural Mozzarella cheese during refrigerated storage

Chetan N Dharaiya, Atanu H Jana, Jarita M Mallik and Rachana B Rathawa

Effect on quality of Paneer using Unripe Mango Powder as a Natural Coagulant

Aditya Kumar, Gokhale AJ, Smitha Balakrishnan, Patel AM and Mallik JM

Optimisation of the *Shrikhand* incorporated with lemongrass (*Cymbopogon citratus*) distillate

Angelika Nelson, SK Aktar Hossain, Sonia Mor, Deodeep Kumar,

Isolation and Characterization of Methicillin Resistant *Staphylococcus aureus* (MRSA) from milk samples

Jubeda Begum and Nasir Akbar Mir

Effect of *Njavara* Rice Bran on Physico-chemical, Sensory, and Textural Properties of Sweetened Yoghurt

Elizabeth Thomas, Ritika and Narender Raju Panjagari

Estimation of β -sitosterol as a tool to detect ghee adulteration with palm oil

Heena Kauser, Shilpashree BG, Neelam Upadhyay and H. Devaraja Naik

Estimation of direct and maternal covariance of production efficiency traits in Murrah buffalo

Smriti Sharma, Surender Singh Dhaka, Chandrashekhar Santosh Patil and Pallavi Rathi

Effect of non-genetic factors on linear type traits score in Sahiwal cattle

Ajay Kumar, Surender Singh Lathwal, Indu Devi, Divyanshu Singh Tomar, Pawan Singh Mamta and Yajuvendra Singh

Dairy innovation portal: A web-based platform to address farmer-led innovations in the Indian dairy sector

Priyajoy Kar, H.R. Meena, B. S. Meena, Gopal Sankhala, A.K. Singh, Rakesh Kumar, Pawan Singh, Salam Jayachitra Devi, Sunil Kumar, Amitava Panja, Vikash Kumar and Romen Sharma