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INVITED REVIEW

Gas chromatographic analysis of triglycerides - The reference method for testing purity of milk fat and perspectives on its use in India: Review

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Abstract Adulteration of milk fat is burning issue, as traditional parameters for testing its purity are becoming ineffective due to advances in practices employed for the adulteration. Gas chromatographic (GC) analysis of intact triglycerides appears as the most promising method for combating menace of the high tech adulteration of milk fat, since it is simple, rapid, robust and comprehensive. Unfortunately, use of this method in India may cause problem of false positive results due to its specifications of parameters suggested on purity of milk fat. Hence, for adopting the method in India, modifications are required in limits of specifications on some parameters. The modifications in specifications of the method require extensive survey to generate database on the parameters. Outcome these efforts will be helpful to recognise Indian milk fat as pure in international market, whenever tested by this method. Until the method gets official endorsement, Indian dairy industry may avail its unmatched advantages by using it as a screening tool. Since, no comprehensive publication on this method is available so far in the literature; an attempt is made in this paper to collate information on its genesis, development, adoption as reference/ standard, basic principle, operational steps, advantage, limitations and evaluation. This review paper serves a navigation tool for concerned scientists and users of the method to understand its fundamental aspects identify reasons for false positive results occur in its use and develop appropriate solutions to resolve the problems as well as improve its application.

Keywords: Milk fat, TG profile, GC analysis, S-values, Testing purity, ISO 17678

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Introduction

Milk fat is an important nutrient in human diet and testing of its purity is very important to ensure constant and well-defined quality of milk and milk products. In purchase of milk from producers or suppliers, its price is determined from %fat content. This pricing policy encourages mixing of cheaper oils and/or fats. Nuisance of adulteration in milk fat is acquiring new dimensions due to adoption of science-based advanced modus operandi for the adulteration (Kala et al. 2016, Sharma et al. 2018, Pathania et al. 2020). Therefore, now fight against the smart adulteration of milk fat requires parameters on its purity, which are very robust against manipulation and highly comprehensive in nature. Moreover, technique used for analysis of these parameters should be simple, rapid, safe, economical and environment friendly. A literature survey of 73 years (Achaya & Hilditch 1950, Si et al. 2023) reveals that the intact triglycerides-based parameters on purity of milk fat and their analysis by GC technique appear as the most promising tool to combat the nuisance (Bosque-Sendra et al. 2012, Cossignani 2019, Csapó et al. 2019). Unfortunately, in India potential of this method for testing purity of milk fat is not fully recognised (Prajapati and Aparnathi 2021) since use of this method may lead to false positive results in Asian countries (ISO/IDF 2010). Reasons for problem of the false positive results in country like India may be attributed to limits suggested in specifications of parameters on purity of milk fat (Pathania et al. 2020, Prajapati and Aparnathi 2021, Sharma et al. 2021). Hence, for implementing the method in India appropriate modifications in limits of some parameters are required.

Though India is the highest milk producer in the world science 1998 (Kalidas et al. 2021), it is very surprising to note that no attempt has been reported so far to find amicable solutions for the problems in limits of some parameters specified in the method. This situation has compelled some quality conscious Indian dairy organisations to adopt the method with only limited use of its specifications. In such implementation of the method only limited parameters are selected for implementation. The parameters and their limits selected in such a way that they are not likely to cause problems of false positive results. Some dairy organisations in India have adopted the method for testing purity of fat present in

supplies of incoming milk, cream and butter before their final acceptance by the dairy plants (Prajapati and Aparnathi 2021).

Although the method is recognised as a standard method by European Union (EU) since 1995 (EC 1995) and as reference method jointly by ISO and IDF since 2010 (ISO/IDF 2010), the survey of literature suggests that no effort is made so far to present information on various aspects of the method in a highly comprehensive form. Therefore, attempt has been made in this review to collate information on (1) problems faced in use of traditional methods for testing purity of milk fat and advanced techniques explored for the same as alternates, (2) significance of analysing intact triglyceride, developments in GC analysis of intact triglyceride to test purity of milk fat and its adoption as reference method and (3) basic principle in working, operational steps, advantage, limitations and evaluation of the method. Moreover, efforts are also made to find information about (a) secret behind stunning success of the method, (b) reasons for false positive results in the method and (c) perspectives on implementation of the method in India. Finally plan of actions is suggested for work required to adopt the method as official reference in India under dairy farming system presently followed in the country.

Traditional methods in testing purity of milk fat

Traditionally, parameters based on butyro refractometer reading at 40°C, Reichert-Meissl value, Polenske value, saponification value, iodine value, fatty acids profile and phytosterol or β-sitosterol test are suggested for testing purity of milk fat. Application of these parameters have number of lacunas and/or suffer from one or more drawbacks. Problems faced in use of the traditional methods are compiled from several sources (Fox et al. 1988, Naviglio & Raia 2003, Azadmard-Damirchi & Torbati 2015, Kala et al. 2016, Sherma et al. 2018, Nilchian et al. 2020, Pathania et al. 2020, Shinde et al. 2021) and summarised below.

- Highly time consuming and very tedious since most of them involve several unit operations and that too under highly precise conditions. As a result, they are not suitable for analysing substantial number of samples in routine.
- Usually necessitate isolation, purification and/or concentration of the compounds targeted as marker in analysis (*e.g.* β-sitosterol or other minor components).
- Manual operations and necessitate human intervention at various stages in analysis. Therefore, they always remain at risk of human bias and/or error
- Destructive in nature and involve use of several corrosive, toxic and/or expensive chemicals. These lead to health hazards, environmental pollution and/or excessive cost of the analysis.
- Difficult to obtain reproducible results, especially when foreign fat is present at low rates. So results remain questionable and situation become difficult to reach a conclusion.
- High limits of detection (LoD) due to large natural variations in parameters, owing to effect of feed given to lactating animals, season of the year and stage of lactation.
- Lack of comprehensive nature and each method detects only limited number of foreign fats. Hence, they necessitate number of different methods to get overall view about the purity.
- Standards on their parameters are mostly specified as minimum or maximum value. Such parameters become highly susceptible to manipulation, as it can be adjusted easily.

Add to all these, practice of adulteration is now becoming very smart because of knowledge about chemistry of milk fat and that of the foreign fats. Application of the science-based information and proficiency gained from experience, a mixture of foreign fats is formulated so precisely that its physical properties and chemical characteristics resembles very well with that of the pure milk fat. Hence, it becomes extremely difficult to detect such well-designed tailer made blend of foreign in milk fat by the conventional parameters on its purity. Consequently, day by day these parameters are becoming ineffective in testing authenticity of milk fat (Bhalerao & Kummerow 1953, Parodi 1969, Kala et al. 2016, Sharma et al. 2018, Pathania et al. 2020, Shinde et al. 2021).

Advanced techniques in testing purity of milk fat

In view of the problems faced in use of traditional methods, different instrumental techniques were explored as alternates for testing purity of milk fat ((Nazrim Marikkar 2022, Mahrous et al. 2023). These advanced analytical techniques are mostly belonged to three different types: (a) GC analysis (EC 2008, ISO/IDF 2010), (b) MIR spectroscopy (Upadhyay et al. 2016 & 2018, Antony et al. 2018a, Gandhi et al. 2022) and NIR spectroscopy (Antony et al. 2018b, Aparnathi et al. 2019) and (c) thermal analysis (Nurrulhidayah et al. 2015, Tomaszewska-Gras 2016, Upadhyay et al. 2017, Farah et al. 2018, Islam et al. 2022). Among the various advanced analytical techniques reported for testing purity of milk fat, the GC analysis of intact triglyceride molecules is the only technique which is developed most systematically, researched very extensively and recognised internationally as the reference method.

Significance of analysing intact triglyceride in testing purity of milk fat

Chemically triglycerides are esters formed by reaction of three fatty acids molecules with one molecule of glycerol. In common edible oils and fats of plants and animals origin triglycerides are present as their most abundant component. While analysis of intact triglycerides, natural distribution of fatty acids in their molecules is retained as such. Therefore, information obtained abut inherent characteristics of natural oils and fats is usually

much higher from analysing intact triglycerides compared to the destructive methods of analysis (Ulberth & Buchgraber 2000, de La Fuente & Juarez 2005, Ruiz-Samblás et al. 2015, Nilchian et al. 2020).

In composition of milk fat triglycerides contribution about 98% (Amaral et al. 2018, Kapoor et al. 2023). Milk fat is most complex among the common edible oils and fats of plants and animals origin. The complexity is attributed to its fatty acid composition, because >400 structurally different fatty acids are identified as components of milk fat. Hence, it can form thousands of structurally different triglycerides (Buchgraber et al. 2004, Liu et al. 2020, Si et al. 2023). Liu et al. (2020) have identified 3454 triglyceride molecules in milk fat. If only 15 major fatty acids occurring @ >1% are considered and their position in triglyceride molecules is ignored, even then 680 compositionally different triglycerides are likely to be present in milk fat (MacGibbon & Taylor 2006). As a result, it is not feasible to exactly duplicate such a complex triglycerides composition of milk fat even by smart techniques. It indicates that, intact triglycerides analysis is a promising option in testing purity of milk fat.

Developments in GC analysis of triglyceride to test purity of milk fat

Significance of analysing intact triglyceride molecules in detecting blend of foreign fats in milk fat was recognized in early 1950s by Bhalerao and Kummerow (1953). However, its application in practice requires suitable technique for separation and quantification of triglyceride molecules and formation of parameters on purity of milk fat based on its triglyceride profile. The development of parameters from information of intact triglycerides was hampered due to problems in achieving their fine resolution. Doors were opened in early 1960s after getting success in separation and quantification of triglycerides by GC technique. The clear separation of triglycerides was achieved using stationary phase stable at high temperatures. The separation by this technique is based on differences in their number of carbon atoms content. The difference as low as two carbon atoms can be resolved successfully (Kuksis and McCarthy 1962). Chronology of developments in intact triglycerides profile-based parameters on purity of milk fat is summarised below. Perusal of this precious literature is very useful to understand fundamentals of the method and evolve constructive ideas to modify the exiting parameters as well as develop new parameters.

- Canadian researchers, Kuksis and McCarthy (1964) were pioneers to use GC analysis of intact triglycerides for detecting foreign fat in milk fat. They employed distortion in elution pattern of triglycerides during their fractionation by GC and relative proportion of specific triglyceride fractions (especially C52 & C54) as criterions in detection of the foreign fat.
- Pardi (1973) carried forward the idea suggested by Kuksis and McCarthy (1964). They worked out eleven ratios of triglyceride fractions in to two groups. Eight ratios comprise individual triglyceride fractions: (1) C54/C52, (2) C54/C36, (3) C52/C50, (4) C52/C42, (5) C52/C40, (6) C52/C38, (7) C52/C36 and (8) C50/C36, Whereas, three ratios comprise group of triglyceride fractions: (1) (C54+C52+C50)/C38, (2) (C54+C52+C50)/C40 and (3) (C54+C52+C50)/(C38+C40). They also worked out minimum and maximum values of the ratios for pure milk fat using authentic samples of Australian butterfat and suggested as criterions of butter fat purity.
- Timms (1980) suggested the following equation for multivariate analysis of three fractions of triglyceride (C40, C42 & C44) as characteristic for pure samples of Australian milk fat.

$$\mathbf{R = (14.197 \times C40) \ (36.396 \times C42) + (32.364 \times C44)}$$

Where, value of **R** should be 100 for the pure milk fat. As per this author, it detects 5% foreign fat with 99% confidence.

- Precht (1991) expanded the idea proposed by Timms (1980) on multiple regression analysis of triglyceride fractions. He standardised five equations to obtain five standardised values (S-values), which can serve as parameters on fat purity of milk fat. A very systematic and highly dedicated work of this author from Germany completed the search for intact triglyceride-based parameters to test purity of milk fat (Precht 1991, 1992a, 1992b). His technique is now used worldwide as reference method. Therefore, procedure followed by him in derivation of equations is shortly described here under.

Precht for his work gathered a required database on triglyceride composition of pure milk fat using carefully planned procedure and taking all relevant technical aspects in to consideration. The data were generated from analysis of 755 samples of milk fat from pure cow milk. The authentic samples of milk were collected from cows under different practices of feeding and stage of lactation as well as from different breeds and different regions of Germany over a period of five years. He made very extensive efforts to derive equations for calculation of S-values of milk fat by applying multiple linear regression in analysis of the database. As many as 32647 equations were derived, which comprise 3 to 15 different fractions of triglyceride from amongst the major fractions ranging (C26–C54). Each equation was examined for its efficacy and efficiency in detection of various foreign fats and five most promising equations were selected (Table 1). These five equations comprise 8 to 10 selected triglyceride fractions and coefficients specifically designed for each fraction of triglycerides in each equation. Precht also established limits of each S-value (*i.e.* S-limits) for pure cow milk fat using authentic samples of cow milk (Table 2). The five S-values are now also designated as S_1, S_2, S_3, S_4 and S_5 or S_{Total} (Povolo et al. 2008, Nilchian et al. 2020). Some Indian authors have designated them S_1 or S_{Total}, S_2, S_3, S_4 and S_5 (Kala 2013, Pathania et al. 2020, Sharma et al. 2021). Among the

two systems of designation the sequence reported as S_1, S_2, S_3, S_4 and S_5 or S_{Total} appears more logical, since it exactly coincides with the sequence of equations suggested for calculation of S-values by EC (2008) and ISO/IDF (2010, 2019). Among the five S-values, S_{Total} detects all type of foreign fats, whereas, rest of the S-values (*i.e.* S_1, S_2, S_3 & S_4) detect specific type of foreign fats.

After successfully developing the method Precht and his co-researchers continue the work on peripheral aspects of the method. Details of their work on some the important aspects and outcome of the work can be referred from their publications such as Precht (1993), Precht et al. (1998), Molkentin and Precht (1994, 1995, 2000), Molkentin (2006, 2007, 2013) as well as Molkentin and Crawford (2009).

Adoption of GC analysis of triglycerides as reference method

The technique developed by Precht for testing purity of milk fat using GC analysis of its triglycerides profile was recognised as a reference method at national and international level. German Institute for Standardization (Deutsches Institut für Normung) adopted it as standard for detection and determination of foreign fats in milk fats vide DIN No. 10336: 1994-09 (DIN, 1994). After considering results of six collaborative trials in member countries, EU adopted the technique as a reference for determination of milk fat purity, vide EC Regulation No. 454/95 (EC 1995) and revised it twice (EC 2001, EC 2008). International collaborative study of the technique was conducted by Molkentin & Crawford (2009). Based on their study ISO and IDF jointly adopted it as reference method for determination of milk fat purity vide ISO 17678: 2010 and IDF 202: 2010 respectively (ISO/IDF 2010). They published its updated version as second edition in 2019 (ISO/IDF 2019). In subsequent part of this article it will be referred as the method.

Basic principle of the method

In biosynthesis of triglycerides in plants and animals, spatial arrangement of fatty acids in their molecules is genetically controlled (Bhalerao & Kummerow 1956). Hence, all naturally occurring edible oils and fats have their unique profile of triglycerides (Buchgraber et al. 2004). Ruminant milk fat is unique in containing substantial amount of short chain fatty acids (C4:0–C14:0) along with most prominent medium chain (C16) and long chain (C18) fatty acids (Bear 1991, MacGibbon & Taylor 2006). Hence, in milk fat of cow and buffalo triglycerides profile ranges from C24 to C54 (Smiddy et al. 2012, Kala 2013, Hazra et al. 2017, Sharma et al. 2018, Pathania et al. 2021). The triglycerides profile of cow and buffalo milk fat has very distinct bimodal distribution pattern with first peak appears between C34 and C42, whereas, second peak appears between C46 and C54. On the other hand, most of the vegetable oil (Edem 2002, Povolo et al. 2008, Park et al. 2014, Dorni et al. 2018) and animal body fats (Wood et al. 2008, Park et al. 2014, Lisitsyn et al. 2017) comprise only medium chain and long chain fatty acids. As a result, in most of the vegetable oils and animal body fats fat major triglycerides generally range from C46 to C54 or C56. Therefore, triglycerides profile of plant oils and animal body fats have only monomodal distribution pattern with only one peak between C50 and C56 (Parodi 1973, Precht 1992a, Gutiérrez et al. 2009, Rohman et al. 2012, Kala 2013, Park et al. 2014). Although, coconut and palm kernel oils do contain short chain fatty acids in substantial amount (Krishna et al. 2010, Rahman et al. 2022), but their medium chain and long chain fatty acids content is very low compared to milk fat. Therefore, their triglycerides distribution pattern is also monomodal with broad

Table 1: Equations suggested for calculation of S-values

S-values	Foreign fat(s) indicated & equation
S_1	Soybean, sunflower, rapeseed, linseed, maize, wheat germ, cottonseed & fish oil = (2.0983C30) + (0.728C34) + (0.6927C36) + (0.6353C38) + (3.7452C40) – (1.2929C42) + (1.3544C44) + (1.7013C46) + (2.5283C50)
S_2	Coconut & palm kernel fat = (3.7453C32) + (1.1134C36) + (1.3648C38) + (2.1544C42) + (0.4273C44) + (0.5809C46) + (1.2926C48) + (1.0306C50) + (0.9953C52) + (1.2396C54)
S_3	Palm oil & beef tallow = (3.6644C28) + (5.2297C30) – (12.5073C32) + (4.4285C34) – (0.2010C36) + (1.2791C38) + (6.7433C40 – (4.2714C42 + (6.3739C46)
S_4	Lard = (6.5125C26) + (1.2052C32) + (1.7336C34) + (1.7557C36) + (2.2325C42) + (2.8006C46) + (2.5432C52) + (0.9892C54)
S_5 (S_{total})	Total (*i.e.* all foreign fats in general) = – (2.7575C26) + (6.4077C28) + (5.5437C30) – (15.3247C32) + (6.2600C34) + (8.0108C40) – (5.336C42) + (0.6356C44) + (6.0171C46)

C26 to C54 represents percentage of each major triglycerides fraction containing respective number of carbon atoms in total triglycerides

Compiled from Precht (1991,1992a, 1992b), EC (2008), ISO/IDF (2019), Sharma et al. (2021)

peak between C32 and C44 (Huebner 1961, Pocklington & Hautfenne 1985, Precht 1992a, Krishna et al. 2010).

The difference in pattern of triglycerides profile of milk fat and that of the foreign fats is depicted in Figure 1. To include one foreign fat from each specific S-value (*i.e.* S_1, S_2, S_3 & S_4) soybean oil, coconut oil, bovine tallow and lard are selected as examples in diagrammatical presentation on triglyceride profile of milk fat and that of the foreign fats. In GC analysis of triglycerides for testing purity of milk fat, the differences exiting between triglyceride profile of milk fat and that of the foreign fats are exploited. The range of each S-value for pure milk fat remains in the vicinity of 100 (Table 2). When foreign fat is mixed with milk fat, it causes deviation in triglyceride profile of the milk fat beyond the natural variation, which in turn causes large shift in its S-value(s). Consequently, S-value(s) of adulterated milk fat cross the specified limit(s) and go below the lower limit or above the upper limit depending on type of foreign fat mixed (Precht 1992a, EC 2008, ISO/IDF 2019).

Operational steps involved in the method

Operation of the method involves eight sequential steps, Outline of these operational steps is compiled from different reports (Precht 1992a & 1992b, EC 2008, ISO/IDF 2010, Kala 2016, Sharma et al. 2021) and summarised below.

Extraction of fat from test sample

The fat from milk is first separated as cream and converted into butter, followed by thorough washing of the resulting butter grains. From butter fat is clarified by melting it at 50°C using a water bath, followed by filtration through folded fine-pored filter paper containing 0.5 to 1.0 g of sodium sulfate in oven maintained at 50°C. Care has to be taken that no serum is transferred. Use of Röse–Gottlieb gravimetric method or silica gel columns are suggested as an alternative method for the extraction of fat.

Preparation of fat solution

The clarified fat is completely dissolved in solvent (*n*-hexane or n-heptane). Amount of fat taken depends on type of column (packed or capillary) used and dimension of the column

Fractionation of triglycerides from the fat

The triglycerides in the fat solution separated into different fractions (i.e. groups) containing same number of carbon atoms in acyl chains of their constituent fatty acids. Fractionation of triglycerides is carried out by programable high temperature low resolution GC.

Quantification of the triglyceride fractions

Quantities of the triglyceride fractions are estimated from their respective peak areas in the chromatogram obtained from GC analysis. Mass of each triglyceride fraction is calculated as % m/m in total triglycerides plus cholesterol.

Calculation of S-values

S-values are calculated by inserting the estimated % mass of the appropriate triglyceride fractions into the standardised equations (Table 1). The results of the S-values to be expressed to two decimal places. Calculate all the S-values irrespective of the kind of foreign fat suspected. Though the S-values are calculated from percentages of triglyceride fractions, they do not represent a percentage themselves. Moreover, the S-values do not have a unit.

Comparison of the S-values with the specified limits

The five S-values obtained from the calculation are compared with their corresponding limits specified as standards (Table 2).

Interpretation of result

The results of the S-values are interpreted in accordance with their success or failure in complying the specified limits. When all five S-values calculated from analysis of the test sample fall within the specified limits, the sample is considered as pure. If one or more S-value(s) fall(s) outside the specified limits, the sample is considered as adulterated. Although individual S-values (*i.e.* S_1, S_2, S_3 & S_4) are more sensitive for certain foreign fats than the general S-value (S_{Total}), the positive result obtained in only one S-value does not allow to draw conclusion on type of foreign fat.

Table 2: Limits specified for S-values of pure fat from cow milk

S-value		Limits of the S-value
S_1	Vegetable oils[#] & Fish oil	98.05 to 101.95
S_2	Coconut fat & Palm kernel fat	99.42 to 100.58
S_3	Beef tallow & Palm oil	95.90 to 104.10
S_4	Lard	97.96 to 102.04
S_{total}	Total (General)	95.68 to 104.32

[#] Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ & Cottonseed

Compiled from Precht (1991, 1992a, 1992b), EC (2008), ISO/IDF (2010), Kala (2013), Povolo et al. (2008), Nilchian et al. (2020) and Sharma et al. (2021)

Quantification of foreign fat (if detected)

When presence of foreign fat is detected in milk fat (*i.e.* at least one of the S-value exceeds the specified limit), its quantity is estimated. Quantity of the foreign fat (% m/m) is calculated using the following formula.

$$\text{Foreign fat in milk fat (\%, m/m)} = \frac{(100 - S)}{(100 - S_f)} \times 100$$

Where, **S** is S-value of the test sample corresponding to the foreign fat detected and $\mathbf{S_f}$ is constant depending on type of foreign fat detected. In other words $\mathbf{S_f}$ is S-value of foreign fat detected in test sample. Its value may be established by analysing the foreign fat or may be taken from literature. In actual practice, particular foreign fat generally remains unknown, since most of the foreign fats are identified by the method as a group and not as a particular foreign fat (except lard). Same problem also arises when blend of foreign fats is mixed in milk fat. Therefore, when foreign fat is not known, $\mathbf{S_{total}}$ is taken **S** and value of $\mathbf{S_f}$ is taken as 7.46 in the formula for calculating quantity of foreign fat (EC 2008, ISO/IDF 2010 & 2019).

Advantages of the method

In testing purity of milk fat by using this method has number of unmatched advantages over the conventional destructive methods. These advantages of the method are compiled from several reports (Precht 1991, 1992a & 1992b, Povolo et al. 1999, Molkentin & Precht 2000, Kamm et al. 2001, de la Fuente & Juarez 2005, Fontecha et al. 2006, Molkentin 2007, Kala 2013, Amaral et al. 2018; Csapó et al. 2019, Cossignani 2019, ISO/IDF 2019, Shinde et al. 2020, Pathania et al. 2020 & 2021) and summarized here.

(1) Highly comprehensive: able to detect entire range of foreign fats including

(a) Plant oils and fats: soybean, sunflower, rapeseed, linseed, maize, wheat germ, cottonseed, coconut, palm kernel, palm and hydrogenated vegetable oil

(b) Animal body fats: Bovine tallow, lard and fish oil

(c) Blends of plant oils as well as fats and/or animal body fats in any combination

(2) Able to quantify the foreign fat or blend of foreign fat with fair degree of accuracy

(3) Better sensitivity: limit of detecting the foreign fat in milk fat ranges from

(a) 2.0 to 5.4%, when specific S-value (i.e. S_1, S_2, S_3 or S_4) employed
(b) 4.0 to 6.1%, when general S-value (i.e. S_{Total}) employed

(4) High reliability: limit of confidence in detection 99% and in quantification 95%

(5) Non-destructive in nature, hence information obtained is much higher

(6) Simple and rapid compared to conventional destructive methods

(7) Except solvent (for dissolving fat), no other chemical is required

(8) Applicable to some milk products e.g. cream, butter, ghee and milk powder

(9) Recognised and accepted internationally as a reference method

Pathania et al. (2021) clarified samples of butter from cow milk as well as buffalo milk at 110, 130 and 150°C and found that there was no significant difference in the content of different triglycerides in ghee on account of temperature of clarification both in case of cow ghee and buffalo ghee. They also stated that their finding can be extrapolated to the fact that temperature of clarification used to prepare ghee from butter will not affect S-limits of milk fat. Thus, it is very fortunate that the method may be applied to test purity of ghee irrespective of temperature employed in the process of clarification.

It has been opined that as on date GC analysis of triglycerides as suggested by EC (2008) and ISO/IDF (2010) is highly effective, most reliable and irreplaceable analytical tool to verify authenticity of fat present in milk as well as in some of the milk products (Bosque-Sendra et al. 2012, Cossignani 2019, Csapó et al. 2019).

Limitations of the method

Though the method is very versatile, its applicability is restricted in specific circumstances due to possibilities for occurrence of false positive results. The circumstances which give rise to false positive results are compiled from different reports (Precht 1992a & 1992b, Battelli & Pellegrino 1994, Precht et al.1998, Kamm et al. 2001, Molkentin 2006, 2007 & 2013, EC 2008, Amaral et al. 2018, ISO/IDF 2019, Pathania et al. 2020, Sharma et al. 2021) and listed here.

(1) Since false positive result may occur, one or more limits of S-values specified in the method may not be applicable to fat obtained from:

(a) Milk of animal species other than cows (*e.g.*, buffalo, goat, sheep, etc.)

(b) Milk of single cow

(c) Milk of cows receiving oilseeds in diet (*e.g.*, cottonseeds)

(d) Milk of cows suffering from acute underfeeding (*i.e*., starvation or malnutrition)

(e) Post-parturient milk (*i.e*. colostrum)

(f) Milk subjected to treatment for removal of cholesterol

(g) Fractionation for alteration in specific properties (*e.g*., softening, melting, etc.)

(h) Skim milk, buttermilk or whey and

(i) Products undergone extensive lipolysis (fat acidity >8 mmol/100 g of fat)

(2) Since phospholipids and partial glycerides overlaps with triglycerides containing short chain fatty acids, hence their presence in large amounts may interfere with the results. Therefore, procedure used for extraction of fat from the test sample is another restriction in applicability of the method.

It is recommended that the fat for testing its purity to be obtained from butter by melting it at 50°C. Therefore, hardly any phospholipids remain present in the test samples of fat. If other extraction methods are used, sufficient care has to be exercised so that the amounts of phospholipids and partial glycerides remain to a minimum (Precht et al.1998, Molkentin 2006 & 2008).

Among the above listed circumstances responsible for false positive results, feeding of lactating milch animals and species of the milch animals are most relevant to some of the Asian countries in general and India in particular. Hence their role in causing the false positive results is covered in depth under separate sub-title 'major causes of false positive results in method'.

Evaluation of the Method

Kala (2013) undertook a study to test the applicability of the method in detection of selected foreign fats in ghee. Adulterated samples of ghee were prepared from control ghee made in the laboratory from mixed milk. The adulterated samples were prepared by mixing beef tallow (@ 2.0 & 6.56%), partially hydrogenated vegetable oil (@ 2.0 & 5.0%), lard (@ 2.0 & 6.27%) and coconut oil (@ 2.0%) in control samples of ghee. The data reported on S-values of adulterated ghee samples suggest that not only the samples containing foreign fats at higher rates, but those containing 2.0% of foreign fat also failed to comply two or more limits of S-values. It proves capability of the method to detect adulteration at the rate as low as 2.0%.

Kala et al (2016) evaluated purity of fat in ghee based sweets using the method, taking *Mysore pak* as one of example of the sweets. Eight market sample of the ghee based sweets were analyzed and compared with laboratory prepared control samples of the sweet. The control samples of sweet were prepared from ghee procured from dairy and also from ghee prepared in laboratory. Fat was extracted from the samples of sweet gravimetrically after removing added spices and nuts. The extracted fat was analysed by the method to get value of its S_{Total}. Examination of the data reported on value S_{Total} suggest that the fat extracted from control samples of sweets complied the limits specified for S_{Total}. Whereas, in case of fat extracted from eight market samples of sweet, seven samples failed in complying the limits of S_{Total}. These results suggest that the method can also be employed in testing purity of ghee used in preparation of tradition Indian sweets like *Mysore pak*.

Shinde et al. (2020) evaluated the method to detect adulteration in ghee. Samples of pure ghee were adulterated with coconut, soya bean, groundnut and sunflower oil @ 1, 2.5, 5, and 10%. The authors found that the method was capable to detect adulteration @ 5% for all the oils, except groundnut which was detected @ 10%. The probable reason for high limit of detection in case of ground oil may be attributed to selection of S-value employed for its detection. In the detection ground oil the authors have employed S_{total}. According to report from Pocklington and Hautfenne (1985), the triglyceride profile of groundnut oil closely resembles to that of the soya bean oil. Therefore, looking to the similarity in triglyceride profile of groundnut oil to that of the soya bean oil, S-value corresponding to soya bean oil might be better option to detection of groundnut oil rather than S_{total}.

Pathania et al. (2020) conducted a study on use of the method to detect highly manipulated fat (*i.e.* designer fat) mixed in ghee as an adulterant. Using limits of S-values specified by ISO/IDF (2010) for cow milk fat. The minimum level for detection of the highly manipulated fat was 7.5%. The authors stated due to lack of the limits specified for S-values for buffalo milk fat, detection of the adulterant fat in buffalo ghee was not possible. Their finding has proved the capability of the method to detect highly complex admixture of foreign fats in cow milk fat with a fairly high degree of sensitivity. Therefore, this report serves as a testimony on robustness of the method.

Nilchian et al. (2020) opined that the detection of animal body fats such as tallow in butter by conventional parameters of purity is more difficult compared to vegetable fats. They mixed tallow in butter @ 0 to 15% and analysed the samples for fatty acids, triacylglycerols and conventional physicochemical parameters. The found that physicochemical parameters and fatty acids could not indicate the adulteration up to 5% level. However, S_1-, S_3-, S_{Total} could detect the adulteration at 5% level. The use of fatty acids and triacylglycerols in combination resulted in the capability to detect the adulteration above 1%.

Secret behind the success of the method

The unmatched advantages of the method may be attributed to way in which (1) idea conceived to use information laying in intact triglycerides, (2) designed of S-values decided, (3) procedure devised to get the S-values, (4) specifications on limits of S-values fixed and (5) natural variations in triglyceride profile of milk fat taken care of. These virtues of the method are compiled from various reports (Precht 992a, Povolo et al. 1999, Molkentin & Precht 2000, Kamm et al. 2001, de la Fuente & Juarez 2005, Fontecha et al. 2006, Molkentin 2007, EC 2008, Amaral et al. 2018; Csapó et al. 2019, Cossignani 2019 and ISO/IDF 2010, 2019) and briefly summarised below.

(1) The idea conceived for utilising inherent information present in intact triglycerides of milk fat is so meticulous that it gives highly robust criterions for testing purity of milk fat. The equations of S-values are designed so carefully that even on mixing different foreign fats with milk fat in any combination, the S-values of adulterated milk fat vary only in one direction. Hence, manipulation of S-values becomes extremely difficult, which makes method highly robust against manipulation.

(2) The designs of five S-values are decided so tactfully that the method capable to detect entire range of foreign fats both from plants and animals origin as well as their blend with high degree of confidence. Even complex admixture of foreign fats can be detected with fair degree of sensitivity. As a result the method can serve as highly comprehensive tool, since it single handedly detects wide variety of foreign fats in milk fat.

(3) Analytical procedure devised to get S-values of test sample is so simple and rapid that all the five S-values can be obtained just from a single run of the GC machine within 30 minutes. The sample of fat extracted from milk or milk product to be just dissolved in solvent and injected in GC machine. Thus, the method is non-destructive, simple, convenient and rapid.

(4) Specifications on limits of all the S-values are fixed in form of a range. Consequently, for each S-value both minimum value and maximum value to be complied, rather than only minimum value or maximum value. The five S-values, each with two limits (lower and upper) leads to formation of ten (5x2) criterions within the single method for testing purity of milk fat.

(5) Effect of natural variations in triglyceride profile of milk fat on efficiency of the method is almost eliminated due to appropriate care taken in designs of the equations employed in calculation of the S-values. It appears that the designs of equations automatically compensating the natural variations occurring in triglyceride profile of milk fat due to normal variations in feed given to the lactating cows, season of the year, breed of the cows and stage of their lactation as well as regional variations. This became possible due to use of very sound database generated for development of the equations. While gathering the database over a period of five years effect of all these factors was very well taken into conidiation.

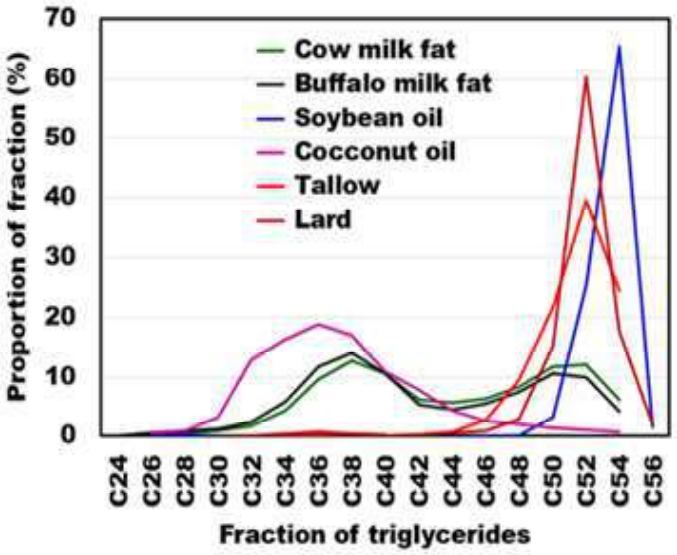

Fig. 1 Pattern of triglyceride profile in milk fat and foreign fats

Data on triglycerides of cow milk fat and buffalo milk fat are compiled from Kala (2013), Hazra et al. (2017), Sharma et al. (2018) and Pathania et al. (2021). Whereas, data on triglycerides of foreign fats are compiled from Parodi (1973), Pocklington & Hautfenne (1985), Precht (1992a), Gutiérrez et al. (2009), Kala (2013) and Park et al. (2014).

Common reasons of false positive results in the method

The S-values of milk fat are calculated from percentage of different triglyceride fractions present in it. In oils and fats fatty acids being integral parts of triglycerides, chain length of the constituent fatty acids determines the number of carbon atoms in their triglyceride molecules. Therefore, in milk fat percentage of short chain (C4 to C14), medium chain (C16:0 & C16:1) and long chain (C18:0, C18:1 and C18:2) fatty acids dictates percentage of its triglyceride fractions and hereby in its S-value. Consequently, wide variation in content of these fatty acids in milk fat leads wide variation in its S-value and there by causes false positive results in the method. Hence, to get idea about causes of the false positive results, it is necessary to understand factors responsible for variation in fatty acids composition of milk fat during its synthesis in mammary gland. To simplify explanation of this complex biological phenomenon, sources for supply of different fatty acids in biosynthesis of milk fat is schematically presented in Figure 2.

For biosynthesis of milk fat in ruminants there are two basic sources of fatty acids: (1) *de novo* synthesis in mammary gland

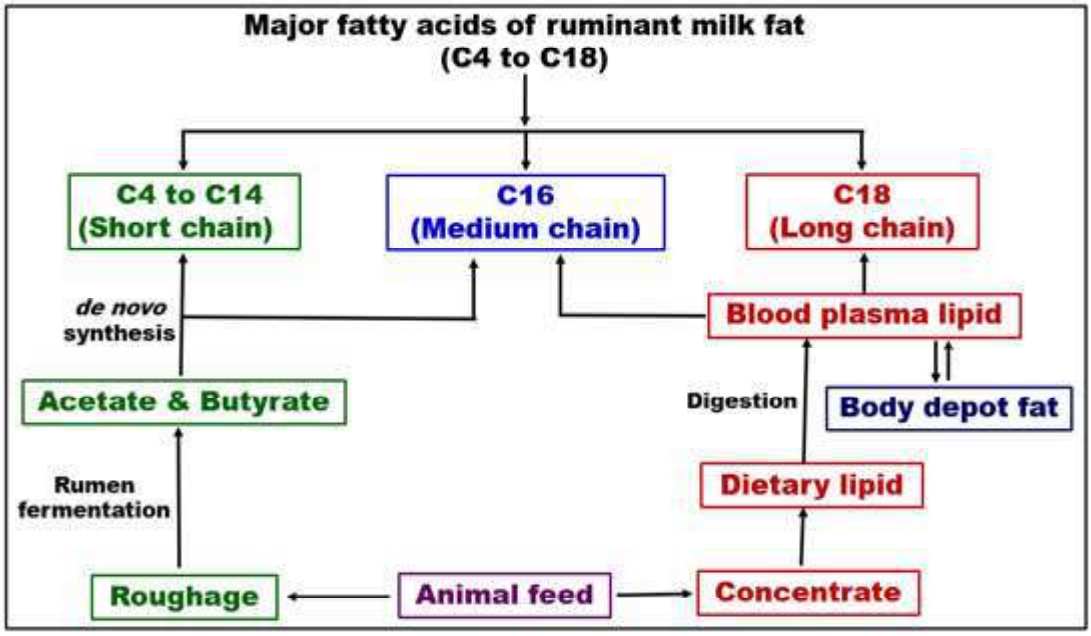

Fig. 2 Sources of different fatty acids for biosynthesis of milk fat in ruminants

Conceptualised from description given by Hawke & Taylor (1983), Grummer (1991), Bear (1991), Palmquis (2006) and Fox et al. (2015)

from acetate and butyrate supplied by rumen fermentation of roughage and (2) uptake from circulating blood plasma lipids which arises from dietary lipid or depot body fat. There is a vast difference in chain length of fatty acids obtained from these two sources. Short chain fatty acids are derived exclusively from the *de novo* synthesis. Whereas, preformed long chain fatty acids are derived exclusively by uptake from circulating blood plasma lipids. However, medium chain fatty acids are derived partly from the *de novo* synthesis and partly from the circulating blood plasma lipids. Proportion of fatty acids from these two sources in biosynthesis of milk fat is greatly influenced by type of feed given to lactating milch animals in their diet and also by species of the milch animals (Hawke & Taylor 1983, Grummer 1991, Bear 1991, Palmquis, 2006). Therefore, variation in type of feed given to lactating milch animals and change in species of the milch animals are generally responsible for occurrence of false positive results in the method.

Type of feed given to lactating ruminants

Table 3: Effect of cottonseed feeding on fatty acid profile of cow milk fat

Sr. No.	Major fatty acids of milk fat	Fatty acids (%)		Difference (CSD – NSD)
		CSD	NSD	
1	Short chain (C4 to C14)	22.76	25.99	–3.23
2	Medium chain (C16:0)	26.81	29.33	–2.52
3	Long chain (C18:0, C18:1, C18:2 & C18:3)	41.62	34.38	+7.24
4	Others (?)	7.93	9.20	–1.27

CSD = Milk fat from cotton seed supplemented diet
NSD = Milk fat from non-supplemented diet (*i.e.* normal or control diet)
Computed from Contarini et al. (1996)

Table 4: Effect of cottonseed feeding on triglyceride profile of cow milk fat

Sr. No.	Major triglyceride fractions of milk fat	Triglyceride fraction (%)		Difference (CSD – NSD)
		CSD	NSD	
1	C28 to C48 (except C40)	58.41	65.37	–6.96
2	C40 + (C50 to C54)	41.56	34.63	+6.93

CSD = Milk fat from cotton seeds supplemented diet
NSD = Milk fat from non-supplemented diet (*i.e.* normal or control diet)
Computed from Contarini et al. (1996)

Most of the plants oils and all animals body fats comprising of long chain fatty acids, followed by medium chain fatty acids and no or negligible short chain fatty acids. Therefore, supplementing any of these oils or fats in feed of lactating ruminant increases the level of long chain fatty acids in blood plasma and their uptake by lactating mammary gland. Elevated level of long chain fatty acids in mammary gland inhibits the *de novo* synthesis of short chain and medium chain fatty acids. As a consequence content of short chain and medium chain fatty acids in milk fat decreases, with simultaneous increase in its long chain fatty acids content (Hawke & Taylor 1983, DePeters et.al. 2001, Onetti et al. 2002, Palmquist 2006). Similar phenomenon also occurs during starvation or acute under feeding due to mobilisation of depot body fat from adipose tissues into blood plasma for compensating shortage of short fatty acids in biosynthesis of milk fat (Precht1991). Such changes in fatty acids composition of milk fat leads to corresponding changes in triglyceride profile of the milk fat.

Effect of supplementing whole cottonseeds in diet of lactating cows on fatty acid composition of their milk fat and associated changes in its triglyceride profile can be realised from excellent work reported by Contarini et al. (1996). Differences in short chain, medium chain and long chain fatty acids contents of milk fat from cows received cottonseed supplemented diet and that from cows received non-supplemented (normal) diet are computed from this repot and presented in Table 3. In the same way differences in contents of different triglycerides fractions also computed and presented in Table 4. The changes in fatty acid profile suggest that on supplementing the cottonseed in diet of lactating cows, contents of short chain and medium chain fatty acids decrease substantially with concomitant increase in long chain fatty acids in their milk fat. Similarly, the changes in triglyceride profile indicate that on supplementing the cottonseed in diet of lactating cows, contents of triglycerides containing lower number of carbon atoms (C28-C48, except C40) decrease considerably with simultaneous increase in triglycerides containing higher number of carbon atoms (C50-C54, together with C40) in their milk fat. The authors concluded that the ratios of different fatty acids cannot be used as indices of purity for butter made from milk from cows fed whole cottonseeds. Therefore, same logic obviously to be hold true in use of triglycerides ratios and S-values in testing purity of milk fat. Because changes in S-values of milk fat is but natural on supplementing the cottonseed other oilseed in diet lactation ruminant.

Contarini et al. (2014) also evaluated efficacy of the EU reference method in testing purity of milk fat from cows kept on mountain feeding. All the samples showed characteristic fatty acid composition of milk fat from mountain pasture-fed cows: high content of linolenic acid, vaccenic acid, *cis*-9, *trans*-11 conjugated linoleic acid and low concentration of saturated fatty acids. All the samples produced false positive results for at least one of the five S-values. The authors suggested that in order to avoid false charges of adulteration, this behaviour should be considered by competent authorities while applying the method to test purity of milk fat.

Alonso et al. (2022) studied fatty acids and triglycerides profile of milk fat from cow receiving ecological v/s conventional pasture in northern of Spain. The author found that among different fatty acids, medium chain fatty acid (C16:0) was significantly lower in milk fat from cow fed ecological pasture, compared to conventional pasture. On the other hand, the long chain fatty acid (C18:1, C18.2, C18.3 & C18:2 c9*t*11) were significantly higher in milk fat from cow fed ecological pasture, compared to conventional pasture. Concomitant significant changes occurred in triglyceride profile of milk fat. Triglycerides containing C40 was significantly lower and those containing C44, C46, C48, C50, C52 and C54 were significant higher in milk fat from cow fed ecological pasture, compared to conventional pasture.

The above reports clearly indicate that following special feeding practices in feeding of ruminants such as feeding oilseeds or their by-products, mountain grasing or feeding ecological pasture result into drastic change in triglyceride profile of their milk fat thereby in its S-value(s), which ultimately leads to false positive results. Thus, the false positive results on testing pure fat from milk lactating ruminants receiving cottonseed or other oilseeds or their by-products like meal and cake in diet is but obvious. This limitation of the method is very clearly reported by ISO/IDF (2010, 2019).

Species of lactating ruminants other than cow

Table 5. Comparative appraisal on triglyceride profile of buffalo and cow milk fat

Triglyceride fraction	Triglycerides fraction (%)[#]		Difference (B – C)
	Buffalo milk fat (B)	Cow milk fat (C)	
C24	0.11	0.10	+0.01
C26	0.55	0.34	+0.21
C28	0.82	0.53	+0.30
C30	1.12	0.90	+0.22
C32	2.40	1.77	+0.63
C34	5.81	4.41	+1.39
C36	11.77	9.55	+2.22
C38	14.02	12.76	+1.26
C40	10.55	10.53	+0.03
C42	5.27	6.20	–0.94
C44	4.51	5.64	–1.13
C46	5.44	6.36	–0.92
C48	7.49	8.29	–0.81
C50	10.64	11.70	–1.06
C52	9.90	12.04	–2.14
C54	4.12	6.07	–1.95

[#] Calculated from Kala (2013), Hazra et al. (2017), Sharma et al. (2018) & Pathania et al. (2021)

In biosynthesis of milk fat proportion of fatty acids used from *de novo* synthesis and that from blood plasma lipids also depends on species of the lactating ruminants (Hawke & Taylor 1983). As a result triglyceride composition of fat from milk of different species will vary and lead to variation in S-values of their milk fat. Differences in triglyceride composition of milk fat from different species and in their S-values are briefly summarised below.

Differences in triglyceride profile of milk fat in ruminants species

Triglyceride composition of fat from milk of various mammalian species (cow, buffalo, sheep, goat, donkey, camel, and/or horse) are reported by Achaya and Hilditch (1950), Breckenridge and Kuksis (1967), Addeo and Kuzdzal-Savoie (1980), Fontecha et al. (1998), Povolo et al. (2008), Goudjil et al. (2003), Smiddy et al. (2012), Tolentino et al. (2015), Bononi et al. (2017) Cossignani (2019) and/or Peyma et al. (2022). This literature suggests wide variation in triglyceride composition of milk fat obtained from different mammalian species.

Triglyceride composition of fat from Indian buffalo milk and that from cow milk has been reported by Kala (2013), Hazra et al. (2017), Sharma et al. (2018) and Pathania et al. (2021). An average triglyceride composition of cow milk fat and that of buffalo milk fat is computed from those reported in publications by these authors. Differences in triglyceride composition of cow milk fat and buffalo milk fat are worked out from their respective average composition derived from the computation and presented in Table 5. The noticeable difference in triglyceride profile of fat of cow milk and that of the buffalo milk is clearly evident. The differences triglyceride profile confirm that contents of triglycerides possessing C24 to C40 is higher buffalo milk fat, whereas, contents of triglycerides possessing C42 to C54 is higher cow milk fat. Therefore, occurrence of differences in S-values of their milk fat is but obvious.

De la Fuente and Juarez (2005) also opined that due to the differences in triglyceride profiles of goat and ewe milk fat compared that of the cow milk fat, the equations proposed by EU regulations are not suitable to monitor purity of goat or ewe milk fat. Therefore, to detect foreign fat in goat and ewe milk fat new multiple regression equations based on their triglyceride profile were developed. Fontecha et al.(1998) proposed two multiple regression equations for detecting mixtures of non-milk fats in goat milk Similar work was carried for ewe milk fat by Goudjil et al. (2003).

Differences in S-values of milk fat from different ruminants species

Romano et al. (2004) carried out work to determine suitability of the official EU method to buffalo milk fat. Their results show that it is necessary to modify the limits of S-values specified in Precht method to determine the purity of buffalo milk fat. Povolo et al. (2008) also carried out investigation on sheep, goat and buffalo milk fat and confirmed that the S ranges, calculated for cow milk fat, are not applicable to the evaluation of genuineness of non-

Table 6: S-values of ghee prepared out of milk collected from farm of the institute

S-value		S-value of ghee from		
		Cow milk[#]	Mixed milk[#]	Buffalo milk[#]
S_1	Vegetable oils[*] & fish oil	100.96	101.47	101.97
S_2	Coconut & Palm kernel fat	99.62	99.10	98.58
S_3	Beef tallow & Palm oil	104.08	107.69	111.29
S_4	Lard	99.43	98.68	97.92
S_{Total}	Total	104.11	107.58	111.05

[*] Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ & Cottonseed
[#] Compiled from Pathania et al. (2020)
[#] Calculated from values of cow & buffalo by average of their respective values

Table 7: S-values of ghee prepared out of milk collected from different regions of India

S-value		S-value of ghee from			
		Cow milk		Buffalo milk	
		Four regions*	Western region	Four regions*	Western region
S_1	Vegetable oils[#] & fish oil	92.82-103.08	97.17-101.77	96.12-103.27	97.20-102.70
S_2	Coconut & Palm kernel fat	99.15-101.41	99.15-100.19	97.75-101.35	98.60-00.78
S_3	Beef tallow & Palm oil	89.53-107.25	97.32-106.48	95.60-117.37	99.58-14.32
S_4	Lard	96.52-105.88	98.16-101.84	94.47-104.81	96.75-101.13
S_{Total}	Total	90.59-106.34	97.27-105.93	94.06-118.21	96.83-112.37

[#] Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ & Cottonseed
*Four regions = Eastern, Western, Northern and Southern
Compiled from Sharma et al. (2021)

bovine milk fats. In analysis of buffalo milk fat these authors found the values of S_1, S_2, S_3, S_4 and S_{Total} as 100.18 (±0.36), 98.88 (±0.13), 107.39 (±0.24), 99.56 (±0.36) and107.46 (±0.55) respectively. Their results indicate that buffalo milk fat failed to comply lower limit of S_2 and upper limit of S_3 as well as S_{Total}.

Pathania et al. (2020) reported work on novel approach to detect highly manipulated fat adulterant in ghee (clarified butter) through signature peaks by GC analysis of its triglycerides. The S-values of pure ghee from cow and buffalo milk are compiled from the results reported by these authors and presented in Table 6. The data suggest that all the five S-values of cow ghee were very well within the limits specified as standards by ISO/IDF (2010). However, in case of pure buffalo ghee, all the five S-values fall out sides the range specified in the standards. Thus, all the five S-values of ghee from buffalo milk failed to comply the specified limits. The authors opined that the limits specified for S-values of cow milk fat are not suitable for the detection of adulterants in ghee made from buffalo milk.

Sharma et al. (2021) conducted a comparative study on the S-values of ghee from cow milk and buffalo milk. The samples of milk were collected from eastern, western, southern, and northern regions of India. The authors reported that in all the four regions all the five S-values of buffalo ghee as well as cow ghee deviated widely from the limits specified by ISO/IDF (2010, 2019). Variation in S-values of cow ghee and buffalo ghee in western region and overall in pooled data of all the four regions are compiled and presented in Table 7. The variations S-value are very large in the pooled data. The rate of compliance by the results to the specification of S-values is most important aspect in this type of study. Such information is required to get correct idea about prospects of the method for its implementation in India and about modifications required in particular limits of specific S-values. However, the rate of compliance by the results not reported by these authors.

Prajapati and Aparnathi (2021) conducted a project work for evaluation of database on S-values of milk fat collected by GCMMF, Anand (Gujarat). The database was collected by analysing 21552 samples of bulk mixed milk over a period of two years. The samples were generally drawn from road tankers containing 10000 to 200000 liters of mixed milk. The main aim in evaluation of the database was to find rate of success in compliance by fat in the milk samples to limits specified by ISO**:** 17678 in the method. The rates of compliance found in samples of low fat milk (≤ 4.0%), medium fat milk (4.01 to 5.99%) and high fat milk (≥6%) are presented in Table 8. The data revealed that the best rates of compliance (>96-100%) are found in samples of low fat milk, which may be attributed to high proportion of its cow milk content. In samples of medium fat milk and high fat milk the rates of compliance for S_1, S_2 and S_4 remained almost ≥ 95%. The rate of compliance for S_{Total} in samples of medium fat milk and high fat milk remained around 93%. In samples of medium fat milk and high fat milk only S_3 has the lowest rate of compliance, which remains in vicinity of 80%. Thus, except S_3 rates of compliance in other four S-values are very promising for their use in application of the method in testing purity of fat in bulk mixed milk in Gujarat.

The rates of compliance observed by Prajapati and Aparnathi (2021), in samples of low fat milk while evaluating the large database, are completely in agreement with S-values of cow ghee and buffalo ghee as reported by Pathania et al. (2020) who obtained the milk samples from institute farm and Sharma et al. (2021) who obtained the obtained the milk samples from western region of India. Similarly, the rates of compliance in medium fat milk and high fat milk are corroborating very well with those reported by Povolo et al. (2008) for S-values of buffalo milk fat.

One of the typical reasons for wide variation in S-values of milk fat reported within the same study or reported between different studies might be attributed to differences in bulk of milk at the time of sampling. The method is basically meant for testing purity of bulk milk, since Prech developed this method using the

Table 8: Compliance rate of milk fat to its specifications on S-values in large database collected by industry

S-value		Samples of milk fat under each category (%)								
		Low fat milk			Medium fat milk			High fat milk		
		BLL	WBLs	AUL	BLL	WBLs	AUL	BLL	WBLs	AUL
S_1	Vegetable oils[#] & Fish oil	0.56	99.44	0	1.10	98.89	0.01	5.02	94.97	0.01
S_2	Coconut & Palm kernel	1.12	98.88	0	4.03	95.78	0.19	3.35	96.19	0.28
S_3	Palm oil & Beef tallow	0	96.07	3.93	0.04	77.80	22.16	0	80.16	19.84
S_4	Lard	0	100	0	0.05	99.93	0.02	0.15	99.72	0.13
S_{Total}	Total	0	98.31	1.69	0.14	92.60	7.26	0.19	93.29	6.52
Total number of samples (21552)			178			11772			9602	

BLL = below lower limit (Fail), WBLs = within both limits (Pass) and AUL = Above upper limit (Fail)

[#]Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ and Cottonseed

Compiled from Aparnathi and Prajapati (2021)

database obtained wherein samples were drawn from bulk milk (Prech 1991, 1992a). Therefore, samples of milk obtained from single cow may leads to false positive results (ISO/IDF 2010, 2019). Similar phenomenon of false positive result may likely to occur when sample is drawn from a very limited bulk of milk. Sharma et al. (2021) also expressed the similar views for variations in S-values of milk fat. The authors stated that it is likely that cow ghee produced commercially by dairy plants meets the limits of S-value specified in the method. In dairy industry ghee is produced from pooled milk collected from large area in chain of milk collection, this nullifies the variations arising from milk of individual cows or a particular route in milk collection.

The forgoing resume of various reports clearly reveals that differences in triglycerides composition of fat from cow milk and that from buffalo milk do exists. These inherent differences in triglycerides composition of their milk fat may create differences in their S-values. Therefore, when specification on S-values meant for cow milk fat are applied to buffalo milk fat may causes false positive results. This limitation of the specification of the method is very clearly and categorically admitted in the method (ISO/IDF 2010, 2019).

From careful and keen examination of the data on (1) S-values of fat in buffalo milk reported by Povolo et al. (2008), (2) S-values of ghee prepared from cow milk and that from buffalo milk reported by Pathania et al. (2020), (3) S-values of ghee from mixed milk computed by averaging respective vales of buffalo ghee and cow ghee reported by Pathania et al. (2020), (4) S-values of ghee prepared from cow milk and that from buffalo milk in western region of India reported by Sharma et al. (2021) and (5) rate in complying S-values of milk fat to limits specified in the method as observed by Prajapati & Aparnathi (2021), the following inferences may be drawn regarding applicability of the limits on various S-values specified in the method for testing purity of fat.

- Major changes in upper limits of S_3 (palm & beef tallow) and S_{Total} (General) are required for application of the method.
- Minor changes in lower limits of S_1 and S_2 (Soy bean oil & other oils) and S_2 (coconut fat & palm kernel fat) are required for application of the method.

After establishing the above referred changes in limits of S-values method may be used very well for testing purity of fat in bulk milk prevailing in Gujarat. However, the problem of false positive results on feeding cottonseed to lactating cows and buffalo in application of limits on S-values specified in the method remains unresolved and to be tackled separately.

Mixing milk of other ruminants species with cow milk

It is also reported that even mixing of milk from other species to cow milk may also lead to a false positive result (Molkentin 2007). Unfortunately, data on S-values of pure fat from mixed milk or pure ghee prepared from mixed milk are not reported in literature. Hence, direct comparison between S-values of fat from cow milk and that from mixed milk is not possible. As an alternate, S-values of ghee from mixed milk are computed from S-values of ghee prepared from buffalo milk and that from cow ghee as reported by Pathania et al. (2020). S-values of ghee from mixed milk are derived by averaging respective S-values of cow ghee and buffalo ghee, assuming their proportion as 1:1 in mixed milk (Table 6). The computed S-values of ghee from mixed milk suggest that among the five values, it failed to comply the specifications on lower limit of S_2 and upper limits of S_3 and S_{total}. These observations are in complete agreement with the opinion expressed by Molkentin (2007).

Perspectives on use of the method in India

In view of practices presently followed in Indian dairy farming system, there are three factual problems of serious concerned in implementing specifications on limits of S-values suggested in the method. These problems include (1) role of buffalo as a major contributor in Indian milk production, (2) practice of mixing of buffalo milk with cow milk and (3) feeding cottonseeds to lactating animals. Each of the three situations may give rise to possibility of false positive results. Therefore, question arises that how to make use of this versatile method in India?

Role of buffalo as a major contributor in Indian milk production

It has been already discussed that substantial difference triglycerides profile of buffalo milk fat and that of the cow milk fat, which causes concomitant differences in S-values of their milk fat and ultimately leads to false positive results. In present system of Indian dairy farming, buffalo is a major milk producing animal. Contribution wise buffalo ranked second in the world milk production with an annual growth rate of 3.5%, compared to 2.1% in the cow milk production. Buffalo has contributed >13% in world, >35% in Asian and >50% in Indian milk production (Balhara et al. 2017). Therefore, possibility of false positive results in the samples of genuine buffalo milk fat is the foremost concern in implementing specification on S-values of the method in some Asin countries in general and in India in particular. Therefore, this fact needs consideration in implementing the method in India for testing purity buffalo milk fat.

Mixing of buffalo milk with cow milk

As mentioned in earlier section, not only buffalo milk, but its mixing with cow milk may also leads problem of false positive results. In present system followed by organized dairy sector for milk collection and its transportation, mixing of buffalo milk with cow milk has become very usual practice. At village milk collection centres cow milk and buffalo milk are generally pooled after collection and stored in bulk milk chilling (BMC) unit. The milk from BMC units of nearby villages is pooled in a road tanker for

transporting it to dairy plant. Therefore, under present system used in collection and transportation, milk received by the organized Indian dairy sector is mostly in form mixed milk, containing buffalo milk and cow milk in different proportion, depending on population of the two species existing in different areas of milk collection. Consequently, possibilities of false positive result in fat from genuine mixed milk is also a matter of serious concerned in India and requires due consideration in implementing the method.

Feeding cottonseeds to lactating cows and buffaloes

It has been established that the feeding cottonseed to lactating cows completely change the of fatty acid profile of their milk fat, thereby concomitant changes in its triglyceride profile of their milk fat. Hence, limits of S-values specified for pure milk in the method may not be applied to milk fat obtained from cows whose diet contain high amount of cottonseed, since it may lead to false positive results. In many parts of India, feeding cottonseeds and/or their by-products such as meal and cake to lactating cows and buffaloes is very common practice. Therefore, the practice of feeding cottonseeds or their by-products is also a serious concern in implementing the specifications of the method on S-values. Even issue of this feeding practise will create a serious problem while forming Indian specifications on limits of S-values. The effect of feeding cottonseed requires consideration in implementing the method in India for testing purity milk fat, obtained from cows and buffalo receiving cottonseed or their byproducts in diet.

It appears from the literature survey that effect of feeding cottonseeds to lactating ruminants on S-values of their milk fat been not reported so far. However, its effect on fatty acid composition of milk fat and consequent changes in physicochemical parameters of the milk fat are given due considerations by AGMARK as well as FSSAI while formation of specification on parameters used in purity of Ghee. This fact is evident from the specification formed on relevant parameters of ghee by AGMARK (Table 9) and that by FSSAI (Table 10). Now FSSAI specifications of ghee on RM value and BR reading are made uniform for the entire country (FSSAI, 2021), considering variations in these parameters in different parts. Somewhat similar considerations are also required while forming Indian specifications on S-values of milk fat for adopting the method in India as an official reference.

Use of the method under present Indian situation

Formation of Indian specifications on S-values of pure milk fat and their adoption as legal standard is very long process and may take several years. Therefore, under present Indian situation an immediate solution for use of the method could be its adoption as a screening tool, by using the specification partially and/or modified as per the local requirement. For modification in the specification of S-value, its limits may be changed as per requirements to suite the pure milk received from the supply. The changes required in limits of S-values may be established by testing genuine samples of milk received from concerned area of milk collection by dairy organisation.

In the use of specifications in partial or modified from it is advisable that doubtful samples found in analysis to be counterchecked through testing it by suitable complimentary analytical technique(s) such as fatty acid profile, β-sitosterol test or any other technique as deemed suitable. Such counter verification of positive samples by suitable complimentary analytical technique(s) may also be adopted to resolve disputes about positive samples. ISO/IDF has also advocates such counter verification of doubtful cases by using suitable complimentary method(s) of analysis (ISO/IDF 2010, 2019).

In cases, where the method is adopted just as a screening or specifications on S-values are not implemented in total, it is very essential to continuously update the database, perform its

Table 9: Agmark specifications on BR reading, RM value & Polenske value of ghee

Parameter	Areas other than cotton tract	Cotton tract area	
		Winter	Summer
BR reading at 40°C	40.0 to 43.0	41.5 to 44.0	42.5 to 45.0
RM value (minimum)	28	23	21
Polenske value	1 to 2	0.5 to 1.2	0.5 to 1.0

Compiled from Agmark (1991)

Table 10: FSSAI specifications on BR reading and RM value of ghee

Parameter	FSSAI (2017)		FSSAI (2021)
	Area other than cotton tract[#]	Cotton tract area	
BR reading at 40°C	40.0-43.0, 40.0-43.5, 40.0-44.0 or 41.0-44.0	41.5 to 45.0	41.0 to 44.0
RM value (min)	28, 26 or 24	21	24

[#] In areas other than cotton tract BR reading & RM value vary from area to area

Compiled from FSSAI, (2017, 2021)

statistical analysis and have a perusal of results. This exercise at regular interval of every one year may provide very important clues for improving use of the method. The changes in S-values over the years may also give indications about possibles weaknesses existing in adopting only partial specifications on limits of S-values. If any weakness is noticed, appropriate measures to be taken to prevent the misuse of the weakness.

Work required to adopt the method as official reference in India

The limits of S-values specified in the method were exclusively developed on the basis of variations found in triglyceride composition of fat from cow milk, that too only from European countries (Molkentin 2007, Povolo et al. 2008, Cottenet et al. 2011). Even in international collaborative study on validation of the method, carried out by Molkentin and Crawford (2009), samples of milk fat from Asian countries were not included. Thus, limits of S-values specified in the method are meant for testing purity of cow milk fat only. Hence, before official adoption the method in India it is essential to (1) ascertain limits of S-values for pure fat from milk of Indian cows and buffaloes as well as from their admixture (*i.e.* mixed milk) thoroughly in entire country and (2) make suitable amendments in the specification on limits of S-values in accordance with the limits of S-values ascertained for the pure fat of Indian cows and buffaloes as well as mixed milk.

In India only limited work is carried out so far to check applicability of the specifications on limits of S-values suggested by ISO/IDF (2010). Hence, very extensive survey is required to ascertain S-values of pure milk fat in India and to form their specification. The survey should cover the following aspects in sufficient length, breadth and depth.

- Different agroclimatic regions of all states and union territories
- Milk from cow and buffalo as well as their mixture in different proportions
- Practices followed in feeding lactating cows and buffaloes, including cottonseed feeding
- Seasonal variations occurring round the year
- Sufficient number of repetitions (replications): data from minimum three years

After collection, compilation and classification of data the specification to be formed by applying appropriate statistical tools. In case of any technical problem faced in formation of specifications on limits of one or more S-values, work may be undertaken for entire revamping of equation(s) of those S-value(s) using the same database generated from the survey. As discussed earlier such exercise on revamping of the equations was carried out for goat milk fat by Fontecha et al. (1998) and for ewe milk by Goudjil et al. (2003). Somewhat similar exercise may also be required for some of the S-values of buffalo milk fat.

The work reported by Prajapati and Aparnathi (2021) for evaluation of large database on S-values of milk fat collected from industry may serve as a useful guide for systematic planning of the work on survey and subsequently for proper classification as well as processing of the data collected from the survey.

The database generated from the exercise on limits of S-values of Indian milk fat should be made available on public domain by publishing it suitably in open access mode. The database to be also submitted to concerned international organisations to consider Indian milk fat as pure on testing its purity by the method and values found in accordance with the Indian standards. It will help to recognised Indian milk fat as pure, in spite of its deviation from the specification suggested by ISO/IDF (2019).

Conclusions

The GC analysis of triglycerides is simple, rapid, robust and internationally approved reference method for testing purity of milk fat. However, specifications suggested in the method for pure milk fat are formed from European cow milk fat only and may cause false positive results in pure fat from buffalo milk and also that from mixed milk. Therefore, it is necessary to establish limits of S-values for pure fat from different types of Indian milk and amend the specifications on S-values accordingly. If technical hitch arises in amending the specifications on some of the S-values, work on redevelopment of equations for those S-values may be undertaken, because application of this versatile method in India is a need of the hour to curb nuisance of mixing cheaper oils and fats in milk fat. Until suitable specifications on milk fat are formed and the method is officially adopted as reference in India, its unmatched advantages may be availed by using it as a screening test.

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RESEARCH ARTICLE

Study on sensory characteristics of *paneer* for process standardization from buffalo milk

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Abstract: Buffalo milk was utilized with the process variables for standardization of processes of manufacturing of *paneer*. The sensory evaluation study was under taken to investigate the effects of treatments on sensory scores for standardizing the manufacturing process. Effect of treatments on flavour score, and body and texture score were significant at $p<0.01$ and $p<0.05$, however, the effect of replication of sensory evaluations by Judges was non-significant. Effect of treatments on colour and appearance score was significant at $p<0.05$.It meant that at 95% level of confidence only, the treatments of colour and appearance was significant i.e. different. It was observed that the *paneer* manufactured from buffalo milk of 6% milk fat and 9% SNF and chilled in 1% NaCl solution was most suitable in regard of good body and texture of *paneer* at the preheating temperature of milk at 80-82°C for 5 minutes.

Keywords: Body and Texture; Colour & appearance; Flavour; *Paneer;* Sensory Evaluation

Introduction

There exist a great potential of entrepreneurship development in dairying including indigenous dairy products for rural youths and dairy farmers. Looking into the status of Bihar in regard of profession in dairying, Bihar has secured ninth position with annual production of 9.818 MMT and per capita availability of milk as 251 g/day in the year 2018-19 (NDDB, 2022).As per the recent statistics, milk production in India reached at 210 million tonnes in the year 2020 (Dwivedi, 2022). The per capita availability of milk in India is projected to rise to 428 g per day in the year 2020-21 from 394 g/day in 2018-19(NDDB, 2022).

The indigenous milk products including *paneer* (i.e. Cottage cheese) plays a great role in the development of Indian economy. *Paneer* is one of the most widely consumed dairy products which have occupied a very important place in various types of Indian cuisines (Kanawjia and Singh 2016). It is generally used after frying to replenish the proteins in vegetarian dishes in place of non- vegetarian proteins. Due to its excellent sensory attributes like cohesiveness and chewiness, the *paneer* is regarded as good replacement of meat for predominantly large vegetarian population of India (Agrawal and Sinha 2014). Apart from its huge domestic market, a large segment of international market also exists. One of the sources of growth of Indian economy may be the export of *paneer* but the main problem is low shelf-life of *paneer*. Even in frozen condition, its shelf-life is reported as 21 days and in vacuum packaging condition, it is 30 days. Besides, a number of indigenous dairy products can also be exported to the ethnic populations settled in North America, Middle East and South East Asia.

Shelf-life extension for a longer period storage and mechanization of *paneer* production process would make its marketing possible at distant places as well as for export (Rajorhia et al. 1984). At ambient temperature, its shelf-life is only 3 to 4 days due to presence of high moisture content and high water activity. Transportation, distribution and storage of *paneer* under frozen condition become difficult task and uneconomical. The sterilization of *paneer* in normal condition produces *paneer* of

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hard body and texture and also causes discolouration. The needs of cold chain for distribution to consumers cannot be omitted even after optimizing the hygienic parameters of processing. The available literature indicated that the *paneer* in the market has variable composition and high load of microbes. *Paneer* has short shelf-life and there is a need of enhancement of shelf-life by hygienic production and process standardization of *paneer*. Therefore, aim of this study was to standardize the manufacturing process of *paneer* from buffalo milk of Bihar origin which will facilitate the transportation and distribution without freezing and enhance the shelf-life of *paneer*.

Materials and Methods

Method of manufacturing the *Paneer*

The experiments of process standardization were carried out during 2018-20 at SGIDT, Patna with the process variables as per plan of study. The methodology for preparation of *paneer* from buffalo milk was used as per standard method (Sachdeva and Singh, 1988; Badshah et al. 2022) with slight modification as per process variables. The buffalo milk was procured from cattle farm of BASU, Patna. The milk were divided in two parts and standardized to 6.0% fat and 9.0% SNF and 4.5% fat and 8.5% SNF for preparation of experimental samples. The experimental samples of *paneer* were prepared as per details given below:

Treatment-T_1- The *paneer* samples prepared from buffalo milk standardized to 6.0% fat and 9.0% SNF with preheating at 90°C for 5 minutes and the fresh *paneer* cubes dipped in plain chilled distilled water for half an hour after pressing at 1 kgf/cm^2 for 30 minutes.

Treatment-T_2- The *paneer* samples prepared from buffalo milk standardized to 6.0% fat and 9.0% SNF with preheating temperature of 90°C for 5 minutes and further the *paneer* cubes dipped with 1% NaCl solution of chilled distilled water for an hour after pressing at 1 kgf/cm^2 for 30 minutes

Treatment-T_3-The *paneer* samples prepared from standardized buffalo milk (4.5% fat and 8.5% SNF) with preheating temperature of 82°C for 5 minutes and the fresh *paneer* cubes dipped in plain chilled water for cooling for half an hour after pressing at 1 kgf/cm^2 for 30 minutes

Treatment-T_4 - The market sample of fresh *paneer* from Patna market.

Physico-Chemical analysis of *Paneer*

The moisture content in samples was estimated by the gravimetric method (AOAC, 2005).Acidity was determined as per method of IS-10484 (1983). The fat content was measured by the Gerber method using Cheese butyrometer as described in AOAC, 2005.The protein of *paneer* was determined by Kjeldahl method (AOAC, 2005). The ash content of *paneer* was estimated by the method of IS-10484 (1983). The lactose content of *paneer* was estimated by the difference of sum total of the major constituents like moisture content, protein, fat and ash from 100 as described by AOAC (2005).

Sensory Evaluation of *Paneer*

The sensory evaluation of samples was done using a BIS score card IS 6273 (Part I) of 100 points recommended by De (1982) for *paneer*. The sensory evaluation of *paneer* samples were carried out by a panel of judges. Each block of *paneer* was cut into one inch small cubes. The *paneer* samples were tempered to room temperature before judging the samples in Petri dishes. The general conditions for sensory evaluation of *paneer* were kept as given in IS 6273 (Part I). Nine panellists were employed in the evaluations to arrive at consistent and valid results.

Statistical Analysis

Data were analyzed using the software Statistical Package for Social Sciences (SPSS) at the 0.05 and 0.01 level of significance following the procedure of Snedecor and Cochran (1994). Data were subjected to one way analysis of variance and Duncan's Multiple Range Test (DMRT) for comparing means of treatments to find the effects between treatments.

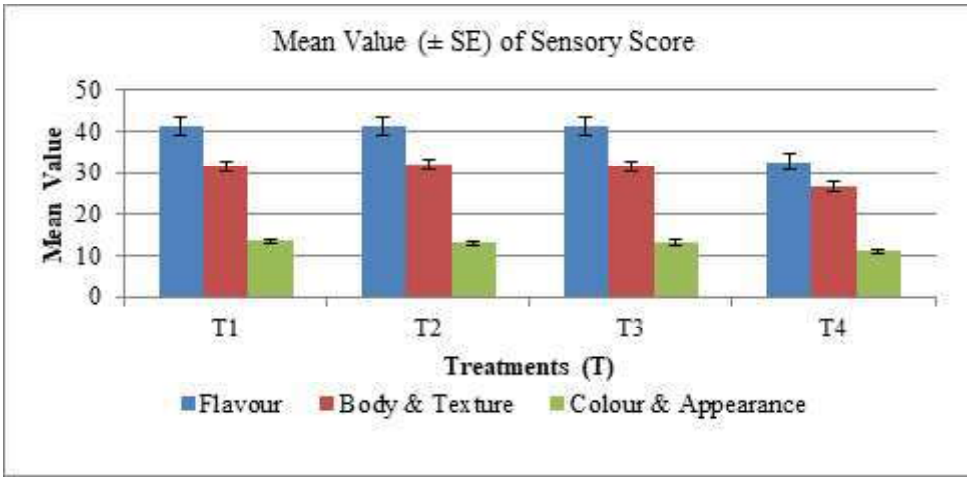
Results and Discussion

Chemical characteristics of *paneer*

The chemical characteristics of different treatment of *paneer* (T_1, T_2, T_3 and T_4) are presented in the Table 1.

The experimental samples of T_2 had maximum yield i.e. 191±1.02 g/litre of buffalo milk and the minimum yield was found with the samples of treatment T_3 (160±0.94). The moisture content of sample T_1 was found maximum (53.48±0.48%). The moisture content of *paneer* in treatment T_3 was 52.8±0.61% and the moisture content in treatment T_2 i.e. after chilling in 1% NaCl solution of T_1 sample was 53.47±0.62 %. It indicated that reduction in moisture content will occur with increasing the salt concentration and will result in high preserving quality due to low moisture and reduced water activity. It was observed that the moisture content decreased with decrease in fat content and preheating temperature. The fat content of treatment T_2 was found maximum (28.07±0.21%) while the fat content of *paneer* prepared with treatment T_3 was observed minimum (20.37±0.15%). The protein content of treatment T_1 was 14.97±0.15% and while the protein content of *paneer* prepared from standard buffalo milk i.e. treatment T_3 was found maximum (22.1±0.20%). The lactose content of treatment T_1 was observed maximum (3.29±0.45%) but lactose content of treatment T_2 was found minimum (1.2±0.43%). The decrease in lactose content is higher in T_2 due to cooling period of 1 hour causing higher solubility of lactose in water in

Fig 1. Sensory Scores of *Paneer* of different Treatments



comparison to 30 minutes in T_1 and T_3. The ash content treatment T_1 was maximum (1.23±0.15%), while the *paneer* cubes dipped in 1% salt solution i.e. in treatment T_2 had higher ash content than T_1 due to absorbed content of Nacl during cooling at a temperature less than 4°C. The ash content of *paneer* of treatment T_3 was measured as 1.94±0.27%, which was higher than that in treatment T_1. The factors affecting ash contents are temperature of preheating and coagulation temperature. Higher the preheating temperature resulted in lower values of ash content in T_1.

Sensory characteristics of *Paneer*

The sensory characteristics of different treatment of *paneer* (T_1, T_2, T_3 and T_4) are presented in the Table 2. The numerical score was an indication of the quality of *paneer* prepared from treatments given as per process variables.

The graph depicting the relation among mean values of BIS sensory score is shown in Fig. 1. The statistical analysis of the sensory evaluation data has been performed to have more effective results. The analysis of variance (ANOVA) for the scores of Flavour, Body and Texture and Colour and appearance are shown in Tables 3, 4 and 5, respectively.

Effect of various treatments on flavour of *Paneer*

Table 2 reveals that the mean values of flavour score in treatments T_1, T_2, T_3 and T_4 were found to be41.11±1.51, 41.11±1.96, 41.11±3.02 and 32.67±5.5, respectively. Highest value of flavour score was recorded in T_3 and lowest in T_4.

It was observed from the result of ANOVA in Table 3 that the effect of treatments on flavour score was significant at $p<0.01$;

Table 1 Chemical quality of *paneer* of different treatments*

Items (%)	Treatment- T_1	Treatment-T_2	Treatment-T_3	Treatment-T_4
Yield (g per litre of milk)	186±0.95	191±1.02	160±0.94	-
Moisture	53.48 ± 0.48	53.47± 0.62	52.80± 0.61	52.25±1.07
Acidity	0.36± 0.01	0.39± 0.05	0.57± 0.05	0.60± 0.05
Fat	27.03± 0.15	28.07± 0.21	20.37± 0.15	25.63± 0.15
Protein	14.97± 0.15	15.4 0± 0.10	22.10± 0.20	18.42± 0.49
Lactose	3.29± 0.45	1.20± 0.43	2.79± 0.38	1.40±0.63
Ash	1.23± 0.15	1.87± 0.43	1.94± 0.27	2.27±0.66

*Data presented in table are average of 3 replicates ± SD.

Table 2 Sensory quality of *Paneer* of different treatment*

Characteristics	PerfectScore	Treatment-T_1	Treatment-T_2	Treatment-T_3	Treatment-T_4
Flavour	45	41.11±1.51	41.11±1.96	41.11±3.02	32.67±5.5
Body and Texture	35	31.56±1.88	31.89±1.90	31.67±2.87	26.89±5.21
Colour and appearance	15	13.44±1.42	12.89±1.54	13.11±1.54	11.00±1.58
Package	5	-	-	-	-

*Data presented in table are average of 3 replicates ± SD.

however, the effect of replication of sensory evaluations was non-significant. That indicates the treatments are different with each other. The difference in mean value of T4with other three treatments mean values was found higher than CD (1%) level; this means the treatment T_4 was quite different from other three treatments. The difference in means of first three treatments were less than CD (5%) value, thus all the three treatments are non-significant. The maximum flavour score was found with T_3, however, the flavour score of T_1 and T_2 were also closer to the maximum value. But, the flavour score of market samples T4 was lowest and this may be due to improper delivery and transport condition or high microbial count. Singh (2022) observed that the flavour score of *paneer* was affected significantly by different temperature of coagulation, coagulants used, fat percentages and different storage period used for preparation and storage of *paneer*. The highest flavour score (8.5) was found in the sample which was coagulated at the temperature of 80°C by lactic acid with 4.0% milk fat at 0 day while lowest score (5.0) was found with coagulation temperature of 70°C using Aonla extract with 4.0% milk fat at 12 days of storage. It is indicated that the temperature of coagulation and preheating should not be kept above 80-82°C during manufacture of *paneer* with buffalo milk of Bihar origin.

However, the *paneer* samples of treatment-T_2 also resulted a flavour score of 41.11±1.96, which was closer to treatment-T_3. Therefore use of milk fat of 6 % and SNF of 9 % was also favourable in regard of flavour score.

Effect of various treatments on body and texture of *Paneer*

The mean values of body and texture score of treatments T_1, T_2, T_3 and T_4 were found to be31.56±1.88, 31.89±1.90, 31.67±2.87and 26.89±5.21, respectively (Table 2). The highest mean score of body and texture was observed31.89 ± 1.90 with *paneer* cubes of treatment-T_2. However, the body and texture scores of *paneer* samples of T_3andT_1 were 31.67±2.87 and 31.56±1.88, respectively which shows similar values.

The result of ANOVA is depicted in Table 4. The body and texture score of market *paneer* was found with value 26.89 ± 5.21, which was lowest as judged by panellist. The effect of treatments on body & Texture score was significant at $p<0.01$ and $p<0.05$, however, the effect of replication of sensory evaluations by judges was non – significant (Table 4). The differences between the mean values of treatment- T_1 to treatment- T_2 and T_3 were lower than CD (5%) value, which indicated that these three were not exhibiting much difference but the difference of mean value of treatment-T_4 with all the three mean values of treatments was higher than CD (1%) which indicated that the means of treatment$T_4$was actually different from all other treatments.The treatment-T_2 was better in regard of body and texture score. As

Table 3 ANOVA of flavour score

Source	D.F.	SS	MSS	Cal. F	TAB F(5%)	TAB F(1%)
Treatment	3	469.42	156.47	13.79	S	S
Replication (No. of Judges)	8	119.00	14.88	1.31	NS	NS
Error	24	272.33	11.35			
TOTAL	35	860.75				
S.EM=	1.12	CD(5%)=	3.28		TAB. F(5%)=	3.01
SE.d=	1.59	CD(1%)=	4.44		TAB. F(1%)=	4.72
CV	8.66					

Table 4 ANOVA of body and texture score

Source	D.F.	SS	MSS	Cal. F	TAB F(5%)	TAB F(1%)
Treatment	3	157.00	52.33	4.99	S	S
Replication (No. of Judges)	8	88.50	11.06	1.06	NS	NS
Error	24	251.50	10.48			
TOTAL	35	497.00				
S.EM=	1.08	CD(5%)=	3.15		TAB. F(5%)=	3.01
SE.d=	1.53	CD(1%)=	4.27		TAB. F(1%)=	4.72
CV	10.61					

Table 5 ANOVA of colour and appearance score

Source	D.F.	SS	MSS	Cal. F	TAB F(5%)	TAB F(1%)
Treatment	3	32.56	10.85	4.49	S	NS
Replication (No. of Judges)	8	16.06	2.01	0.83	NS	NS
Error	24	57.94	2.41			
TOTAL	35	106.56				
S.EM=	0.52	CD(5%)=	1.51		TAB. F(5%)=	3.01
SE.d=	0.73	CD(1%)=	2.05		TAB. F(1%)=	4.72
CV	12.32					

the highest means of treatment-T_2 which was superior treatment and chilling in 1 % Nacl solution of distilled/pasteurized water if occur in treatment-T_3also, it would have given the most significant result in improving the body and texture.

Singh (2022) found that the highest mean body and texture score (7.11) of *paneer* with temperature of coagulation of 80°C and lowest mean score (6.38) was observed with coagulation temperature of 70°C. Percent fat in milk also affected the body and texture score. Singh (2022) reported that the highest score (7.12) was found in 4.0% milk fat, while it was observed as minimum score (6.46) with 3.5 % milk fat in respect of body and texture. Therefore, buffalo milk of 6.0 % milk fat and 9.0 % SNF chilled in 1% Nacl solution was most suitable in regard of good body and texture of *paneer* that can be observed from highest score of treatment-T_2 but the preheating temperature should be kept to 80-82°C similar to treatment-T_3 with coagulation temperature of 70°C.

Effect of various treatments on colour and appearance of *Paneer*

The mean value of colour and appearance score in Treatments T_1, T_2, T_3 and T_4 were found to be13.44 ±1.42, 12.89 ±1.54, 13.11±1.54 and 11.00±1.58, respectively (Table 2). The effect of treatments on colour and appearance score was significant at $p<0.05$ and not significant at $p<0.01$. However, the effect of replication of sensory evaluations by Judges was non-significant (Table 5).The colour and appearance score of control samples (TreatmenT_1) was highest i.e. 13.44 ± 1.42.

However, there was no mark difference in colour and appearance score except lowest value of 11.00±1.58 in market *paneer* sample (treatment-T_4). The difference between means of sample of T_1 and T_4 was higher than the critical difference (CD 1%) that indicated the treatment-T_1 and treatment-T_4 were significantly different. The colour and appearance score of treatment-T_1 was highest with maximum fat content. Singh (2022) reported that the colour and appearance score of *paneer* was significantly affected by temperature of coagulation, types of coagulants and fat percentage at 0.1 % level of significance. The highest mean score (7.08) was found with 80°C coagulation temperature in place of lowest score (6.45) at 70°C coagulation temperature. The mean maximum score (7.01) and minimum score (6.47) were observed in case of *paneer* prepared from 4.0% and 5.5% milk fat, respectively.

The effects of fat content and coagulation temperature and cooling with salt solution have been found significant in controlling the sensory scores. It has been found that use of treatment-T_2 with preheating temperature of 82°C instead of 90°C showed better result in giving high flavour, body and texture and colour & appearance score along with low bacterial counts and all chemical characteristics as per the FSSAI (2006) standards.

Conclusions

The *paneer* manufactured from buffalo milk of 6.0% milk fat and 9.0% SNF and chilled in 1% NaCl solution was found most suitable in regard of good flavour, body and texture and colour and appearance scores of *paneer*. It can also be observed that the highest sensory scores was found with treatment-T_2 but the preheating temperature should be kept to 80-82°C similar to treatment-T_3 with coagulation temperature of 70°C.The buffalo milk with preheating temperature of 82°C and coagulation temperature of 70°C with citric acid (2%) coagulation and cooling in 1-3% NaCl solution of distilled/ pasteurized chilled water was found as best parameter.

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RESEARCH ARTICLE

Evaluation of selected characteristics of market *Dhap Khoa*

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Abstract: *Dhap khoa* is heat desiccated traditional Indian milk product. It has higher moisture content than other two *khoa* types (*pindi* and *danedar*). In this paper, some important characteristics (chemical, colour and textural) of six market *dhap khoa* samples were evaluated. Moisture of *dhap khoa* was statistically highly significant ($p<0.001$) whereas its fat content was found statically non-significant ($p>0.05$). Its moisture content ranges from 40.77 ± 0.33 % to 43.77 ± 0.15 %. Colour characteristics (*L*, a*, b**) of market *dhap khoa* samples were found statistically highly significant from each other i.e., between market sample group ($p<0.001$). *L*, a** and *b** values varied from 81.39±0.03 to 84.84±0.55, -5.43 ±0.03 to -4.29 ±0.10 and 15.24 ± 0.12 to 15.96 ± 0.02, respectively. The textural characteristics (hardness, adhesiveness, cohesiveness, springiness, gumminess and resilience) of market *dhap khoa* were found statistically highly significant ($p<0.001$). Their chewiness was found statistically significant ($p<0.01$). The hardness, adhesiveness, cohesiveness, springiness, gumminess, chewiness and resilience, ranged from 4.58 ± 0.87 N to 11.37 ± 0.15 N, -198.90 ± 4.31 to -139.56 ± 3.69 g.s, 0.087±0.001 to 0.140±0.002, 0.05±0.002 to 0.12±0.003 m, 1.04±0.12 to 1.58±0.10 N, 0.04±0.0095 to 0.13±0.0034 Nm and 0.019±0.0093 to 0.028±0.0010, respectively. The characteristics showed the variation in *dhap khoa* quality in the market and it may be useful for improvisation in process equipment and process parameter selection for *dhap khoa* preparation.

Keywords : *Dhap khoa*, market sample, characterstics, texture, colour, moisture

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Introduction

India is the largest milk-producing nation in the world contributing over 23% of the world's total milk production. India's total milk output is about 221.06 million tonnes (MT) in 2021-22 (DAH & D, 2022-23). About half of the milk produced is consumed in liquid form, and the other half is used to make various milk products, including ice cream, milk powders, ghee, curd, butter, *khoa* and *khoa*-based products, *paneer*, cheese, and *chhana* and *chhana*-based products. The dairy products that are native to India and certain Asian nations, including Nepal, Bangladesh, and Pakistan, are referred to as traditional Indian dairy products i.e. TIDPs (Aneja et al. 2002). In different parts of the country, various sweets are made and categorized on the basis of production process, taste and names. Numerous efforts have been made during their production process, including heat desiccation, heat coagulation, fermentation, and the use of diverse ingredients and therefore there are several TIDPs, their variants, and brands available in the market.

Typically, *khoa* and *channa* are the two important base materials to make variety of sweets. *Khoa* is used in preparation of several sweets such as *peda*, *burfi*, *kalakand*, and milk cakes whereas the sweets made with *channa are rasogolla, rasomalai,* and *sandesh*, *kala-jamun, pantooa* etc. There are some other sweets which are made with the combination of both *channa* and *khoa*. It is estimated that *khoa* is produced annually in India which utilize 7% of total milk production (Prasad et al. 2015).

Khoa is a heat desiccated milk product produced by continuous heating of milk until desired total solids (55-65%) obtained with a semi solid consistency. A good quality *khoa* has uniform white colour with tinge of brown colour with sweet taste so buffalo milk is preferred over cow milk for production of *khoa* to get high quality superior and acceptable *khoa* (Aneja et al. 2002). It is classified into three major types' viz. *pindi*, *danedar* and *dhap* (IS 4883, 1980). This classification is based on their chemical composition and end uses. *Pindi* is dry kind of *khoa* which is used to manufacture of *burfi* and *peda* kind products. *Danedar khoa* have granular texture and it is used for production of milk cake and granular heat desiccated products. *Dhap khoa* is a kind of heat desiccated milk product.

Dhap khoa have solid content (55% minimum) with fat content (about 37% minimum) on the basis of dry matter content. It is characterized by a loose, sticky body and a smooth texture. It has higher moisture content than *pindi* and *danedar* types of *khoa*. *Dhap khoa* is preferred for making *gulabjamun* because after frying and soaking in sugar syrup, it creates homogenous balls with the appropriate rheological qualities (IS 4883, 1980). *Dhap khoa* is also preferred for preparation of *kalajamun*, *pantooa*, *carrot halwa* etc. (Aneja et al. 2002). It is also utilized for making different kind of products like *jalebi* and *bottle gourd halwa etc.*

The production of *dhap khoa* is mostly in hands of non-organized milk handling system such as local vendors etc. There are very few market suppliers for *dhap khoa*. The shelf-life of *dhap khoa* is very less due to higher moisture content and few products may made up of this kind of *khoa*.

In published literature, the characteristics of some market TIDPs are available such as *brown peda* (Londhe and Pal, 2008), *khoajalebi* (Pagote and Rao, 2012), *kheer mohan* (Meena et al. 2014), and *khoa-peda* (Singh et al. 2018) and *gulabjamun* (Sukre et al. 2021). However, systematic study on determination of various characteristics of *dhap khoa,* available in market*,* is rarely found and mentioned in published literature. These characterestics may be useful to better comprehend the consumer prospectives about *dhap khoa* and improvisation in process equipment for *dhap khoa* manufacturing. Therefore, the aim of present study was to characterize the selected chemical, colour, and textural attributes of the six market *dhap khoa* samples, collected from different places.

Materials and Methods

Collection of *Dhap khoa* Samples

The different places of India especially North India is famous for production of *khoa* and *khoa* based different sweets. For this study, the market *dhap khoa* samples were collected from six different places of India like Jaipur (Rajasthan), Mathura (Uttar Pradesh), Ludhiana (Punjab), Ambala (Haryana), Karnal (Haryana), and Delhi. Collected samples were randomly designated as M1, M2, M3, M4, M5 and M6 for study. *Dhap khoa* samples were procured carefully and hygienically for determination of its characteristics.

Fat

Fat content of the samples was estimated using acid digestion method (Werner Schmidt Method) (FSSAI, 2015). Fat (%) in the samples was calculated by following equation:

$$Fat, \%\ w/w = \left(\frac{W_1 - W_2}{W_3} \right) \times 100$$

Where W_1 = weight of contents in the flask or metal dish or glass bowl before removal of fat (g); W_2 = weight of contents in the flask or metal dish or glass bowl after removal of fat (g) and W_3 = weight of material taken for the test (g)

Moisture

Moisture content of the samples was estimated using gravimetric method (FSSAI, 2015).

Moisture (%) in the samples was calculated by following equation:

$$Moisture, \%\ by\ mass = \left(\frac{M_1 - M_2}{M_1 - M} \right) \times 100$$

Where M =mass of the empty dish (g); M_1 = initial mass of the dish, along with the material taken for analysis (g) and M_2=final mass in g of the dish, along with the material after drying (g)

Colour Characteristics

The International Commission on Illumination created the CIE *L* a* b** system in 1976. This technique depicts colour in accordance with how the human eye perceives it. It is a device-independent method that is widely regarded as a standard in the dairy and food industries. The system is made up of a three-dimensional colour space with the letters *L*, a*,* and *b**, which stand for the degrees of lightness, redness-greenness, and yellowness-blueness, respectively where *L** ((lightness: 0 (black) to 100 (white)), *a** ((redness: +60 (red) to -60 (green)) and *b** ((yellowness: +60 (yellow) to -60 (blue)). The colour properties of market *dhap khoa* (*L*, a*,* and *b**) were assessed using the reflectance spectroscopic technique utilizing the reflectance meter, Colour flex (Hunter lab, Reston, Virginia, USA). Prior to colour measurement, the instrument was calibrated with a standard black glass and white glass tiles as specified by the manufactures (Barnwal et al. 2014; Srinivasa et al. 2017). To ensure there are no air bubbles in the *dhap khoa* sample, it is blended before being transferred to a sample beaker to measure the colour. The readings for *L**, *a**, and *b** were noted.

Textural Characteristic

Textural characteristics of market *dhap khoa* were determined using a texture analyzer (TA-XT2i, Stable Micro Systems Ltd., Surrey, England) equipped with a 25 kg load cell and calibrated with 5 kg standard dead weight (Sukre, 2021). Before testing, sample was cut into cylindrical form (1 cm dia. ×1 cm height) by cutting tool provided with equipment. Compression probe (P-75)

was used to compress the *dhap khoa* samples up to 80 per cent of its original height (80 per cent of its strain), using a double compression test. The constant probe speeds (5 mm/s pre-test speed, 2.5 mm/s speed during test and 5 mm/s post-test speed) were used throughout the study and temperature of the samples were maintained at 25±1°C. Force distance compression curve was obtained and analyzed to estimate the hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness, resilience of the samples.

Statistical Analysis

The Analysis of Variance (ANOVA) for all various characteristics of market *dhap khoa* samples were analyzed by using IBM SPSS Statistics 27.0.1 software and 2D-graphs of various characteristics were prepared by using Microsoft Excel 2016 MSO (Version 2303).

Results and Discussion

Chemical characteristics

Table 1 represents the ANOVA for moisture, fat and colour characteristics of market *dhap khoa.*

The moisture content of market *dhap khoa* samples were statistically significantly different ($p<0.001$). Moisture content of market *dhap khoa* (Fig.1) ranged from 40.77 ± 0.33 (M3) to 43.77 ± 0.15 (M5). Mehta (2015) reported that *khoa* had moisture between 18 and 42%. *Dhap khoa* has solid content (55% minimum) in accordance with Bureau of Indian Standards. So, the moisture content should be lower than 45%.

The fat content of the six market samples (M1, M2, M3, M4, M5, and M6) were statistically non-significant (Table 1). The variation between the various *dhap khoa* samples is minimal (Fig.1) and varies from 21.93 ± 0.52 (M5) to 23.10 ± 0.13 (M3). Fat content of *dhap khoa* was reported between 20 to 23% (Prasad et al. 2015).

Colour Characteristics

The *L**, *a**, and *b** colour features of the *Dhap khoa* samples were found to be statistically highly significant ($p\leq0.001$) with respect to sample type (Table 1). The values of *L** (Fig. 2) ranged from 81.39 ± 0.03 (M5) to 84.84 ± 0.55 (M6). The *a**-value ranged from (-5.43) ±0.03 (M1) to (-4.29) ± 0.10 (M4) whereas *b**-value ranged from 15.24 ± 0.12 (M6) to 15.96 ± 0.02 (M3). Kumar et al. (2006) observed that *gulabjamun* balls' lightness value (*L**) ranged from 23.48 to 79.86 when deep-fried. Arora et al. (2022) studied heat desiccated *chhana-murki* and found that it's *L*, a** and *b** values ranged from 62.39 and 79.27, -2.06 and -1.74, and 14.66, to 14.82, respectively. So, *L** values of *dhap khoa* was on higher side of lightness than desiccated c*hanna-murki*. The *a** value of *dhap khoa* was more towards greenish side than the *channa murki*. The b* of *dhap khoa* had more yellowness than desiccated *channa murki*.

Textural characteristics

ANOVA for textural characteristics of market *dhap khoa* are depicted in Table 2. It showed that hardness, adhesiveness, cohesiveness, gumminess and resilience were statistically highly significantly different ($p<0.001$) whereas chewiness and springiness were statistically significantly different ($p<0.01$). Figure 3 shows the variation of springiness, cohesiveness, chewiness and resilience of dhap khoa among different market samples. The values for springiness, cohesiveness, chewiness and resilience ranged between, 0.05 ± 0.002 (M3) to 0.12 ± 0.003 m (M4), 0.087 ± 0.001 (M5) to 0.140 ± 0.002 (M4), 0.04 ± 0.0095 N (M2) to 0.13 ± 0.0034 Nm (M1), and 0.019 ± 0.0093 (M3) to 0.028 ±

Table 1 ANOVA for moisture, fat and colour characteristics of market *dhap khoa*

Market *dhap khoa*	Moisture, %	Fat, %	*L**	*a**	*b**
DF	5	5	5	5	5
SS	18.49	2.814	26.024	4.039	1.473
MS	3.698	0.563	5.205	0.808	0.295
F-Value	12.703	1.46	2081.917	276.991	146.921
Prob	<0.001***	0.273^{NS}	<0.001***	<0.001***	<0.001***

^{NS} Non-significant; *** $p\leq0.001$, n=3

Table 2 ANOVA for textural characteristics of market *dhap khoa*

Market *dhap khoa*	Hardness (N)	Adhesiveness (g.s)	Cohesiveness	Gumminess (N)	Chewiness (N)	Resilience	Springiness
DF	5	5	5	5	5	5	5
SS	324.145	8576.802	0.011	0.007	0.571	0.021	0.011
MS	64.829	1715.36	0.002	0.001	0.114	0.004	0.002
F-Value	156.958	129.822	144.426	278.375	6.253	97.68	144.426
Prob	<0.001***	<0.001***	<0.001***	<0.001***	0.004**	<0.001***	<0.01**

**$p\leq0.01$; ***$p\leq0.001$, n=3

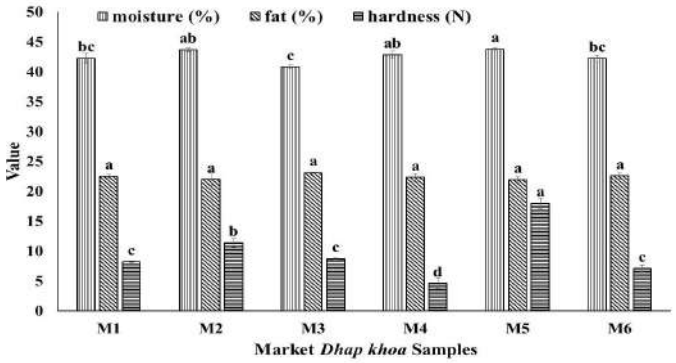

Fig. 1 Moisture , fat and hardness of market *dhap khoa*

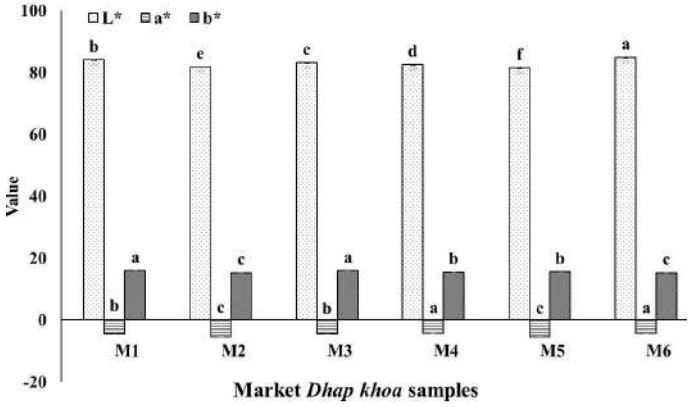

Fig. 2 Colour characterestics (*L**, *a**, and *b**) of market *dhap khoa*

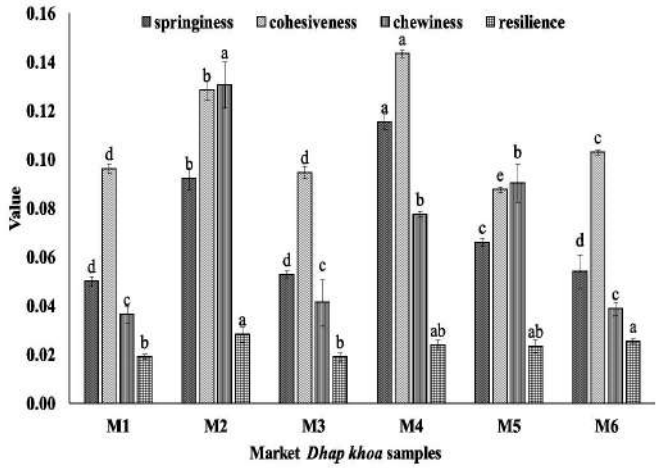

Fig. 3 Springiness, cohesiveness, chewiness and resilience of market *dhap khoa*

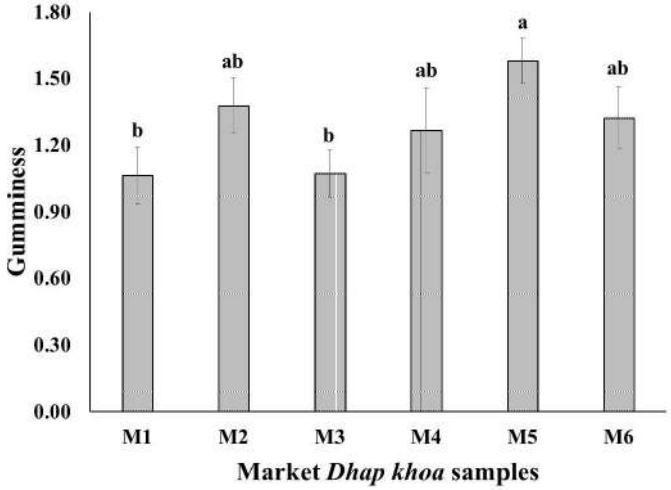

Fig. 4 Gumminess of market *dhap khoa*

0.0010 (M1), respectively. Springiness is unaffected by the characteristics of the composition (Gupta et al. 1990). As the amount of total solids increased, *khoa*'s cohesiveness tended to decrease. About 50% of the cohesiveness is contributed by total solids (Gupta et al. 1990). Additionally, cohesiveness and moisture content in *khoa* were found to be negatively correlated (Adhikari et al. 1994). Gupta et al. (1990) noted that the increase in total solids caused the instron chewiness in *khoa* to increase.

Adhikari et al. (1994) observed a negative correlation between the moisture content and internal chewiness of *khoa*. Hardness (Fig.1) ranged from 4.58 ± 0.87 N (M4) to 11.37 ± 0.15 N (M1). Gupta et al. (1990) reported that total solids (TS) have an impact on *khoa*'s hardness and with increase in its total solids, the hardness increases. Total solids alone accounts for around 78% of the *khoa*'s hardness. A negative association between moisture and the instron hardness of *khoa* was also reported by Adhikari et al. (1994). When cow milk *khoa* was converted into *gulabjamun,* there was about 50 per cent decrease in its hardness (Adhikari, 1993).

Gumminess (Fig. 4) ranged between 1.04 ± 0.12 N (M2) to 1.58 ± 0.10 N (M5). Compositional characteristics of *khoa* have a significant impact on gumminess and chewiness as well. Gupta et al. (1990) found with an increase in total solids, there was an increase in the instron gumminess in *khoa*. Moisture and instron gumminess have a negative connection (Adhikari et al. 1994).

The values for adhesiveness (Fig. 5) ranged between -198.90 ± 4.31 (M5) to -139.56 ± 3.69 (M6) g.s. Londhe et al. (2008) reported that the decrease in free moisture during storage may be the cause of the decreased adhesion in brown peda. Adhesiveness of brown peda market samples were reported in the range of -460 to -1522.65 g.s. Higher adhesiveness values might be linked to higher moisture content in the peda (Londhe et al. 2008).

Conclusions

Six market *dhap khoa* samples were studied to determine their some important characteristics (chemical, colour and textural). The moisture content was statistically highly significantly different between market samples. Moisture content ranged from

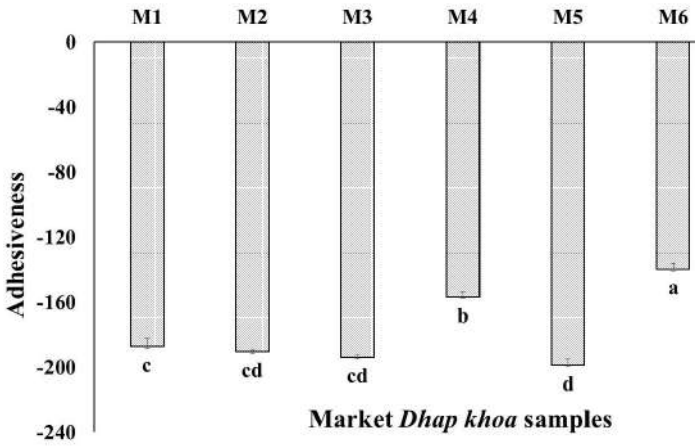

Fig. 5 Adhesiveness of market *dhap khoa*

21.93 ± 0.52 % (M5) to 23.10 ± 0.13 % (M3). Their fat content was statically non-significantly different from each other. Colour charatestics (*L*, a*, b**) was statistically highly significantly different from each other. The *L** value ranged between 81.39±0.03 to 84.84±0.55. Texture charaterstics such as hardness, cohesiveness, gumminess, chewiness, springiness and resilience were statistically highly significant. Hardness and cohesivness ranged from 4.58 ± 0.87 N to 11.37 ± 0.15 N and 0.087±0.001 N to 0.140±0.002 N, respectively. These characterstics may be useful for improvisation in process equipment design, process parameter selection and to get consumer prospective about *dhap khoa*.

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RESEARCH ARTICLE

Preparation of *Basundi* using *Ashwagandha* for value addition

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Abstract: The objective of the present study was to develop an acceptable quality *Basundi* incorporated with *Ashwagandha* powder**.** Four batches of *Basundi* were prepared using three different levels of *Ashwagandha* powder viz. 0 (P1), 0.20 (P2), 0.25 (P3) and 0.30 (P4) % (w/w of milk). Addition of ashwagandha powder up to 0.25% i.e. P3 was found to be acceptable. In order to evaluate the effect of level of sucrose on its acceptability, P3 *Basundi* was incorporated with three level of sugar viz. 5, 6 and 7 % (w/w of milk) and it was found that *Basundi* prepared using 6 % sugar was most acceptable. Cardamom was found most suitable background flavour on basis of sensory score among three different flavors viz. cardamom, saffron and nutmeg when added at the rate of 0.25 % w/w of *Basundi*. Based on the results obtained in this study a method for preparing *Basundi* using *Ashwagandha* powder was developed. The standardized method involved use of mixed milk (fat: SNF ratio of 0.5), addition of 0.25 % *Ashwagandha powder and* 6 % sugar (w/w of milk) and using cardamom flavour @ 0.02% w/w of *Basundi*. The total score of the developed product was 90.13 indicating that the product could be graded as excellent quality based on the 100 point score card suggest by BIS for sensory evaluation of milk.

Keywords: *Basundi*, *Ashwagandha*, Cardamom, Herbal

Introduction

Basundi is a heat-desiccated, thickened milk dessert, having white to light caramel colour, creamy consistency with soft textured flakes uniformly suspended throughout the matrix of product (Aneja 2002a). This product is well known in western and southern part of India, particularly in Andhra Pradesh, Gujarat, Karnataka, Kerala, Maharashtra and Tamil Nadu. It is analogous to *Rabri* and *Khurchan*, which are popular in the northern and central parts of India (Pandya, 2006; Patange et al. 2006). It has a sweetish caramel aroma, consumed directly as a dessert; it contains all the solids of milk in an approximate two-fold concentration plus additional sugar, with food and nutritive value. Additives (sugar, flavours and nuts) increase the calorific value of the product (Pal, 1997; Aneja et al. 2002b).

Ashwagandha (*Withania somnifera*), is one of the prime medicinal plants which is highly valued for its medicinal and nutraceutical properties (Sangwan et al. 2004; Misra et al. 2005; Hussain et al. 2011). This plant is known to synthesize withasteroids which have shown antioxidant, anti-tumour, adaptogenic, anti-stress, anti-convulsant, immuno-modulatory and neurological effects (Misra et al. 2005). It improves learning ability and memory capacity (Pawar et al. 2014). Numerous phytochemical studies have established *Ashwagandha* as a source of steroidal lactones known collectively as Withanolides (Jayaprakasam et al. 2004) of which withaferin-A has been recognized as the most important bioactive constituent (Xu et al. 2011). It finds extensive use in Ayurvedic system of medicine as a rasayana and medhyarasayana. The roots are extensively used in most of the Indian herbal pharmaceuticals and

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nutraceuticals and are well described in Ayurveda, the ancient Indian system of plant medicine for immuno-modulation and anti-ageing (Bhattacharya et al. 2001; Chaurasiya et al. 2008). Aureli et al. (1992) reported about the antimicrobial property of some essential oils against *Listeria monocytogens*. According to Kumar et al. (2009) antibacterial activity of aqueous and ethanolic extracts of *Ashwagandha* was determined against Staphylococcus aureus and Escherichia coli in terms of minimum lethal concentration. The *Ashwagandha* is capable of improving immune function. It increases haemoglobin concentration, red blood cell count, white blood cell count, platelet count, and body weight, in addition to providing immune-stimulatory activity (Ghosal et al. 1989).

With rapid expansion of urban and semi-urban areas, the demand for traditional dairy products is increasing and nowadays the popularity and demand of *Basundi* is increasing due to its delicacy. Moreover, addition of *Ashwagandha* powder in *Basundi* would result in a safe, physical and mental health promoting, value added traditional Indian dairy product, since natural substances are generally preferred over chemical ones and are generally seen as healthy (Gruenwald, 2009). Therefore, keeping in view the above medicinal and health benefits of *Ashwagandha*, the present research work was planned to develop a sensorily acceptable product by incorporating *Ashwagandha* powder in *Basundi* for its value addition.

Materials and Methods

Fresh mixed milk (cow: buffalo) used for manufacturing *Basundi* was procured from Vidya Dairy, Anand. The milk was standardised to desired Fat: SNF ratio 0.5±0.01. Good quality cane sugar, nutmeg, saffron and cardamom were procured from the local market. The *Ashwagandha* powder (root) was collected from Medicinal and Aromatic Plant, Processing Centre, AAU Anand, India. Cardamom, saffron and nutmeg were procured from local market.

Sensory Evaluation

Basundi prepared using *Ashwagandha* powder was evaluated for its sensory characteristics by a panel of 7 judges selected from Dairy Technology staff of the college**.** The selection criterion was that the subject had to be familiar with the product as well as show consistent results between sensory evaluation sessions. Each sample of product (~ 50 ml) was served in polypropylene (PP) cups for sensory evaluation.

Preparation of *Basundi*

Basundi was prepared in Dairy Technology department, AAU, Anand according to the method developed by Patel and Upadhyay (2003a). For selecting the rate of addition of *Ashwagandha* powder, it was incorporated in the product @ 0% (P1), 0.2% (P2), 0.25% (P3) and 0.3% (P4) (w/w of milk). Total four replications were taken for deciding the rate of addition of herb. *Ashwagandha* powder was incorporated during the addition of sugar i.e., at stage of 2X concentration of *Basundi*, on basis of preliminary trials. With a view to get optimum sweetness in the finished product, sugar was added into milk at three levels, viz., 5, 6 and 7 % (w/w of the milk).

Quality Analysis of *Basundi*

Representative samples of *Basundi* were analysed for total solids and fat content as per FSSAI (2012). The SNF content of *Basundi* was calculated by subtracting fat and sucrose from TS content. Total protein of *Basundi* was determined by Semi-Microkjeldahl method (IS: 1479 (Part-II), 1961), using Kjel-plus Digestion System (Model-KPS 006L, M/s. Pelican Instruments, Chennai) and Kjel-plus Semi-Automatic Distillation System (Model-Distil M, M/s. Pelican Instruments, Chennai). Lactose content of the milk, and *Basundi* samples was determined as per IS:10029 (1981) with slight change in quantity of sample taken. Sucrose content of *Basundi* samples was determined by the Colorimetric method suggested by Pantulu et al. (1976). The Ash content was estimated by using the standard method described for milk (IS: 10029, 1981). Withanolides content of *Ashwagandha* root powder was analysed by Colorimetric method, developed by Mishra (1994) and Mishra and Pocnori (1994). Microbiological analysis for total viable count, coliform, yeast & mold and spore count was carried out according to the standard methods described in FSSAI (2011).

Milk and *Basundi* was subjected to various physico-chemical analyses. Titratable acidity of all the samples was determined by using the method described in IS: 1479 (Part I), (1960). The pH of milk and *Basundi* was measured using digital pH meter (Mettler-Toledo AG, 8603 Schwerzenbach, Switzerland). The water activity of *Basundi* samples, tempered at 25°C temperature, was measured using Rotronic Hygroskop Model: Hygrolab-3 (M/s. Rotronic ag, Switzerland) connected to a sensing element (AW-DIO) with a measuring range of 0-100 % relative humidity. Free Fatty Acids (FFA) content (measured in terms of oleic acid) of milk/ *Basundi* samples was determined by the method suggested by Deeth and Fitz-Gerald (1975). The quantitative method presented by Keeney and Bassette (1959) for quantifying HMF by spectrophotometric measurement of the 2-thiobarbituric acid (TBA) reaction product was used to assess the extent of browning in milk and *Basundi* samples. The specific gravity of milk and *Basundi* samples was determined at 20°C using a specific gravity bottle according to the method described by Ling (1956). Viscosity of *Basundi* was determined by using "Brook field" viscometer (DV II + Pro viscometer, Model- LVDV-II + P, USA) at 20°C. Insolubility index of *Basundi* samples was determined using the procedure recommended by Haugaard et al. (1978) meant for finding solubility index of milk concentrates.

Statistical Analysis

The mean values obtained during the analyses of *Basundi* samples, were subjected to statistical analysis using completely randomized design (CRD) using software developed at Anand Agricultural University.

Results and Discussion

Effect of rate of addition of *Ashwagandha* powder on proximate composition

A comparative appraisal of proximate composition of *Ashwagandha* added *Basundi* (AAB) manufactured using different rate of addition of *Ashwagandha* in *Basundi* on proximate compositional attributes is collated in Table 1. In all the three experimental samples sugar was added into milk at 5% (w/w of the milk). The tabulated values showed that with the increased rate of addition of *Ashwagandha* powder viz. 0, 0.2, 0.25 and 0.30 % (w/w basis of milk) fat and lactose content decreased significantly whereas ash content of *Basundi* were increased significantly (P<0.05). Whereas in other compositional attributes the effect of *Ashwagandha* was found statistically non-significant on TS, protein, sucrose, and fat: SNF ratio. The significantly higher ash content could be due to result of *Ashwagandha* addition, as reported by Boone (1998) that *Ashwagandha* is rich in iron. Significant decrease of sucrose and fat content with increase in *Ashwagandha* could be due to slight lowered TS content of the *Basundi*. However, total solids content was non-significant affected, data indicates that TS content is slightly decreased when the increased rate of *Ashwagandha* was used. Addition of *Ashwagandha* with increased rate of addition was significantly (P<0.05) decreased the fat % of the *AAB*. This effect might be due to addition of *Ashwagandha*.

Ashwagandha with increased rate of addition was statistically not significantly affected the protein content but, it can be envisaged that *Ashwagandha* powder addition in *Basundi* decreased the protein % linearly. From tabulated data, it was noticed that, P4 is significantly differed from P1, P2 and P3 with respect to ash content. This effect might be due to addition of

Table 1: Effect of addition of *Ashwagandha* powder on the proximate composition and physico-chemical properties of experimental *Basundi*

Constituents (%)	Rate of addition of *Ashwagandha* powder (%)				CD (0.05)
	0 (P1)	0.20 (P2)	0.25 (P3)	0.30 (P4)	
Total Solids	47.38±0.05	47.36±0.08	47.32±0.04	47.30±0.06	NS
Fat	11.80±0.06	11.67±0.08	11.59±0.06	11.48±0.05	0.10
Protein	10.30±0.06	10.20±0.05	10.16±0.09	10.11±0.17	NS
Lactose	11.18±0.12	11.11±0.12	10.96±0.09	10.83±0.09	0.17
Sucrose	12.70±0.01	12.66±0.02	12.64±0.02	12.64±0.07	NS
Ash	1.85±0.07	1.93±0.05	2.04±0.05	2.14±0.05	0.09
Fat : SNF ratio	0.50±0.004	0.50±0.002	0.50±0.005	0.50±0.003	NS
Acidity (%LA)	0.42±0.01	0.43±0.01	0.45±0.02	0.45±0.02	NS
pH	6.59±0.04	6.53±0.04	6.52±0.08	6.45±0.05	0.08
FFA (µ eq/ml) (oleic acid)	1.40±0.05	1.36±0.02	1.34±0.04	1.32±0.05	NS
HMF (µ mol/litre)	15.54±0.02	15.56±0.08	15.69±0.09	15.71±0.11	0.13
Water activity (a_w)	0.98±0.001	0.97±0.002	0.97±0.002	0.97±0.006	NS
Specific gravity	1.13±0.01	1.13±0.01	1.14±0.01	1.15±0.01	NS
Viscosity (mPa.s)	52.90±0.04	54.23±0.52	54.55±0.40	54.19±0.50	0.72
Insolubility index (ml)	0.24±0.05	0.31±0.03	0.34±0.03	0.40±0.01	0.05

Figures placed after ± indicates standard deviation (n=3), NS – Non-significant, CD (0.05) – Critical difference at 5.0 % level of significance

Ashwagandha, as it contains various constituents like, alkaloids, phenolic compound and it is also high in iron level, which might be contributing the increased ash content. Using the same method, Patel and Upadhyay (2003b) had reported the average composition of *Basundi* i.e. fat 11.61 %, SNF 23.05 %, protein 9.86 %, lactose 10.79 %, sucrose 12.69 %, ash 1.72 %, total solids 47.35 %, and fat: SNF ratio 0.50; the values appear to be similar for all the attributes of control. Whereas with increasing the rate of addition of *Ashwagandha*, compositional attributes were tend to slightly change.

Effect of rate of addition *Ashwagandha* powder on Physico-chemical Properties

The effect of *Ashwagandha* powder addition on physico-chemical properties of the *Basundi* is presented in Table 1. On comparing the physico-chemical attributes of *Basundi* samples prepared using different rate of addition, it observed that the products manufactured, irrespective of the different levels had statistically similar values for acidity, free fatty acids, water activity and specific gravity, whereas pH, HMF, viscosity and insolubility index values differed significantly (P<0.05). The minimal inclination of acidity may be compositional effect of *Ashwagandha* or assigned by the slight lowering of TS level as observed in Table 1. Similarly, slightly depression of FFA content, might be either due to its antioxidative characteristics or due to decrease in fat content as observed in Table 1. It can be observed that with the increasing the rate of addition of *Ashwagandha* in the product HMF content tends to escalate significantly. This, significant rise of HMF value can be contributed by *Ashwagandha*, as it is having light brown color and also it contains starch, which possibly increase the HMF content of product. Patel and Upadhyay (2004b) reported that replacement of 25 % sweet cream buttermilk (SCBM) solids significantly increased the HMF content from 20.96 to 27.16 μ mol/litre. The viscosity of the *AAB* samples were significantly (P<0.05) higher at each incremental level of *Ashwagandha* addition. Control is significantly differed from all the *Ashwagandha* added products. P4 is statistically differed from P2 but it was at par with P3. This, effect of *Ashwagandha* in *Basundi* might be due to its water holding ability, as it has increased viscosity, with its increasing rate of addition. Similarly, significant increase in insolubility index exhibited when *Ashwagandha* level was increased. P4 is significantly differed from all the products. The higher level insolubility index with higher rate of addition of *Ashwagandha*, might be, due to addition of *Ashwagandha* powder as, it has lower solubility. However, the pH value remained statistically same up to addition of 0.25 % *Ashwagandha*. The slight decline of pH might be affected due to compositional effect of *Ashwagandha* or slight lowered TS level. The water activity of product remained statistically same for the *Basundi* added with different rate of addition of *Ashwagandha* powder in *Basundi*. This slight decrease in water activity (aw) can be due to its water holding property of *Ashwagandha*. Statistically, the effect of rate of addition of *Ashwagandha* on the specific gravity was not significant and slight transient may be due to it compositional attributes.

Patel and Upadhyay (2004b) reported that use of SCBM solids for substitution of BM solids in manufacture of *Basundi* had an adverse effect on FFA and HMF contents. The *Ashwagandha* can contribute in the variation in physico-chemical properties of *Basundi* when, added at different rate of addition, as it is characterised by the presence of steroidal lactones, alkaloids and flavonoids. According to Purohit (2011) *Ashwagandha* roots contain alkaloids, starch, reducing sugar, glycosides, dulcitol, withaniol acid and a neutral compound.

Effect of rate of addition *Ashwagandha* powder on sensory attributes

Table 2: Effect of addition of *Ashwagandha* powder on sensory quality of the experimental *Basundi*

Rate of addition of *Ashwagandha* (%)	Sensory score of *Basundi*			
	Flavor (Max. 45)	Body and texture (Max. 35)	Color and appearance (Max 15)	Total score* (Max. 100)
0.0 (P1)	39.96±0.29	28.25±0.45	11.96±0.61	85.43±0.98
0.20 (P2)	37.86±0.57	28.68±0.57	11.75±0.21	83.54±0.97
0.25 (P3)	37.96±0.75	30.31±0.78	12.71±0.44	86.29±0.96
0.30 (P4)	36.50±0.63	29.11±0.59	11.36±0.47	81.25±0.58
CD (0.05)	0.86	0.94	0.71	2.48

* Including full packaging score (5). Figures placed after ± indicates standard deviation (n=3), NS – Non-significant, CD (0.05) – Critical difference at 5.0 % level of significance

The data obtained for changes in sensory attributes of *Basundi* with increasing rate of *Ashwagandha* presented in Table 2. It was noticed that, flavor score of *Basundi* were significantly (P<0.05) differed when *Ashwagandha* added at escalating rates. The flavor score of P3 was statistically, at par with P2. Although, addition of *Ashwagandha* in *Basundi* resulted in decline in the flavor score, slight nutty and caramelized flavor was perceived in the product due to addition of *Ashwagandha* powder. The body and texture score of *Basundi* increased by addition of *Ashwagandha* powder up to 0.25 %. However, further addition resulted into slightly reduction in the body and texture score. This might be due to very thick consistency attributed by *Ashwagandha* powder. The changes in color and appearance score indicates that there was a slight improvement in the color and appearance score of *Basundi* on addition of *Ashwagandha* up to 0.25 % i.e., P3 and it had highest score, might be this imparted slight desired caramelised color to the product. However, further addition resulted into decline in the color and appearance score. There was a significant difference between total score of the *Basundi*, when *Ashwagandha* powder was added at different levels. P3 had the highest total score and it was statistically at par (P<0.05) with P1 (control). Often, the addition of *Ashwagandha* resulted in some medicinal flavor and bitter aftertaste, it imparted desired body and texture and color and appearance at 0.25 % level of addition (P3). But, further addition to 0.30 % *Ashwagandha* in the product (P4) results declination in the total score of the *Basundi*.

Dubey et al. (2013) incorporated *Ashwagandha* roots powder in beverages namely banana milkshake, mango lassi and pineapple drink. The prepared beverages were accepted with regard to sensory characteristics. 3 % *Ashwagandha* scored the best in overall acceptability in Pineapple drink, whereas 1 % *Ashwagandha* scored the best in both, Banana Milk Shake and Mango lassi. They concluded that the medicinal value of the beverages increased with addition of this herb.

Ashwagandha powder, at rate of 0.0 % (T0), 0.3% (T1), 0.5% (T2) and 0.7% (T3) with 40 % cane sugar (by weight of chakka), was mixed for manufacture of Shrikhand. Product prepared by addition of 0.5 % was superior in organoleptic parameter followed by T3, T1 and T0, respectively (Landge et al. 2011).

Effect of level of addition of sugar in *AAB* on the proximate composition

Three different rate of addition of sugar levels viz. 5, 6 and 7 % (w/w of milk) were selected so that sucrose content in the final product would be in vicinity of average value of sugar reported in literature. The effect of addition of different level of sugar in *AAB* on composition is as influenced by the sugar levels is portrayed in Table 3. It can be seen that, different level of addition of sugar in *Basundi* had significantly (P<0.05) increased the TS content. It could be observed that TS content was being increased proportionately with incremental level of sugar in product, and all the three products were significantly different from each other. Patel and Upadhyay (2004a) reported that addition of 5, 6 and 7 % of sugar had resulted in increased TS content up to 47.95, 49.09 and 51.70 % respectively in buffalo milk *Basundi*. Incremental rate of sugar in pre-concentrated milk, progressively and significantly increased the TS content of *Basundi*. The different level of sugar in *Basundi* making had non-significant effect on the fat content of the product. Tabulated value indicated that the increased rate of sugar addition increased the fat content of the product. However, the effect was found statistically non-significant. Patel and Upadhyay (2004a) also noticed that sugar addition had non-significant effect on fat content of buffalo milk *Basundi*. Present data were slightly differ from reported values by Patel and Upadhyay (2004a), might be due to addition of *Ashwagandha* in *Basundi*. It can be noticed that increasing the rate of addition of sugar significantly declined the protein content of product. However, the protein content of *AAB* at the addition of 5 % and 6 % sugar were statistically found unaffected. Patel and Upadhyay (2004a) reported significant effect of different level of sugar on protein content in *Basundi*.

It can be seen that sucrose content of the product was affected significantly with the increased level of sugar addition. It was revealed that increased rate of sugar addition had significantly (P<0.05) increased the sucrose content. In present study, sucrose level is quite lower than sugar content found in *Basundi* made by Patel and Upadhyay (2004a). This is might be due to addition of *Ashwagandha* and maintaining the TS level in the final product. It can be seen that sugar level had statistically not affected the lactose and ash content. So, sugar level had slightly increased the lactose and ash content of resultant product, but they were statistically non-significant. It is evident that fat: SNF ratio was decreased from 0.50 at 5 % sugar addition to 0.49 at 7 % sugar addition in *AAB*. However, the difference was found statistically non-significant. This decrease may be occurred might be due to increase in sucrose % in the product.

It was evident from the aforesaid that the increasing the extent of addition of sugar in pre-concentrated milk during manufacture of *Basundi* led to progressively significant increase in sucrose, and thus, TS content of experimental *Basundi*. The ratio of concentration maintained at the end of *Basundi* making being the same (i.e. 2.5 X the total milk solids including sugar), the contribution by sucrose to the TS of *AAB* increased proportionately.

Effect of sugar levels on the physico-chemical properties

The physico-chemical properties of the *Basundi* influenced by addition of sugar are collated in Table 3. It is evident that rate of sugar addition had non-significant (P<0.05) influence on the acidity (% LA) of product. From table values, it was noticed that the pH was not affected significantly with the addition of sugar

in *AAB*. However, statistically the pH was observed same for different rate of added sugar in *AAB.* Patel and Upadhyay (2004a) were reported that influence on pH of *Basundi* prepared using buffalo milk, was not affected significantly by sugar addition. The FFA content was decreased with increasing the sugar level of *AAB*, but the reduction in FFA was found non-significant. The HMF content was found increased non-significantly (P<0.05) with the increased rate of addition of sugar in *AAB*. But it could be noticed that HMF was slightly increased with the increased sugar level in product. The similar trend was observed by Patel and Upadhyay (2004a). According to them, the addition of sugar from 5-7% in *Basundi* resulted in non-significant increase in HMF content. Slight variation in the HMF values of the *AAB* might be affected due to addition of *Ashwagandha* powder. Comparison of data indicated that the initial HMF content is slightly more in present data, might be due to additional color imparted by *Ashwagandha* powder itself, as it was slight yellow-brown colored substance. As expected water activity (aw) decreased with increasing sugar level from 5 to 7 %. It can be seen that *AAB* had showed statistically similar water activity at different rate of sugar addition in product. Such effect of sugar addition on water activity (a_w) is an established phenomenon observed in other similar dairy and food products (Walstra and Jenness, 1984). Patel and Upadhyay (2004a) observed the significantly (P<0.05) decreased water activity of *Basundi* prepared from buffalo milk with addition of sugar. Slight variation in water activity values of the *AAB* might be influenced by addition of *Ashwagandha* powder. This could be possibly advantageous to use higher level of sugar for enhancing the shelf life of the *AAB*. The specific gravity of *Basundi* samples increased significantly (P<0.05) with increased in sugar level from 5 to 7 %. These tabulated values revealed that specific gravity of 5 and 6 % sugar added *Basundi* were statistically alike. Similarly, use of 6 and 7 % sugar level in product were observed significantly at par in their specific gravity. But specific gravity of 5 and 7 % rate of added sugar in *Basundi* were noticed significantly different. Patel and Upadhyay (2004a) also reported the similar influence of sugar levels on specific gravity of the buffalo milk *Basundi*.

The viscosity of the *AAB* samples were significantly (P<0.05) higher at each incremental level of sugar addition. Thus, it can be clear that viscosity of each level of sugar addition was significantly differed from each other. According to Patel and Upadhyay (2004a) the viscosity of the *Basundi* samples was significantly higher at each incremental level of sugar addition, mainly on account of increased solids content, mainly on account of increased solids content. This implies that a nominal rise in sugar level can be used to obtain adequate viscosity, without rendering the product too sweet. From Table 3 it is evident that, there was a significant decreased in the insolubility index of the *AAB* with the increased level of sugar. It was noticed that a significant decreased in insolubility index exhibited when the sugar level was raised from 5 to 6 % and beyond that level, the insolubility index remained unaffected i.e. at 6 and 7 % addition of sugar, the insolubility index was found statistically at par. Patel and Upadhyay (2004a) suggested that this implies beneficial effect of increased sugar level on the protein stability in buffalo milk *Basundi*. Data delineated by them are lower than the present data (Table 3) for *AAB.* The higher level of insolubility index is due to addition of *Ashwagandha* powder in the product. Gaikwad and Hembade (2011) reported that best Ujani *Basundi* product with optimum consistency and optimum sweet with characteristic brown colour can be prepared by using the standardized Buffalo milk with 6% fat and 9% SNF by adding the 8% sugar and

Table 3: Effect of levels of sugar on the proximate composition and physico chemical properties of *AAB*

Constituents (%)	Rate of addition of sugar (%)			CD (0.05)
	5	6	7	
Total Solids	47.21±0.03	47.78±0.08	49.06±0.11	0.11
Fat	11.71±0.17	11.70±0.06	11.44±0.06	NS
Protein	10.16±0.04	10.12±0.07	10.03±0.06	0.08
Lactose	10.94±0.03	10.83±0.16	10.74±0.11	NS
Sucrose	12.31±0.12	13.01±0.33	14.73±0.38	0.41
Ash	2.09±0.10	2.10±0.04	2.11±0.08	NS
Fat : SNF ratio	0.50±0.004	0.50±0.002	0.49±0.001	NS
Acidity (%LA)	0.45±0.01	0.44±0.01	0.44±0.01	NS
pH	6.51±0.01	6.53±0.01	6.54±0.04	NS
FFA (µ eq/ml)	1.34±0.01	1.29±0.03	1.25±0.08	NS
HMF (µ mol/litre)	15.65±0.05	16.22±0.18	16.72±0.19	NS
Water activity (a_w)	0.973±0.001	0.963±0.010	0.964±0.022	0.010
Specific gravity	1.14±0.01	1.15±0.01	1.16±0.01	0.01
Viscosity (mPa.s)	54.72±0.25	57.86±0.54	60.21±0.74	0.76
Insolubility index (ml)	0.34±0.01	0.32±0.01	0.31±0.01	0.01

Figures placed after ± indicates standard deviation (n=4), NS – Non-significant, CD (0.05) – Critical difference at 5.0 % level of significance

concentrating to 3.0X to its original total solids including sugar. Patel and Upadhyay (2003a) and Patel and Upadhyay (2004a) also reported the similar effect of sugar addition and found non-significant (P<0.05) effect on the acidity of *Basundi* prepared from buffalo milk. So reported values were appearing to be alike to the present acidity of *AAB.*

Therefore, it is evident that the level of sugar did not significantly influenced the acidity, pH, FFA, HMF whereas, water activity (a_w), specific gravity, viscosity and insolubility index were markedly influenced. Based on these physico-chemical properties, it seems that a level of 6 % sugar addition is beneficial with regard to protein stability, FFA development and viscosity.

Effect of sugar addition on the sensory attributes of *AAB*

The prepared samples of *Basundi* with 5, 6 and 7 % sugar (w/w of milk) were subjected to sensory evaluation by panel of seven judges using sensory score card for *Basundi*. Total five replications were conducted for the each rate of addition. The data obtained for changes in sensory attributes of *Aswagandha* added *Basundi* with increasing rate of sugar are presented in Table 4. It is evident that flavor score was significantly (P<0.05) influenced by the sugar addition at different rate in the *AAB.* Flavor and total scores in product were significantly higher at 6 % rate of sugar addition. Body and texture score of *AAB* prepared using different level of sugar were alike statistically. Therefore, it could be perceived that 6 % addition in product was most acceptable on account of body and texture of product. The body and texture score of the product diminished on further raising the sugar addition which had tendency to give thick consistency. Total score was significantly (P<0.05) influenced by the level of sugar addition in the *AAB.* The total score allotted to 6 % sugar level was highest (88.29) followed by 5 % (86.57) and 7 % (86.05) of sugar addition in the product.

The *AAB* prepared using 5 % level sugar was perceived to be some typical flavor, medicinal, bitter or groundnut flavor in the product, as mentioned by judges. On contrary, *Basundi* made using 7 % sugar perceived to be sweeter. The sensory quality of

Table 4: Effect of rate of addition of sugar on sensory score of *AAB*

Rate of sugar addition (%)	Sensory score of *Basundi*			
	Flavor (Max. 45)	Body and texture (Max. 35)	Color and appearance (Max 15)	Total score* (Max. 100)
5.0	39.10±0.57	30.21±0.40	12.27±0.25	86.57±0.93
6.0	40.61±0.11	30.53±0.31	12.14±0.43	88.29±0.59
7.0	38.92±0.38	30.09±0.48	12.05±0.40	86.05±0.88
CD(0.05)	0.55	NS	NS	1.17

* Including full package score (5); Figures placed after ± indicates standard deviation (n=4), NS – Non-significant, CD (0.05) – Critical difference at 5.0 % level of significance

Table 5 Effect of addition of flavoring on the sensory attributes of the *AAB*

Addition of Flavors	Sensory Score			
	Flavor (Max. 45)	Body and Texture (Max. 35)	Color and Appearance (Max 15)	Total Score* (Max. 100)
Control	38.89±0.37	30.56±0.88	12.32±0.29	86.77±0.99
Cardamom	42.23±0.49	31.16±0.47	12.57±0.31	90.97±0.82
Saffron	40.66±0.26	30.96±0.34	12.79±0.52	89.41±0.95
Nutmeg	39.57±0.76	30.97±0.65	12.14±0.51	87.68±0.99
CD (0.05)	0.78	NS	NS	1.52

* Including full package score (5); Figures placed after ± indicates standard deviation (n=3), NS – Non-significant, CD (0.05) – Critical difference at 5.0 % level of significance

the product diminished on further raising the sugar addition rate mainly on account to excessive sweetness and partly due to thick consistency. It can be inferred that the addition of sugar at 6 % by weight of milk yielded organoleptically superior quality *Basundi*. Thus, taking cognizance, sensory score and judge's preference, 6 % sugar addition was considered to be optimum.

Effect of addition of flavorings in the *AAB*

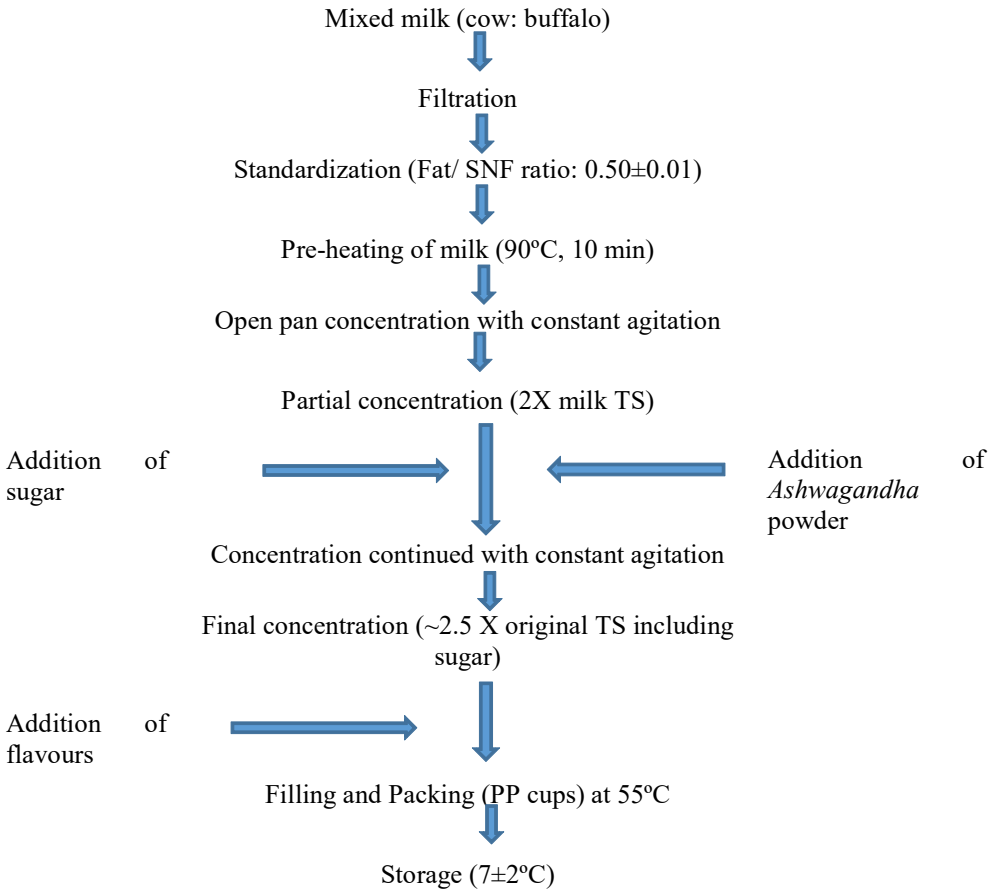

Fig.1 Flow Diagram for Preparation of *Basundi* using *Ashwagandha* powder

Table 6 Average Chemical Composition, Physicochemical Properties and Microbiological Quality of *AAB*

Attributes of Standardised *Basundi* Prepared Using *Ashwagandha* Powder			
a. Proximate Composition%		b. Physico-chemical Properties	
Total Solids	47.78	Acidity (%LA)	0.45
Fat	11.73	pH	6.52
Protein	10.12	FFA (µ eq/ml) (oleic acid)	1.34
Lactose	10.83	HMF (µ mol/ litre)	16.32
Sucrose	13.03	Water activity (a_w)	0.96
Ash	2.10	Specific gravity	1.15
Fat : SNF ratio	0.50	Viscosity (mPa.s)	56.41
Withanolides, on dry matter basis	0.29	Insolubility index (ml)	0.33
c. Sensory Attributes		d. Microbiological Quality	
Flavor	41.33	Standard Plate Count	$2.24x10^3$ cfu*/g
Body and Texture	31.28	Thermoduric Count	Nil
Color and Appearance	12.52	Yeast and Mould	Nil
Total score	90.13	E. coli Count	Nil

*Colony forming unit

One of the important aspects considered in the acceptability of dairy product is the enticing flavoring ingredients. The flavoring ingredients utilized in *Basundi* are cardamom (about 0.02 % of concentrated milk), saffron and borneol (edible camphor) etc. (Aneja et al. 2002a). Hence, to improve the flavor profile and to enhance the overall acceptability of *AAB*, suitable flavor could be added as background flavor. Therefore, three different flavorings viz., cardamom, saffron and nutmeg at rate of about 0.02 % of concentrated milk were used to assess their compatibility with *AAB*. *AAB* without a flavor addition was used as control. The data obtained for changes in sensory attributes of *Basundi* with addition of different flavor in *AAB* is presented in Table 5. From the tabulated values it was revealed that different flavoring had a significant ($P<0.05$) effect on the flavor, and total score of the product, whereas, body and texture and color and appearance score was almost unaffected with respect to addition of background flavors in product. Similarly, use of nutmeg flavor given the flavor score comparable to control. Incorporation of cardamom to the *AAB* as background flavor resulted masking effect as it reduced the bitter/ medicinal after taste in the prepared *AAB*. It can be seen that the *Ashwagandha Basundi* prepared with addition of cardamom, saffron and nutmeg as flavors ingredients; the total score found were 90.97, 89.41 and 87.68 respectively. Owing the highest sensory acceptance and its masking property, cardamom was selected for the final product.

Standardized method for preparation of *AAB*

For manufacture of sensorily acceptable, value added *Basundi*, the standardized method of manufacture is developed from the above study. For this purpose, fresh mixed milk, skim milk, cream, *Ashwagandha* powder, sugar and flavoring were used.

For preparation of *AAB* the standardized process was comprised of standardization of mixed milk (0.50±0.01, fat: SNF ratio), fore-warming of milk (90ºC for 10 min), partial concentration to approx. 2X the original milk TS, addition of sugar (6 %, w/w of milk) and *Ashwagandha* powder (0.25 %, w/w of milk), final concentration to approx. 2.5X the original milk TS inclusive of sugar, filling and packing in polypropylene (PP) cups at 55ºC, cooling and storage (7±2ºC). For preparation of *Basundi* using *Ashwagandha* for value addition the standardized method is given in Fig. 1. The Average Chemical Composition, Physicochemical Properties and Microbiological Quality of standardized *AAB* is depicted in Table 6. The proximate composition of standardized *Basundi* added with Ashwagandha at 0.25 %, sugar 6 % and Cardamom flavor had the proximate composition having total solids 47.78 %, fat 11.73 %, protein 10.12 %, lactose 10.83 %, sucrose 13.03 %, ash 2.10 % and fat: SNF ratio 0.5. Withanolides content of the product was 0.29 % (on dry matter basis). The proximate physico-chemical properties were having acidity 0.45 % lactic acid, pH 6.52, FFA 1.34 µ eq/ml, HMF 16.32 µ mol/litre, water activity 0.97, specific gravity 1.15, viscosity 56.41 mPa.s and insolubility index 0.33 ml. Sensory score for the standardized *Basundi* prepared using *Ashwagandha* powder were 41.33, 31.28, 12.52 and 90.13 for flavor, body and texture, color and appearance and total score, respectively. Microbiological quality assessed in standardized *Basundi* (fresh) was found to be standard plate count (SPC) $2.24x10^3$ cfu/g, whereas thermoduric count, yeast and mould and E. coli count were nil.

Conclusion

Hence, it can be concluded that a sensorily acceptable, delectable, novel and value added *Basundi* can be prepared using 0.25 % *Ashwagandha* powder, 6 % sugar and cardamom flavor. Commercialization of such *Basundi* will promote the production and utilization of the Indian traditional dairy product *Basundi* and the therapeutic herb *Ashwagandha*.

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RESEARCH ARTICLE

Development and characterization of herbal Kulfi (Ice Cream) using Tulsi, Ginger, and Clove

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Abstract: Herbs and spices are used for imparting flavor, the aroma of the products and also possess various therapeutic properties like anti-stress, anti-hypertensive, anti-tumor, anti-oxidant, anti-microbial, and anti-inflammatory properties. The present study was aimed to develop herbal kulfi using tulsi paste, ginger juice, and clove extract and the effects of the addition of herbs on its compositional, physicochemical properties, nutritional parameters, sensory attributes, and microbial quality. Initial trials were conducted to adjudge the most acceptable levels using tulsi paste (2.5%, 5.0% and 7.5%), ginger juice (2.0%, 3.0% and 4.0%) and clove extract (2.0%, 3.0% and 4.0%). An optimized herbal kulfi was developed using 2.5% tulsi paste, 2.0% ginger juice, and 4.0% clove extract. The kulfi prepared without any addition of tulsi, ginger, and clove was treated as control. The optimized herbal kulfi reported a fat content of 12.0%, protein 3.53 %, total solids 42.80%, ash 1.0233%, carbohydrate 23.04%. The developed herbal kulfi reported 10.01% anti-oxidant activity and total phenolic content of 56.96 mg GAE/100*g*. The products possess a good level of anti-oxidant and total phenolic content. The herbal kulfi conforms to the FSSA requirements for 'kulfi'. The standard plate count was found 3.85 cfu/*g*. The yeast and mold and coliform count were absent in the product as per FSSA standard.

Keywords: Antioxidants, Total phenolic content, Specific gravity, Melting resistance, Viscosity, Sensory

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Introduction

Herbs have been used by humans since ancient times. They promote human health and prevent the occurrence of diseases. India is popularly known as the botanical garden of the world because of the huge varieties of herbs and medicinal plants found in the country. Different parts of the herbal plant such as the plant's seeds, berries, roots, leaves, bark, or flowers can be used for medicinal purposes. Spices may be different portions of the plant such as bud (clove), bark (cinnamon), root (ginger), aromatic seed (cumin), and flower stigma (saffron) of a plant. In addition to taste enhancement of food, culinary spices have been also used as food preservatives and for their health-enhancing properties for centuries. Tulsi (*Ocimum sanctum*) is the most common herb in India. Its extract or paste is used for the treatment of a sore throat, cough, and tonsil problems. The leaves of tulsi contain volatile oil eugenol, euginal (also called eugenic acid), urosolic acid, carvacrol, linalool, limatrol, caryophyllene, and methyl carvicol (Kelm et al. 2000). The botanical name of ginger is *Zingiber officinale Roscoe* which comes under the Zingiberaceae family of plants. This rhizome can be processed into powder, syrup, volatile oil, and oleoresin (Ajav and Ogunlade, 2014). The rhizome contains fats, carbohydrates, protein, fiber, water, and volatile oil (Singletary, 2010). The ginger extract could have potent protective effects against nephrotoxicity induced by various toxicants (Gabr et al. 2017). It has also been found useful in curing ulcers and preventing heart attack and stroke (Malhotra and Singh, 2003). Clove refers to a sealed flower bud which grows on a tree belonging to *Myrtaceae* family similar to guavas (Milind and Deepa, 2011). Clove is rich in phenolic compounds (e.g., flavonoids, hidroxicinamic acids, etc). Clove is also a good source of gallic acid. It contains around 783.50 mg of gallic acid per 100 g of clove weight (Cortés-Rojas et al. 2014). *Caryophylli Flos* is one of the most commonly used materials in Chinese medicine, it possesses several therapeutic properties, such as antiseptic, analgesic, anti-phlogistic, anti-vomiting, anti-spasmodic, anti-carminative and kidney reinforcement effects (Lin et al. 2016). Kulfi, an Indian traditional frozen dairy product, has a composition almost similar to that of ice cream (Giri et al. 2014). It is made from concentrated sweetened milk with or without the addition of nuts and flavor and is known for its refreshing cool and delightful taste (Ramachandran et al. 2005). At the present

the dairy industry is actively involved in novel product development with health benefits (Mudgil and Barak, 2016). The development of novel formulations and imitation dairy products is practiced by dairy processors to achieve ease of use and quality of the products. Kulfi is a widely accepted frozen dairy product and is popular among all age groups. The herbs and spices could be added to existing products to enhance the shelf life, add value and increase the anti-oxidative and anti-microbial potential. Thus, the present study was taken to develop herbal kulfi by the incorporation of tulsi, ginger, and clove--.

Materials and Methods

Materials

Amul brand of high fat milk (fat 6.0% and S.N.F. 9.0 %) was purchased from local market. Mix stabilizer and emulsifier (pectin, agar agar and guar gum) was obtained from Brion Fine Chem., Bombay. Sugar was obtained from local market. Tulsi, ginger and clove were obtained from local market of Prayagraj. Stainless steel vessels were used for condensation of milk.

Preparation of tulsi paste

Tulsi paste was prepared as per the method discussed by (Trivedi et al. 2014) in that first wash the tulsi leaves with potable water thoroughly to remove impurities and heated at 65°C for 5 min. The heat-treated tulsi leaves were crushed in the juice maker along with the water to obtain a fine paste. It was then filtered through a clean, sanitized fine double layered muslin cloth to obtain tulsi paste and kept at refrigeration temperature (7±1°C) and the final paste contains 11% total solids.

Preparation of ginger juice

The ginger juice was prepared as per (Ahammed et al. 2014) method firstly fresh raw ginger rhizomes were washed in running tap water, peeled and shredded. Then extracted the juice by using blender and then the juice was filtered through two-fold muslin cloth and kept at refrigeration temperature (7±1°C) until used.

Preparation of clove extract

For the preparation of clove extract, clove, and water were taken in a ratio of 1:15. Thereafter, they were heated at 70°C for 2 min. The heat-treated cloves are filtered through a clean, sanitized, fine double layer muslin cloth to obtain clove juice and kept at refrigeration temperature (7±1°C) until used.

Preparation of herbal kulfi using tulsi paste, ginger juice and clove extract

Kulfi was prepared from whole milk having fat 6.0 % and SNF 9.0 %. The milk was condensed in an open pan to half its original volume (2:1). At this stage the flame was reduced and 0.2 % stabilizer and sugar at the rate of 15% of condensed product were added to the pan and mixed thoroughly while heating slowly. After thorough mixing of the sugar, and stabilizer the heating was stopped. This was referred to as control kulfi. For the preparation of herbal kulfi, a similar process was followed and after the addition and mixing of sugar and stabilizer, a concentrated mix of tulsi paste, ginger juice, and clove extract was added as per treatments and the kulfi mix was held at 70°C for 5 min and thereafter the mix was cooled down. The prepared mix was filled into plastic molds, usually of conical shape. The freezing of kulfi is conventionally carried out by liquid brine solution.

Sensory evaluation

The sensory analysis of the prepared herbal kulfi was carried out by means of seven selected panelists of Warner College of Dairy Technology, Prayagraj. They scored the sample on the basis of a 9-point hedonic scale, ranging from 'like extremely = 9' to 'neither like nor dislike = 5' to 'dislike extremely = 1' (Annexure I). The sensory evaluation attributes were color and appearance, flavor, body, and texture melting resistance, and overall acceptability (Stone and Sidel, 2004).

Physico-chemical analysis

Acidity

The acidity of kulfi was determined by the method described in IS: 1964.

Specific gravity

Specific gravity (at 30°C) of kulfi mix samples was determined using a standard specific gravity bottle (25 ml capacity) and distilled water was considered as the standard liquid.

Melt down time

The melting rate (Melt down time) of the kulfi was observed by drawing 50 g of the sample onto a wire net placed on a funnel over a beaker, immediately after removal from the hardening chamber. The time taken by the sample for the complete meltdown and dripping into the beaker at room temperature was noted (27°C). The melting rate was expressed as ml/15 min (Giri et al. 2014).

Fat

The fat content of kulfi was determined as per the method outlined in FSSAI (2015).

Total solids

The total solid content of kulfi was determined by hot air oven according to the method outlined in FSSA manual (FSSAI, 2015).

Protein

Microkjeldahl method was used for the determination of total nitrogen content. The percentage of protein was obtained by multiplying total nitrogen by a factor of 6.38 as per FSSA manual (FSSAI, 2015).

Ash

Ash content of kulfi was analyzed according to the method outlined in FSSAI (2015).

Carbohydrates

Carbohydrates of kulfi were determined by using the difference method (subtracting ash, fat, protein from total solids).

Anti-oxidant activity

The anti-oxidant activity was determined by scavenging of the radical 2, 2-diphenyl-1-picryhydrazyl (DPPH) as described by (Tabart et al. 2009) the stock solution was prepared by stirring 75 mg DPPH in 1 l methanol overnight. In the assay, 0.75 ml sample, standard (0–0.1 mmol Trolox), or blank (methanol) and 1.5 ml DPPH solution were mixed. The absorbance of samples, standards, and blanks at 517 nm was determined after 5 min. For each sample, a blank with 1.5 ml methanol, instead of the DPPH reagent was included to correct for any sample absorbance at 517 nm.

Total phenolic content

The total phenolic content was analyzed by Folin Ciocalteu's method (Zhang et al. 2006) using gallic acid as standard. For this, the sample (0.2 mL) was mixed with 0.1 mL of 0.2 N Folin–Ciocalteu's phenol reagent. After 2–5 min, 0.8 mL of 20% sodium carbonate solution was added to the mixture and incubated for 10 min at room temperature. After this, the mixture was subjected to centrifugation carried out at 150*g for the duration of 8 minutes and the absorbance of the supernatant was measured at 730 nm against a blank (distilled water). The total phenolic content was expressed in terms of µg gallic acid equivalent (GAE) per 100gm of kulfi.

Microbiological analysis

Standard plate count, yeast and mold, and the coliform count were analyzed as per manual microbiological testing (FSSAI, 2012).

Statistical analysis

All experiments were performed in triplicate. Average values of the parameters obtained for the trial samples were compared with the control sample. The results were tested by using Microsoft excel by employing analysis of variance (ANOVA) and a comparison between means was made by critical difference (CD) value.

Results and Discussion

Physico-chemical properties of kulfi/kulfi mix

Effect of tulsi paste on physico-chemical properties of kulfi/ kulfi mix

Table 1 shows the effect of different levels of tulsi paste on the acidity of kulfi. Statistical analysis using ANOVA revealed that there was insignificant increases up to 2.5% of tulsi paste then after it was deceased. The acidity of control sample T_0 was (0.235 % LA); followed by T_1 (0.35% LA), T_2 (0.305 % LA) and T_3 (0.30 % LA). Goraya and Bajwa (2015) had incorporation of processed amla caused a significant ($p<0.01$) rise in acidity of all ice cream samples. This was due to the presence of ascorbic acid and phenolic substances in amla. The specific gravity of the kulfi mix sample insignificantly decreased to the control sample. The specific gravity of the kulfi mix was T_0 (1.289), T_1 (1.248), T_2 (1.218) and T_3 (1.212), respectively. Similar result was observed by Salem and Massoud (2003) reported that an increased level of replacement of sugar with stevia in fiber fortified frozen yogurt decreased the specific gravity of the product due to decreases in the total solids content of the final products. The meltdown time of kulfi was significantly deceased to the control sample. The meltdown time of kulfi was T_0 (12.20), T_1 (6.80), T_2 (7.31), and T_3 (7.78) respectively. A similar result was observed by (Trivedi et al. 2014) that had a significant effect on meltdown property, which decreased significantly ($p<0.05$) with an increase in level of basil juice in the ice cream samples. This could be due to the dilution effect of basil juice in the ice cream samples.

Effect of ginger juice on physico-chemical properties of kulfi/ kulfi mix

Table 1 shows the effect of different levels of ginger juice on the acidity of kulfi. Statistical analysis using ANOVA revealed that there was a non-significant difference among all treatments. The acidity was increases followed by T_0 (0.235 %LA), T_1 (0.32 %LA), T_2 (0.33 %LA) and T_3 (0.34 %LA) respectively. (Gabbi et al. 2017) had observed ginger juice and powder inclusion at increasing levels caused a significant ($P < 0.01$) increase in acidity of the ice cream samples due to the presence of ascorbic acid and phenolic substances in the ginger. The specific gravity of the kulfi mix sample insignificantly decreased to the control sample. The specific gravity of the kulfi mix was T_0 (1.289), T_1 (1.288), T_2 (1.252), and T_3 (1.232) respectively, in Table (1). A similar result was observed by Gaur et al. (2019) had prepared herbal milk in which the control sample (1.2373) was recorded higher than the experimental sample (1.2207). The meltdown time of kulfi was significantly deceased to the control sample. The meltdown time of the kulfi was T_0 (12.20), T_1 (6.53), T_2 (7.20), and T_3 (6.62)

respectively, in Table (1). Agrawal et al. (2016) reported that the increases of ginger juice in the treatments resulted in the decreasing of melting characteristics of the ice cream.

Effect of clove extract on physico-chemical properties of kulfi/kulfi mix

Table 1 shows the effect of different levels of clove extract on the acidity of kulfi. Statistical analysis using ANOVA revealed that there was an insignificantly difference among all treatments. The acidity was increases followed by T_0(0.235 %LA), T_1(0.28 %LA), T_2(0.32 %LA), and T_3 (0.365 %LA), respectively. Sagdic et al. (2012) reported that the addition of phenolic-rich substances, like ellagic acid and gallic acid, enhanced the acidity of ice cream due to acidic nature of these components. The specific gravity of the kulfi mix sample insignificantly decreased to the control sample. The specific gravity of the kulfi mix was T_0(1.289), T_1(1.234), T_2(1.231) and T_3(1.220) respectively, (Table 1). (Goraya and Bajwa, 2015) had prepared ice cream using processed amla they found that the control sample had the lowest specific gravity of 0.533 and it highest for ice cream samples with high levels of processed amla product due to the low level to total solid was present in processed amla. The meltdown time of the kulfi was significantly deceased to the control sample up to 2.0% of clove extract after that it was increased. The meltdown time of kulfi was T_0(12.20), T_1 (7.61), T_2(7.76), and T_3(7.93), rSespectively, (Table 1). Murtaza et al. (2004) had added figs as replacement of fat in ice cream decreased the meltdown time of the ice cream gradually, with the highest value for plain ice cream sample. The reason is that the meltdown of ice cream is influenced by its composition and additives and by fat globule size. Storage also significantly decreased the meltdown time of the ice cream.

Sensory evaluation

Effect of tulsi paste in kulfi on sensory attributes

The result of the effect of addition of tulsi paste on sensory attributes of kulfi was presented in Table 1 & Figure 1. The sensory attributes such as color, flavor, and overall acceptability were significantly different. Upon increasing the level of tulsi paste, the color score decreased significantly ($p<0.05$) due to the dark green color of the herbal kulfi. The addition of tulsi paste at the level of 2.5% reported the highest score for flavor while the addition at 7.5% level lowered the flavor score considerably because of the undesirable intense flavor of tulsi. The scores for melting resistance increased upon the addition of 5% tulsi paste. The decrease in body and

Table 1: Effect of addition of different levels of tulsi paste, ginger juice and clove extract on physico-chemical and sensory parameters

Treatment	Level (%)	Physico-chemical properties			Sensory Score (9-point scale)				Overall Acceptability
		Acidity (% LA)	Specific Gravity	Melt down time (Min)	Color and Appearance	Flavor	Body and Texture	Melting characteristics	
T_0 (control)	0	0.235^a±0.006	1.289^a±0.080	12.20^b±0.652	7.80^a±0.109	7.57^a±0.090	7.92^a±0.113	8.30^a±0.114	7.54^a±0.104
T_1	2.5	0.35^a±0.016	1.248^a±0.010	6.80^a±0.281	8.76^b±0.072	8.64^b±0.09	8.61^a±0.088	7.83^a±0.067	8.66^b±0.079
T_2	5.0	0.305^a±0.022	1.218^a±0.011	7.31^a±0.357	7.80^a±0.114	7.73^a±0.152	7.82^a±0.102	7.85^a±0.090	7.76^a±0.086
T_3	7.5	0.30^a±0.024	1.212^a±0.024	7.78^a±0.594	7.71^a±0.119	7.20^a±0.116	7.90^a±0.086	7.58^a±0.108	7.58^a±0.078
C.D.		0.322	0.483	1.652	0.924	0.968	0.893	0.886	0.844
T_0 (control)	0	0.235^a±0.006	1.289^a±0.080	12.20^b±0.652	7.80^a±0.109	7.57^a±0.090	7.92^a±0.113	8.30^a±0.114	7.54^a±0.104
T_4	2.0	0.32^a±0.025	1.288^a±0.022	6.53^a±0.565	8.40^a±0.086	8.59^b±0.054	7.90^a±0.137	8.21^a±0.072	8.71^b±0.063
T_5	3.0	0.33^a±0.023	1.252^a±0.012	7.20^a±0.490	8.16^a±0.091	7.61^a±0.088	8.02^a±0.096	8.28^a±0.079	7.70^a±0.085
T_6	4.0	0.34^a±0.026	1.232^a±0.017	6.62^a±0.599	7.73^a±0.098	7.30^a±0.085	8.11^a±0.100	8.11^a±0.100	7.71^a±0.085
C.D.		0.347	0.485	1.770	0.886	0.811	0.958	0.868	0.836
T_0 (control)	0	0.235^a±0.006	1.289^a±0.080	12.20±0.652	7.80^a±0.109	7.57^a±0.090	7.92^a±0.113	8.30^a±0.114	7.54^a±0.104
T_7 (2.0%)	2	0.28^a±0.015	1.224^a±0.006	7.61^b±0.706	7.28^a±0.072	7.12^a±0.078	7.18^a±0.054	7.87^a±0.081	7.38^a±0.066
T_8 (3.0%)	3	0.32^a±0.026	1.231^a±0.014	7.76^c±0.841	7.61^a±0.066	7.45^a±0.074	7.50^a±0.072	8.01^a±0.104	7.47^a±0.071
T_9 (4.0%)	4	0.365^a±0.020	1.220^a±0.009	7.93^d±0.782	8.21^b±0.072	8.28^b±0.079	7.97^a±0.092	8.22^a±0.074	8.30^b±0.078
C.D.		0.322	0.475	2.030	0.814	0.810	0.834	0.877	0.814

Mean±S.E., n=3;* Different letters indicated the significant difference ($p<0.05$) among the column. ; * Different letters indicated the significant difference ($p<0.05$) among the column; C.D = S.E. * t (5%) df, 5%, Where, S.E. = Standard error; T_0=Control; T_1, T_2, T_3 (Tulsi Kulfi); T_4, T_5, T_6 (Ginger Kulfi), T_7, T_8, T_9 (Clove Kulfi)

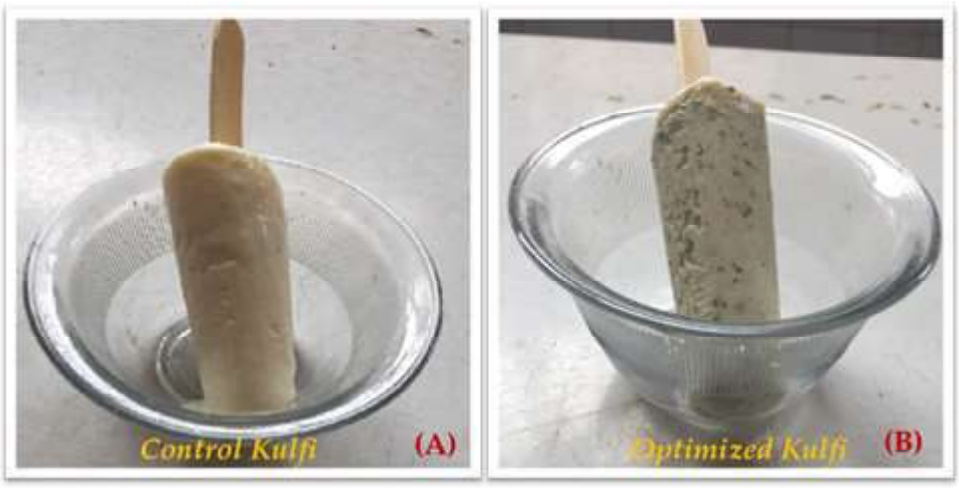

Fig. 1 (A) Control Kulfi without additives (B) Optimized Kulfi with additives

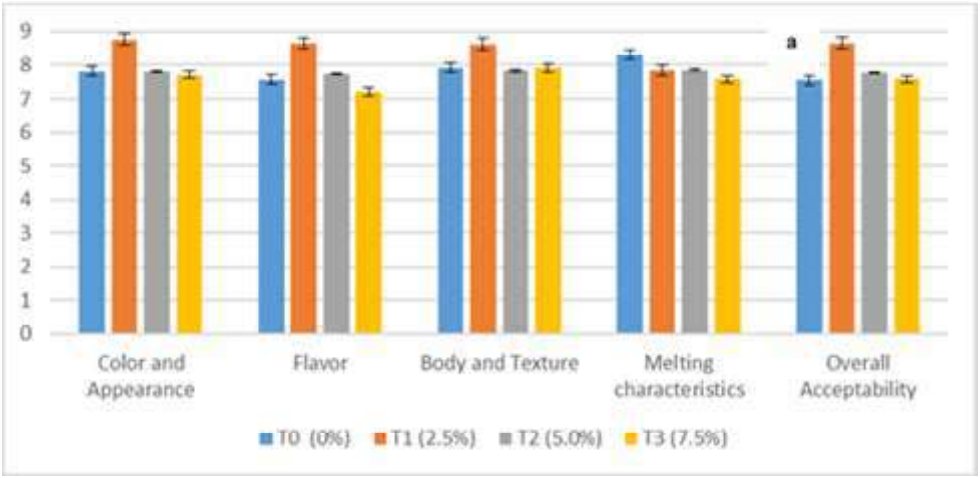

Fig. 2 Effect of addition of tulsi paste on sensory parameters of herbal kulfi.

texture scores insignificantly ($p<0.05$) in experimental samples was mainly attributed to the presence of basil particles at higher levels. The coarseness associated with the experimental samples was ascribed to the tulsi paste which was detrimental to 'eating quality'. The presence of a higher amount of paste made the kulfi hard in texture. A similar trend was observed in overall acceptability as well. The scores decreased beyond the 2.5% level of tulsi paste due to the high and intense flavor of tulsi which was disliked by the panelists. Similar results were reported by (Kumar et al. 2013) who found that a higher level of addition of tulsi extract to ice cream decreased the sensory scores for color and appearance. Kumari et al. (2011) reported that the addition of tulsi paste improved color, and appearance, body and texture and also overall acceptability of herbal yoghurt. Kumar et al. (2013) prepared herbal ice cream using tulsi extract compared with plain ice-cream and found flavor preference at 3 % level. (Trivedi et al. 2014) incorporated basil powder in ice cream and observed that the body and texture scores decreased marginally when basil powder at a level of 0.5% was added to ice cream, whereas at 2.0% level the scores for the body and texture of ice cream decreased drastically due to presence of particles of basil. The melting quality score of samples also decreased with the increase in the levels of tulsi powder.

Table 2: Different trials conducted for selection of levels of variables

Variables	Levels of variation (%)			Level selected (%)
Tulsi paste	2.5	5.0	7.5	2.5
Ginger juice	2.0	3.0	4.0	2.0
Clove extract	2.0	3.0	4.0	4.0

Effect of ginger juice on sensory attributes of kulfi

There was an insignificant ($p<0.05$) difference in the color and appearance score upon the addition of ginger juice at varying levels (Table 1). Flavor and overall acceptability scores were significantly ($p<0.05$) different for the herbal kulfi containing different levels of ginger juice. The flavor score decreased significantly ($p<0.05$) upon the addition of ginger juice. The highest scores for flavor were observed for kulfi with 2% added ginger juice, thereafter the scores decreased due to the high

pungency of ginger. Similar results were observed by Gavhane et al. (2014) who prepared ginger-flavored peda in which the color and flavor scores decreased with an increase in levels of ginger addition. Further (Agrawal et al. 2016) also used ginger juice in ice cream and observed that the flavor preference was increased up to 4% only, beyond that the scores decreased. Ginger juice had no significant effect on the body and texture of kulfi as evident from their scores. Gabbi et al. (2017) concluded that increase dose of ginger juice improved body and texture due to the presence of appreciable amounts of starch (3.87%). The melting resistance score was highest at level 3.0% of addition of ginger juice but thereafter, the score decreased. (Pinto et al. 2004) observed that ice cream containing 4% ginger juice had the highest overall acceptability scores. Similarly, Regu et al. (2016) observed that as per increasing the level of ginger in cottage cheese the overall acceptability was declined due to the high pungent flavor of ginger which is not liked by the panelists. In the present study, the overall acceptability scores revealed that 2.0% ginger juice addition is acceptable by panelists (Figure 2).

Effect of clove extract in kulfi sensory attributes kulfi

The results of the effect of addition of clove extract on the sensory properties of kulfi are presented in Table 1 & Figure 3. Results revealed that upon addition of clove extract at 4.0% level, maximum scores of color and appearance were reported. Similar results were observed for flavor, taste, and body & texture maximum score at 4.0%. The melting resistance was not significantly ($p<0.05$) affected by the addition of clove extract. Maximum overall acceptability was obtained for the kulfi samples containing 4% clove extract and its value was significantly different ($p<0.05$) as compared to the control sample (T_0). Singh et al. (2017) prepared kulfi supplemented with a wood apple in which the color score increased with increasing the level of pulp to 15%. (Ali et al. 2015) reported improvement in color score with an increased level of pomegranate seed powder in ice cream. Badola et al. (2018) observed that the flavor and overall acceptability scores significantly decreased ($P<0.05$) with an increase in the level of curry leaf oil and clove oil in burfi.

Optimized Kulfi

From the above results, the optimum level selected for tulsi paste was 2.5%, ginger juice was 2.0% and clove extract was 4.0% for the preparation of herbal kulfi. Selected levels of tulsi paste, ginger juice, and clove extract are given in Table 2.

Physico-chemical properties of control and optimized kulfi mix/ kulfi

The data on the physicochemical properties of kulfi mix/kulfi are presented in Table 3.

Acidity

The acidity of control kulfi was reported to be 0.225 % L.A., while optimized herbal kulfi had 0.28% L.A. The slight increase in acidity could be due to the presence of ginger juice in herbal kulfi which is slightly acidic in nature. Darade et al. (2016) had prepared kulfi by using mango pulp in which acidity increased with increasing the level of mango pulp i.e., 0% > 10% > 15% > 20%. A similar result was reported by Husain et al. (2018) in which the acidity of

Table 3: Physicochemical, nutritional and microbiological parameters of herbal and control kulfi

Parameters	Control	Optimized herbal kulfi
Physico-chemical parameters		
Acidity (% L.A.)	$0.225^{a} \pm 0.0001$	$0.28^{a} \pm 0.01$
Specific gravity	$1.3176^{a} \pm 0.006$	$1.2884^{a} \pm 0.006$
Melt down time (ml/15min)	$10.96^{a} \pm 0.07$	$6.33^{b} \pm 0.07$
Fat (%)	$10.24^{a} \pm 0.07$	$12.00^{b} \pm 0.11$
Total solids (%)	$44.20^{a} \pm 0.67$	$42.80^{a} \pm 0.43$
Protein (%)	$3^{a} \pm 0.10$	$3.53^{b} \pm 0.11$
Ash (%)	$0.97^{a} \pm .02$	$1.02^{a} \pm 0.02$
Carbohydrates (%)	$29.97^{a} \pm 0.65$	$26.28^{b} \pm 0.48$
Nutritional parameters		
Anti-oxidant (%DPHH activity)	-	10.01*g*/100*g*
Total phenolic content	-	56.96 mg GAE/100*g*
Microbial parameters		
SPC (Cfu/*g*)	$3.69^{a} \pm 3.28$	$3.85^{b} \pm 3.43$
Coliform (Cfu/*g*)	Nil	Nil
Yeast and mold count (Cfu/*g*)	Nil	Nil

Mean±S.E., n=3; * Different letters indicated the significant difference ($p<0.05$) among the column

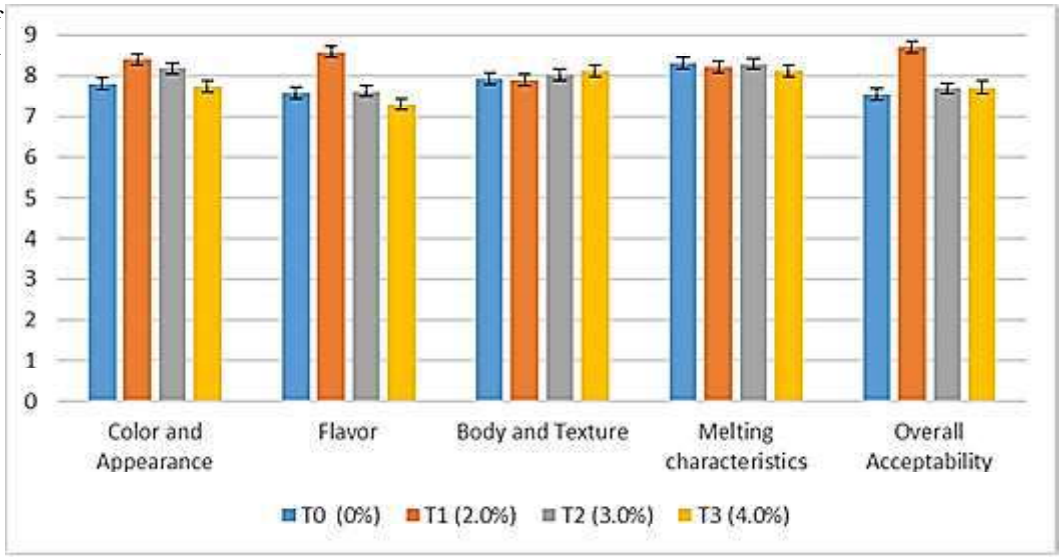

Fig. 3 Effect of addition of ginger juice on sensory parameters of herbal kulfi

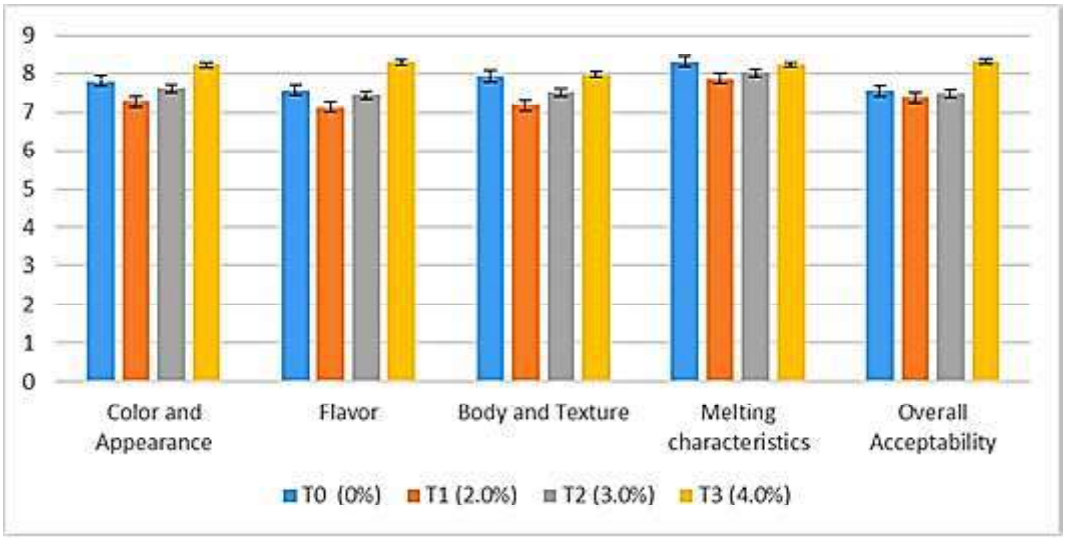

Fig. 4 Effect of addition of clove extract on sensory parameters of herbal kulfi

sandesh upon addition of ashwagandha and tulsi increased due to the acidity of tulsi extract.

Specific gravity

The specific gravity of the optimized kulfi mix sample did not differ significantly from the control kulfi mix. The specific gravity of the control and optimized sample were 1.3176 and 1.2884. The specific gravity of the optimized sample decreased slightly due to the low solids content of ginger juice and clove extract. A similar result was observed by Gaur et al. (2019) who had prepared herbal milk in which the control sample (1.2373) recorded higher specific gravity than the optimized sample (1.2207). Giri et al. (2014) prepared diabetic kulfi in which the specific gravity of the control sample was significantly higher than that of all treated kulfi mix samples. Among the treated samples, as the level of stevia increased from 0.05 to 0.07%, a significant decrease in specific gravity was noticed i.e., 1.098, as against 1.086, 1.080, and 1.076 for 50, 60, and 70% sugar reduction through 0.05, 0.06, and 0.07% stevia addition respectively.

Melt down time

The meltdown time was significantly ($p<0.05$) higher in the control sample as compare to the optimized sample. It was observed that the optimized sample had a lower meltdown (6.33 ml/ 15 min) followed by the control sample (10.96 ml/ 15 min). There are several factors that affect the meltdown rate of kulfi such as fat, total solids, amount of protein, emulsifier, and stabilizers, freezing and storage temperature, etc. Similarly, Giri et al. (2014) reported that the melting rate (ml/15 min) of control kulfi was 18.1, as against 14.8, 12.4, and 12.2 for 50, 60, and 70% sugar reduction through 0.05, 0.06, and 0.07% stevia addition respectively. In the present study, the melting rate of the control sample was significantly higher than that of all treated kulfi samples. Fat aggregation appeared to be the major contributor to the melting resistance of

kulfi through the existence of networks resulting from the presence of fat, proteins, or other stabilizers.

Proximate analysis of control and optimized kulfi

Fat

The average fat content of herbal kulfi was 12.0%. The fat content was higher than the control kulfi (10.24%). By the addition of tulsi, ginger, and clove the fat content increased significantly ($P<0.05$). A similar result was observed by Siddhu et al. (2017) where the incorporated different combination of pineapple pomace, orange pomace, and pomegranate pomace viz. 3%, 4%, and 5% respectively had content higher fat as compared to control kulfi.

Total solids

The total solids content in optimized herbal kulfi did not differ significantly ($P > 0.05$) from control kulfi. For control kulfi total solid content was 44.20% and for optimized herbal kulfi 42.80%. The decrease in total solids could be due to low total solids content in ginger juice and clove extract. Bhadakawad et al. (2009) found that the golden kulfi prepared from 40:60 blends of buffalo milk and safflower milk had lower total solid content compared to the control golden kulfi. Similarly, David (2016) observed that ice cream having 2.0% ginger juice had lower total solids compared to control. A similar result was observed by (Jadhav et al. 2017) who reported that the total solids content decreased with the increased level of ginger juice in ice cream. Chorage et al. (2018) found that the total solids content of "*shrikhand"* decreased due to a lower amount of total solids content in ginger juice.

Protein

The protein content observed in control kulfi was 3.0% and optimized herbal kulfi was 3.53%. The protein content of control kulfi was significantly different from optimized herbal kulfi. Darade et al. (2016) observed that the protein content of mango kulfi in all treatments with mango pulp was lower than control kulfi. Similar result was found by Ojha et al. (2018) who observed that upon addition of tulsi powder and turmeric powder at the rate of 0.3% to "*shrikhand"*, the protein content in herbal "*shrikhand"* was higher as compared to the control sample. Also, (Gabbi et al. 2017) observed that ice cream prepared by incorporating processed ginger powder, had higher protein content as the ginger powder had higher protein (5.82%) content.

Ash

The ash content of 0.9793% was observed for control kulfi (T_0) and 1.0233% was observed for treatment T_1. Trivedi et al. (2014) reported an increased ash content of ice cream added with basil powder. Ubale et al. (2014) observed that the ash content increased with an increase in levels of pulp i.e., T1 (7%), T2 (8%) and T3 (9%). Also, Misra (2016) found that the ash content of ice lolly added with 2.0% tulsi paste was higher than control. David (2016) also reported that the ice cream with added ginger juice at 2% level had higher ash content than the control. Ash content was higher in the optimized sample due to the presence of minerals in the herbs which affected the ash percent in the optimized product.

Carbohydrates

The addition of herbal preparations had a significant effect ($P<0.05$) on the carbohydrates content of optimized herbal kulfi. The carbohydrates content of optimized herbal kulfi showed a decreasing trend with the addition of tulsi, ginger, and clove. It may be due to the dilution by the addition of ginger juice and clove extract. The carbohydrate content was 29.97% for control kulfi (T_0) and 26.28% for optimized herbal kulfi. A similar result was found by Giri et al. (2014) who found that the carbohydrate percentage decreased significantly with increased levels of sugar replacement.

Antioxidant activity of kulfi

The antioxidant activity of optimized herbal kulfi as a % DPPH activity was found to be 10.01 and the total phenolic content of optimized herbal kulfi was found to be 56.96 mg GAE (Gallic acid equivalent) /100g. Badola et al. (2018) observed that the antioxidant activity of burfi increased with an increase in the concentration of clove bud oil. Similarly, Palthur et al. (2014) studied the antioxidant activity by DPPH method of milk prepared by partial substitution of *Ocimum sanctum* powder and reported 40% activity. (Samaddar et al. 2015) observed that the antioxidant activity of Trans- Cinnamaldehyde and Eugenol enriched flavored milk were 0.1495 and 1.2860 µM of Trolox/ml of milk respectively of the product and ultimately enhanced the shelf life of the product. Srivastava et al. (2015) used different levels of ginger extract and beetroot extract to produce herbal yogurt from cow, buffalo, and goat milk and reported that the antioxidant activity of goat milk yogurt containing 2% each ginger and beetroot extracts and cow milk yogurt containing 2% ginger extract had highest antioxidant activities measured by DPPH and FRAP methods.

Total phenolic content in kulfi

In the present study, the total phenolic content of optimized herbal kulfi was found to be 56.96 mg GAE/100g. The total phenolic content in clove bud essential oil incorporated burfi also increased from 1.07 to 5.09 µg/gm as reported by Badola et al. (2018). Chanmchan et al. (2017) obtained the total phenolic content of reduced sugar ice cream with ginger was the highest, and it was 2.60 times higher than the control formula and 2.32 times higher than the lemongrass formula.

Microbiological analysis of kulfi

The temperature at which kulfi is produced, stored, and served are below freezing and thus microbial growth is no concern. The viability of many microorganisms is preserved by freezing. Freezing and frozen storage are detrimental to some microorganisms. Although kulfi itself does not suffer direct microbial spoilage, several ingredients of kulfi are susceptible to spoilage, because they are held at temperatures suitable for microbial growth. In the present study, kulfi samples were evaluated for microbial quality in terms of SPC, coliform, yeast, and mold count. The standard plate count of 3.69cfu/*g* was observed for control kulfi (T_0) and 3.85Cfu/*g* in the optimized product. The standard plate count of herbal kulfi showed an increasing trend with the addition of tulsi, ginger, and clove. Trivedi et al. (2014) in which the average SPC of control (P0) sample was lowered as compared to ice cream samples containing basil powder. The coliform was absent in the product. A similar result was reported by Kumari et al. (2011) in which the experimental yogurt samples reported the lowest in yeast, and molds count due to anti-microbial properties of herbal paste (tulsi) added in low-fat herbal yogurt. There was no growth of coliform in kulfi upon adding tulsi, ginger, and clove. The yeast and mold count were also absent in the product. Both the control and experimental sample gave the nil result.

Conclusion

It can be concluded that a nutritionally rich for routine consumption can be made via the incorporation of tulsi paste, ginger juice, and clove extract at 2.5%, 2.0%, and 4.0% respectively. It possesses good appreciable anti-oxidant activity and phenolic content which are associated with various health benefits. The herbal kulfi prepared in the study is not only nutrients rich but also acceptable to the consumers at a similar scale as compared to the control samples. For some attributes, herbal kulfi attained higher sensory scores as compared to the control kulfi sample. The herbs and spices possess various therapeutic properties, such as anti-septic, analgesic, anti-vomiting, anti-spasmodic, anti-carminative, anti-tumor, and anti-bacterial, and also used as a natural flavoring agent in the kulfi. The use of tulsi paste, ginger juice, and clove extract also improves the techno-functional properties of kulfi such as specific gravity and meltdown. The incorporation of herbs and spices in kulfi improved the melting resistance of kulfi. Hence, this product has potential for preparation at the commercial level due to its improved nutritional, organoleptic, and techno-functional characteristics.

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Conflict of interest

There is no conflict of interest among authors

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RESEARCH ARTICLE

Physicochemical, colour, textural, rheological and sensory properties of goat milk mozzarella cheeses as affected for acidulants

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Abstract: The present study aimed at investigating the effect of four acidulants, *viz.,* acetic acid (CAA), citric acid (CCA), hydrochloric acid (CHA) and lactic acid (CLA) on quality and acceptability of goat milk mozzarella cheese. Four different treatments were prepared using 25% (w/v) of acidulants and their physicochemical, functional, colour, textural, sensory and rheological properties were evaluated. No significant difference in cheese yield, pH, protein and fat were observed among treatments. However, titratable acidity for CCA was significantly higher ($p<0.05$) than for other treatments. Moisture and ash contents in CAA were significantly higher ($p<0.05$) than in CLA and CHA. Cheese CAA had significantly lower ($p<0.05$) meltability than the other treatments. The fat leakage for treatment CCA was significantly higher ($p<0.05$) as compared to other cheeses. The Hunter colour redness (a^*) value for CAA was significantly higher ($p<0.05$) compared to the other treatments. The yellowness (b^*) value of treatment CAA was significantly higher ($p<0.05$) than the treatment CLA. The hardness and chewiness values for the treatments CAA and CHA were significantly higher ($p<0.05$) compared to the other two treatments. Cohesiveness and gumminess values were the highest for the treatment CAA, while these were the lowest for the treatment CLA. The evaluation of frequency and temperature sweeps of cheese samples revealed that the storage modulus (G') and loss modulus (G") for the treatment CHA was the highest, while the treatment CLA had the lowest values. The sensory characteristics of the cheeses from different treatments were statistically similar ($p>0.05$) except colour and appearance score, which was the highest for CCA. It can be concluded that though the type of acidulants had significant effects on various quality parameters of goat milk mozzarella cheese, their overall acceptability remained unaffected.

Keywords: Acidulants; Goat milk; Mozzarella cheese; Rheological behaviour; Sensory evaluation; Texture profile

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Introduction

Mozzarella cheese is one of the most consumed cheeses worldwide since it is relished by consumers when eaten alone and as a pizza ingredient (Francolino et al. 2010). Acidification is a critical stage in cheese making that ensures the desired cheese curd characteristics. Because it does not depend on starter efficiency, the direct acidification method for cheese production has gained significant commercial interest (Seth and Bajwa, 2015). The chemical composition and structure of the para-casein matrix obtained during the manufacturing process have a significant impact on the properties of mozzarella cheese (Martínez-Martínez and Vélez-Ruiz, 2019). Also, it has been reported that the type of acid used for pre-acidification affects curd characteristics, rate of curd formation, milk coagulation from the rennet, the recovery of milk solid-not-fat, yield, moisture content, mineral retention, elasticity, textural and rheological properties of cheese (Breene et al. 1964; Ernstrom, 1965; Quarne et al. 1968; Keller et al. 1974; Najafi et al. 2006). Different acids/acidulants, such as lactic acid, acetic acid, hydrochloric acid, phosphoric acid, citric acid, malic acid and glucono-delta-lactone have been used for the preparation of mozzarella cheese (Breene et al. 1964; Quarne et al. 1968; Keller et al. 1974; Patel et al. 1985; Dave et al. 2003; Najafi et al. 2006). Seth and Bajwa (2015) used acetic acid, citric acid and lactic acid as acidulants for the preparation of buffalo milk mozzarella cheese. Moreover, Abd El Aziz and Abo-sera (2015) used organic acids and fruit juices for direct acidification while preparing cow milk mozzarella cheese.

Buffalo milk is generally used to prepare mozzarella cheese, however, now it is also produced from cow milk in many countries. Goat milk can also be attempted for the preparation of high-quality mozzarella cheese, as goat milk products receive special preferences due to their characteristic flavour, texture and delicacy.

The recent trend of growth in the consumption of goat milk and milk products is driven by their beneficial effects such as lower allergenicity, better digestibility and many shared anecdotal health claims on human health (Hanelein, 2004; Lima et al. 2018). The preparation of mozzarella cheese from goat milk either alone or in combination with buffalo milk has been attempted in the past with limited studied parameters (Sabikhi and Kanawjia, 1992; Pal and Agnihotri, 2000). However, to the best of our knowledge, there is no report on the application of different acidulants for the preparation of goat milk mozzarella cheese. Since goat milk has compositional differences from cow and buffalo milk as far as protein and fat are concerned, it would be interesting to investigate the processing approach and the quality of goat milk mozzarella cheeses prepared using different acidulants. Therefore, the present study was envisaged to use acetic acid, citric acid, hydrochloric acid and lactic acid as an acidulant in the preparation of goat milk mozzarella cheese and observe their effect on physicochemical, colour, textural, rheological and sensory properties of the products.

Materials and Methods

Ingredients

The fresh goat milk (Fat: 4.5%; SNF: 9.37%) was procured from the Jamunapari unit of the ICAR-Central Institute for Research on Goats, Makhdoom and brought to the laboratory. The milk was filtered, pasteurized at 72 ºC for 30 sec and cooled down to below 10 ºC. The pasteurized milk was used for the preparation of mozzarella cheeses. Analytical grade acidulants like acetic acid, citric acid, hydrochloric acid and lactic and other analytical chemicals and reagents were procured from the standard firm. Microbial Meito® rennet in powder form having an activity of 300,000 Mu/g was purchased from Meito Sangyo Co., Ltd. (Nishi-ku Nagoya 451–8520, Japan) for cheese preparation.

Experimental details

In the present study, the rennet concentration and the process (pre-acidification pH, renneting temperature, coagulation time, cooking/scalding time and plasticization temperature) for the preparation of goat milk mozzarella cheese were standardized. This was followed by the preparation of goat milk mozzarella cheese using a 25% concentration of different acidulants, namely acetic acid, citric acid, hydrochloric acid and lactic acid. The prepared mozzarella cheeses were packed in low-density polyethylene (LDPE) pouches and analysed for the physicochemical, colour, textural, rheological and sensory properties.

Preparation of mozzarella cheese

Pasteurized goat milk (Fat: 4.5%; SNF: 9.37%) was taken and pre-acidified to pH 5.40±0.02 using acetic acid (CAA), citric acid (CCA), hydrochloric acid (CHA) and lactic acid (CLA). This was followed by the addition of rennet (40mg/L) into the milk and proper mixing. The milk was left undisturbed for setting at 31ºC for 1h. Thereafter, the curd was cut into 1 cm^3 pieces. The temperature of the cheese vat having curd pieces was gradually raised from 31ºC to 39ºC in 40-45 min with simultaneous stirring. After cooking whey was drained off through a strainer and the cheese curd was subjected to manual plasticization at 72±2 ºC. Subsequently, plasticized cheese was dipped into a brine solution (6%) and then dried at a refrigerated temperature. The weight of the prepared mozzarella cheese was recorded and packed in an LDPE for further study.

Product analyses

Cheese yield, pH and acidity

The cheese yield was measured by dividing the weight of the cheese by the total weight of the milk.

$$\text{Cheese yield (\%)} = \frac{\text{Weight of cheese}}{\text{Weight of milk}} \times 100$$

The pH of cheese samples was determined by homogenizing 10 g of the sample with 20 ml of distilled water, and the reading was recorded at 25 °C using a microprocessor-controlled pH analyser (Mettler Toledo®, Ohio, USA). The titratable acidity of the cheese, expressed as a lactic acid (% by weight), was evaluated by mixing 10 g of grated cheese with 90 ml distilled water, and subsequent titration of the filtrate.

Proximal composition

The moisture, fat, protein and ash content of the grated cheese samples were determined using a moisture analyser, Gerber method, Kjeldahl assembly, and Muffle furnace, respectively as per methods described by IS: SP18(Part XI) 1981.

Functional properties

The meltability of cheese samples was determined by the method of Savello et al. (1989). Here, a pyrex glass tube (25cm long and 3cm diameter) was taken and the grated cheese was plugged at one end. After incubation at 30 °C for 2 h, the tube with cheese was heated at 110°C for 50 min in a horizontal posture, and the distance travelled by molten cheese from the reference line was measured in centimetres. The fat leakage or oiling off property of cheese was determined by the method of Breene et al. (1964). The percentage area ratio of the cheese disc and oil ring was calculated as the fat leakage.

Hunter colour parameters

The colour values of the cheese samples were recorded by evaluating Hunter lightness (L^*), redness (a^*) and yellowness

(b^*) values using Color Tec PCM+ (ColorTecAssociates, Inc., Clinton, NJ) at six different places of samples.

Texture profile analysis

The Stable Micro System (Model TA.XT 2i/25 Surrey, U.K.) was used to assess the textural properties of goat milk mozzarella cheeses. The analysis was carried out using the central cores of two pieces of each sample (1.5 cm^3), that were compressed twice to 60% of their original height. A 75 mm compression platen (P75) connected to a 30 kg load cell was applied at a crosshead speed of 2 mm/s to press the samples.

Rheological property

The rheological properties of goat milk mozzarella cheese samples were assessed using a dynamic rheometer (MCR72, Anton Paar Ltd., Austria) connected to a 25 mm diameter parallel stainless steel plate geometry with a 2 mm gap (Sharma et al. 2016). The cheese samples were placed on the platform and equilibrated at the test temperature for 2 min. To prevent moisture loss during measurements, vegetable oil was applied around the sample. The linear viscoelastic (LVE) limit of the cheeses was determined using strain amplitude sweeps ranging from 0.01 to 100% at 1 Hz and 70 °C. The frequency sweeps of the cheese samples were measured at 70 °C using 0.2% strain amplitude and frequencies ranging from 100 Hz to 0.01 Hz. The temperature sweeps of the samples were determined by increasing the temperature from 20 ºC to 90 ºC, and amplitude and frequency were kept fixed at 0.2% and 1 Hz, respectively.

Sensory properties

Sensory analysis of mozzarella cheese samples was carried out by a panel of 8 panellists comprised of scientists and students aged between 20 to 60 years. After briefing the panellists about the experiment they were requested to rate the coded cheese samples on the sensory evaluation proforma using a 9-point hedonic scale (1 = extremely dislike and 9 = extremely like) for different sensory attributes like colour and appearance, flavour, body and texture and overall acceptability.

Statistical analysis

The experiment was independently replicated thrice to generate the data and each analysis was performed in duplicate (n=3). Eight trained sensory panellists were selected for the organoleptic evaluation of the cheese during each experimental trial (n=24). The generated data were analysed using one-way analysis of variance (ANOVA) in SPSS for Windows (version 17.0, SPSS, Inc., Chicago, IL) to determine the mean and standard error (SE) for each parameter. The obtained means were compared by Duncan's multiple range test, considering significant differences when $p< 0.05$.

Results and Discussion

Cheese yield, pH and acidity

The yields of mozzarella cheese prepared with different acidulants were statistically similar and ranged from 11.91% to 12.69% (Table 1). This is probably because of the use of the same milk and processing approach for all the treatments. Lower total solids (13.26%) in goat milk than in buffalo milk might have contributed to the lesser cheese yield. Similar to our finding, Martínez-Martínez and Vélez-Ruiz (2019) reported a yield of 11.7 to 13.1% for cheeses manufactured following different formulations. Chatli et al. (2019) also observed the yield of low-fat and full-fat buffalo milk mozzarella cheeses in the range of 8.90-13.81%. However, a comparatively lower product yield (7.71-9.44%) for low-fat mozzarella cheeses was obtained by Zisu and Shah (2005). There were no significant differences in the pH value (5.94-5.98) among treatments. However, treatment CCA had a slightly lower pH value as compared with other treatments. Guinee et al. (2002) reported pH values in the range of 5.4-5.9 for low-moisture mozzarella cheeses. Titratable acidity for treatment CCA was significantly higher ($p<0.05$) in relation to other treatments, which could be attributed to the non-significantly lower pH value for the mozzarella cheese prepared using citric acid.

Proximal composition

The analysis of the proximate composition of the mozzarella cheeses prepared using four acidulants showed that treatment CAA had significantly higher ($p<0.05$) moisture content compared with the treatment CLA (Table 1). The moisture content (50.82%-52.01%) reported in the present study is in agreement with the findings of Abd El Aziz and Abo-srea (2014) in cow milk mozzarella cheese. Seth and Bajwa (2015) observed a significant effect of acidulants on moisture and moisture in non-fat substances contents of mozzarella pre-cheeses. There were no significant differences in the protein (19.02%-20.77%) and fat (21.50%-21.83%) contents among the four treatments. This could be attributed to similar protein and fat recovery in all the treatments, as followed processing conditions were alike for all the cheeses. Similar fat content was reported by Abd El Aziz and Abo-srea (2014) in cow milk mozzarella cheese. The treatments CHA and CLA had significantly lower ($p<0.05$) ash contents than the treatment CAA. The ash content (2.37-2.72%) recorded in the present study is lower than the values (2.9-3.0%) reported by Seth and Bajwa (2015) in buffalo milk mozzarella cheese prepared by using different acidulants. A significantly higher moisture and ash contents for the treatment CAA could be due to more uptake of brine. The treatment CCA had statistically similar ash content to other treatments.

Functional properties

The functional attributes of mozzarella cheese important for pizza include the desired degrees of flow and stringiness on baking (Guinee et al. 2002). Cheese meltability is an important functional attribute, particularly for cheese used in food eaten after heating (Altan et al. 2005), and it is directly influenced by calcium, fat content, moisture content, pH, and nitrogen fractions (Machuca et al. 2015; Zisu and Shah, 2005). The meltability of cheese prepared using acetic acid was significantly lower ($p<0.05$) than cheeses from other treatments (Table 1). However, the meltability of the mozzarella cheese from treatments CCA, CHA and CLA were statistically similar. A significantly higher meltability for the treatments CAA, CHA and CLA might be attributed to a larger decrease in the Ca and Ca: protein during pre-acidification by citric acid, HCl and lactic acid as compared to acetic acid, as the differences in the binding affinity of calcium for the acidulants have been previously reported (Inczéy, 1976). Keller et al. (1974) also observed a significant effect of the type of acidulants on cheese meltability. The meltability recorded in the present study (17.17-21.37 cm) was much higher than the values (5.1-12.2 cm) reported by Abd El Aziz and Abo-srea (2014) in cow milk mozzarella cheese.

Table 1 Effect of different acidulants on physicochemical, functional, Hunter colour, textural and sensory properties of goat milk mozzarella cheese

Parameter	CAA	CCA	CHA	CLA
		Physicochemical properties		
Cheese Yield (%)	12.06±0.29	11.91±0.25	12.69±0.06	11.98±0.41
pH	5.98±0.01	5.94±0.04	5.97±0.00	5.98±0.01
TA (% by wt)	0.33±0.01^{b}	0.37±0.01^{a}	0.31±0.01^{b}	0.32±0.01b^{b}
Moisture (%)	52.01±0.24^{a}	51.62±0.27^{ab}	51.08±0.32^{ab}	50.82±0.45^{b}
Protein (%)	20.77±0.64	19.02±0.93	19.18±0.11	20.10±0.44
Fat (%)	21.83±1.28	21.50±1.43	21.67±0.61	21.83±0.98
Ash (%)	2.72±0.07^{a}	2.57±0.12^{ab}	2.49±0.03^{b}	2.37±0.04^{b}
		Functional properties		
Meltability (cm)	17.17±0.95^{b}	20.75±0.31^{a}	21.37±0.23^{a}	20.97±0.07^{a}
Fat leakage (%)	105.03±1.39^{c}	135.14±2.33^{a}	113.36±2.44^{b}	107.58±1.38^{c}
		Hunter colour and Texture profile analysis		
Lightness (L^*)	83.93±0.27	83.02±1.07	83.28±0.31	83.26±0.31
Redness (a^*)	3.60±0.19^{a}	2.46±0.12^{c}	2.94±0.11^{b}	2.93±0.15^{b}
Yellowness (b^*)	17.89±0.71^{a}	16.25±0.52^{ab}	16.90±0.67^{ab}	15.96±0.11^{b}
Hardness (N/cm^2)	51.01±2.03^{a}	41.45±2.85^{b}	52.08±2.07^{a}	38.32±1.32^{b}
Adhesiveness (Ns)	-0.38±0.11	-0.69±0.11	-0.27±0.10	-0.25±0.07
Springiness (cm)	0.81±0.03^{a}	0.72±0.02^{b}	0.84±0.03^{a}	0.87±0.02^{a}
Cohesiveness (ratio)	0.54±0.01^{a}	0.54±0.02^{a}	0.49±0.02^{ab}	0.46±0.02^{b}
Gumminess (N/cm^2)	27.41±1.52^{a}	22.61±2.02^{b}	25.53±0.83^{ab}	17.54±0.65^{c}
Chewiness (N/cm)	22.13±0.76^{a}	16.39±1.63^{b}	21.38±1.03^{a}	15.19±0.42^{b}
		Sensory properties*		
Colour & appearance	8.08±0.14^{ab}	8.25±0.17^{a}	7.71±0.16^{b}	7.81±0.17^{ab}
Flavour	8.13±0.17	8.27±0.15	7.85±0.18	7.86±0.18
Body & Texture	7.75±0.20	8.04±0.20	7.69±0.14	7.89±0.16
Overall acceptability	7.98±0.16	8.17±0.21	7.76±0.15	7.80±0.16

n=6; *n=24; Means bearing different superscripts in a row differ significantly ($P<0.05$)

CAA: Mozzarella cheese with acetic acid; CCA: Mozzarella cheese with citric acid; CHA: Mozzarella cheese with hydrochloric acid; CLA: Mozzarella cheese with lactic acid

Heat influences fat leakage, which is affected by heating temperature and time. When cheese is heated, it melts and releases fat, which can coalesce and result in fat separation from the protein matrix. Fat leakage, like meltability, is an important factor to consider if cheese is going to be consumed after heating. A moderate oiling off is preferable because it protects the cheese shreds from surface dehydration, case hardening, and scorching during pizza baking, whereas excessive free oil may appear greasy and unappealing melt (Kindstedt, 2007). The fat leakage for the treatment CCA was significantly higher ($p<0.05$) than the cheeses from other treatments (Table 1). Also, fat leakage for the treatment CHA was significantly higher ($p<0.05$) as compared to treatments CAA and CLA. The fat leakage (105.03-135.14%) for the different cheeses observed in the present study was lower than the average value (163%) reported in four mozzarella cheeses by Wang and Sun (2004).

Hunter colour parameters

The Hunter colour parameters of prepared cheeses were evaluated to know the effect of the studied acidulants and are presented in table 1. The lightness or brightness (*L**) values (83.02-83.93) of cheeses among treatments did not differ significantly. Martínez-Martínez and Vélez-Ruiz (2019) also observed high luminosity in fresh cheese with lightness values greater than 78. According to Alvarez et al. (2007), the high moisture content in the cheeses was related to their increased luminosity. There were significant differences ($p<0.05$) in the Hunter colour redness (*a**) values of different treatments, and the redness value of different cheeses decreased in the following order CAA>CHA=CLA>CCA. The yellowness (*b**) for the treatment CAA was significantly higher ($p<0.05$) than the treatment CLA. However, yellowness values for treatments CAA, CCA and CHA were statistically similar. Also, there were no significant differences in the yellowness values of treatments CCA, CHA and CLA. Martínez-Martínez and Vélez-Ruiz (2019) reported the redness value of the cheese systems in the negative (green) and yellowness value in the positive. Although, the redness values of the studied cheeses were low (2.46-3.60), these values were not on the negative side to consider the colour green.

Texture profile analysis

The texture profile analysis of goat milk mozzarella cheese revealed that hardness values for treatments CAA and CHA were significantly higher ($p<0.05$) in relation to treatments CCA and CLA, indicating that cheeses prepared using the citric acid and lactic acid were softer (Table 1). The lower hardness values for mozzarella cheeses prepared using citric acid and lactic acid might be ascribed to low calcium content and a high degree of casein hydration (Lawrence et al. 1984). According to Metzger et al. (2001), calcium plays a crucial role in cheese texture by crosslinking protein, and a reduction in cheese calcium decreases crosslinking among protein fibres, resulting in softer texture. Among treatments, no significant differences in adhesiveness values were observed. The springiness value for the treatment CCA was significantly lower ($p<0.05$) than for other treatments. However, the differences in the springiness values of treatments CAA, CHA and CLA were non-significant. The springiness values (0.72-0.87) of the mozzarella cheeses observed in the present study were higher than the values (0.41-0.73) observed by Martínez-Martínez and Vélez-Ruiz (2019). The treatment CLA had a significantly lower ($p<0.05$) cohesiveness value as compared to CAA and CCA. However, the cohesiveness values of CHA and CLA were statistically similar. In agreement with our results, Martínez-Martínez and Vélez-Ruiz (2019) reported the cohesiveness value of functional mozzarella cheese in the range of 0.41-0.73. However, Zisu and Shah (2005) reported a higher cohesiveness value (0.63-0.74) for low-fat mozzarella cheese. Gumminess and chewiness values are secondary data and they varied according to the values of hardness, cohesiveness and springiness. The gumminess and chewiness values for the treatment CAA were the highest, while treatment CLA had the lowest value. Seth and Bajwa (2015) reported significantly different gumminess and chewiness values for the cheeses prepared using three acidulants.

Rheological

According to Keller et al. (1974), the type of acid used to prepare direct acid low-moisture part-skim mozzarella affects cheese calcium content and rheological characteristics. They further stated that the type of acid used for pre-acidification is important as it may influence the functionality of low-fat mozzarella cheese. The strain sweeps of mozzarella cheeses from different treatments showed that the limit of linear viscoelastic (LVE) was about 1 % (Fig. 1a). The application of strain beyond LVE resulted in a gradual decrease in the value of storage modulus (G') for all the cheeses. The storage modulus for the treatment CCA was lower than other treatments. Frequency sweeps of mozzarella cheeses exhibit how viscous and elastic properties change with the rate of applied strain or with a timescale of deformation. The storage (G') and loss (G") moduli for all the treatments increased with increasing frequency from 0.01 to 100 Hz (Fig. 1b, 1c). The dynamic moduli (G' and G") for the treatment CHA was the highest, whereas for the treatment CLA was the lowest throughout the studied frequency range. Temperature sweeps in the range of 20-90 °C were used to study the viscoelastic properties of goat milk mozzarella cheeses during melting (Fig. 1d). Across the entire test temperature range, the dynamic moduli decreased with increasing temperature in all treatments. There was a steep decrease in the storage modulus of all the treatments as the temperature was raised from 20ºC to about 40-45ºC, and this was followed by a more gradual decline in its values, to the lowest at 90ºC. The initial decline in the value of G' reflects softening of the cheese probably due to liquefaction of the lipid phase, which is fully liquid at about 40°C. Storage and loss moduli values represent the extent and strength of protein-protein bonds in the

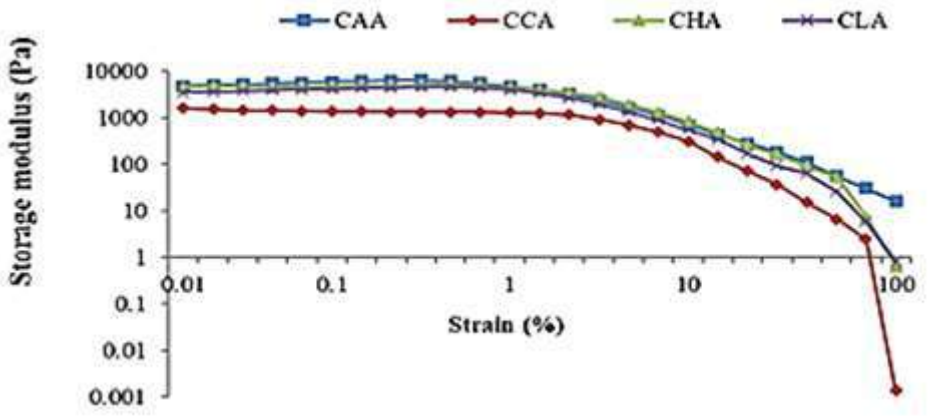

Fig. 1a Strain sweep of goat milk mozzarella cheese prepared by different acidulants

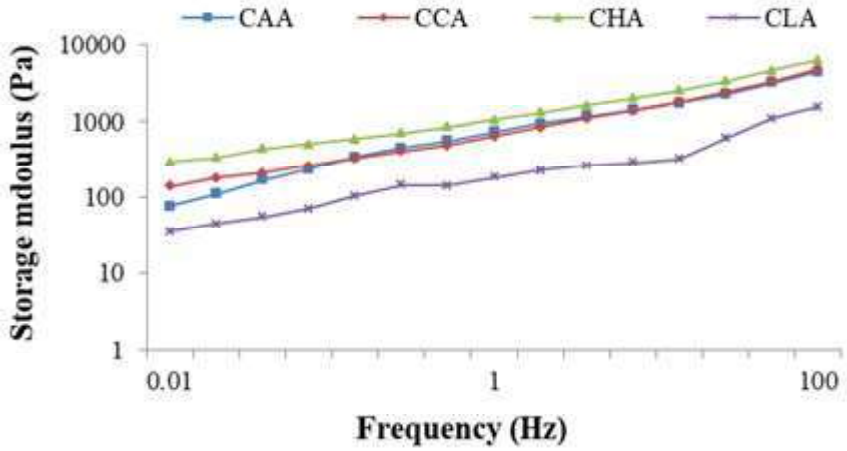

Fig. 1b Storage modulus of goat milk mozzarella cheese prepared by different acidulants as a function of frequency

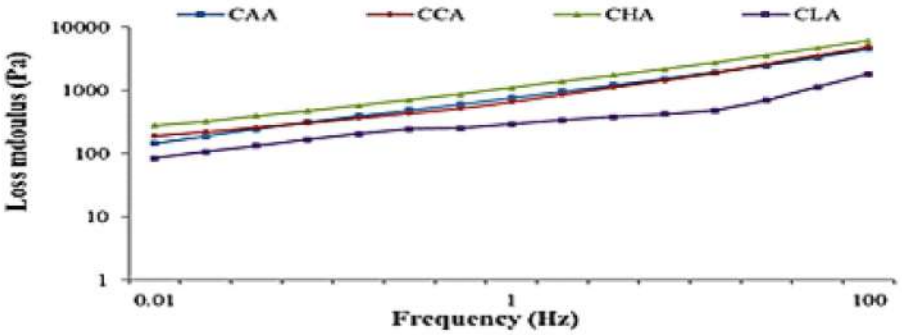

Fig. 1c Loss modulus of goat milk mozzarella cheese prepared by different acidulants as a function of frequency

cheese, so a decrease in their values indicates weak protein-protein interactions. Similar to the frequency sweeps, the dynamic moduli for the treatment CHA was the highest, while for the treatment CLA was the lowest throughout the studied temperature range. The heat-induced changes in the viscoelastic property of cheeses suggest the alteration in their structure, and the cheese showing a lesser degree of structural modifications in the studied temperature range is expected to have low functional properties (Guineeet al. 2002).

Sensory properties

The organoleptic evaluation of the goat milk mozzarella cheeses showed that treatment CCA had significantly higher ($p<0.05$) colour and appearance score than CHA (Table 1). The treatment CHA had a colour and appearance score statistically similar to treatments CAA and CLA. Similarly, differences in the colour and appearance scores of treatments CAA, CCA and CLA were non-significant. There were no significant differences in the flavour, body and texture, and overall acceptability scores among treatments. However, the scores for these sensory parameters for the treatment CCA were non-significantly higher than other treatments. In their study on buffalo milk mozzarella cheese, Seth and Bajwa (2015) also found that products prepared using citric

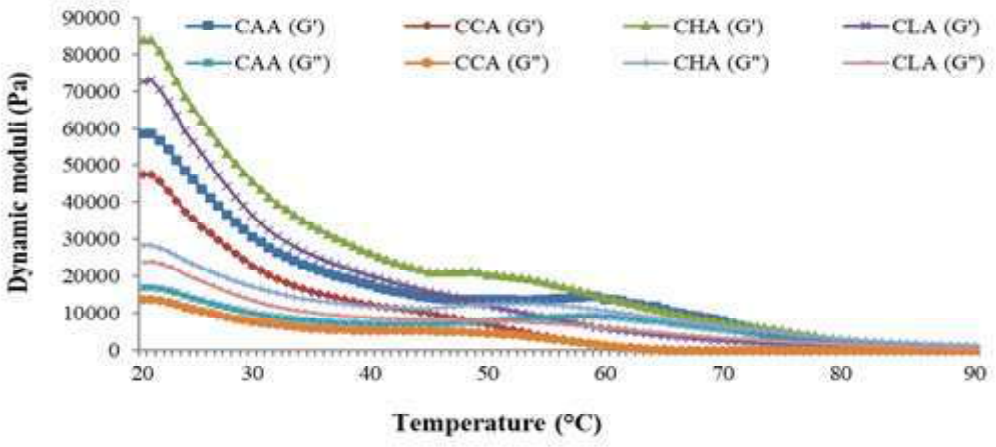

Fig. 1d Temperature sweeps of goat milk mozzarella cheese prepared by different acidulants

acid had a higher appearance and overall acceptability scores as compared to cheeses made with acetic acid and lactic acid.

Conclusions

The findings of the present study showed that the acidulants used for pre-acidification of milk to prepare goat milk mozzarella cheese had significant effects on acidity, moisture, ash, meltability and fat leakage. Hunter colour parameters, especially redness and yellowness values were affected by the used acidulants. Also, acidulants had a significant effect on the textural properties of the mozzarella cheese, and cheeses prepared with acetic acid and hydrochloric acid were harder in relation to citric acid and lactic acid. The evaluation of the rheological parameters of cheeses revealed a similar pattern of structural changes in applied frequency and temperature range. Although cheeses from all the treatments were highly acceptable as rated by the panellists, the assessment of functional and textural properties showed that treatments CCA and CLA were superior to others.

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RESEARCH ARTICLE

Inhibitory effect of spices on beta lactamase enzyme of resistant bacteria isolated from milk of healthy cattle

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Abstract: Present study was undertaken to observe the effect of spices on the extended Spectrum beta lactamase enzyme of ESBL Producing Enterobacteriaceae Group of Bacteria isolated from healthy cattle milk. Out of 100 samples collected randomly from various dairy farms located at the different areas of Rewa 14 samples characterized by the phenotypic standard methods were found to be ESBL positive giving a prevalence rate of 14%. Inhibitory potential and per cent inhibition of beta lactamase enzyme were observed against dry powder of Methi seeds (*Trigonella foenum-graecum*), dry zinger, (*Zingiberofficinale*), Ajwain seeds (*Trachyspermum ammi)* Kalonji seeds (*Nigella sativa*), Black pepper (*Piper nigrum*), Clove bud (*Syzigium aromaticum*) (10mg/ml) and one test drug Tazobactum by colorimetric assay method performed on the individual spices and in their different combinations on the basis of absorbent value. The absorbance level of each spice was observed using NITROCEFIN as a chromogenic substrate at 405nm wavelength. Minimum absorbance value was observed by *Zingiber officinale* (0.48 ± 0.009) and the maximum absorbance value of *Syzgium aromaticum* (1.698 ± 0.069). The inhibitory effect of combination of the spices showed maximum absorbance value (0.46 ± 0.06) by *Piper nigrum* and *Zingiber officinale* No significant difference was seen between the absorbance value of *Z.officinalis*, *N.sativa* (0.61± 0.05) and *P.nigrum T.faenum graecum* (0.62±0.03). Study observed the *in vitro* inhibitory potential present in the spices and could be used in near future to combat antimicrobial resistance to some extent.

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Introduction

Extended spectrum beta lactamase are enzymes that hydrolyze most penicillins and cephalosporins, including oxyimino-beta lactam compounds (cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but not cephamycins or carbapenems (Rahman et al. 2004) Antimicrobial therapies may hasten the emergence of antimicrobial resistance due to these enzyme producing organisms that would, otherwise, be delayed. Exchange of resistance genes between bacteria from different sources can also occur in the environment (Batabyal et al. 2018). Since 2000, the European Antimicrobial Resistance Surveillance Network has reported a steady increase in the rates of invasive *E. coli* and *Klebsiella pneumonia* isolates resistant to third-generation and fourth-generation cephalosporins (Tenover et al. 1999, Paterson and Bonomo,2005)

Milk is a major part of human food and plays a prominent role in the diet. The presence of pathogenic bacteria in milk is of considerable public health concern, especially for those individuals who still drink raw milk. *Escherichia coli* and *Klebsiella pneumoniae* are humans and animals opportunistic pathogens, responsible for a wide range of infections. Milk is an ideal medium for the rapid multiplication of bacteria, particularly under unhygienic production and storage.

Spices have been defined as plant substances from indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste of foods. Spices include leaves (coriander, mint), flower (clove), bulbs (garlic, onion), fruits (red chilli, black pepper), stem (cinnamon), rhizomes (ginger, turmeric) and other plant parts. Apart from providing aroma and flavour spices have been recognized for their properties of preserving foods and medicinal values due to the presence of bioactive compounds (Dhiman et al. 2015, Faujdar et al. 2020). Resistance among pathogenic microbes against various antimicrobial drugs has been an increasingly important and most appalling problem, globally. Synthetic chemicals can be toxic in nature; hence, these spices containing phytochemical, which have both antimicrobial and antioxidant properties, must be taken to control this problem and

could be a better alternative with growing concern of antimicrobial resistance.

Material and Methods

Isolation of ESBL producing *Enterobacteriaceae* group of bacteria

Milk samples were collected from various dairy farms for isolation of ESBL producing *Enterobacteriaceae* group of bacteria. Milk sample (100 samples) enriched in Tryptose soya broth (10 ml/1 ml sample) fresh overnight broth culture was streaked in Tryptone bile glucuronic agar media. Broth and media both were supplemented with cefotaxime(2µgm/ml) and aztreonam(4µgm/ml) to isolate ESBL producing *Enterobacteriaceae* bacteria.

Phenotypic Characterization

Double Disc Synergy Test (DDST): Two cephalosporins (cefotaxime) disc with (amoxicillin-clavulanic acid disc in the centre were placed in the plates. Augmentation of the inhibition zones around any of the cephalosporin discs in the direction of the disc containing amoxicillin-clavulanic acid indicated the positive result. The distance between the discs was kept 20 mm centre-to-centre however it could be reduced or expanded for strains with very high or low levels of resistance(EUCAST, 2013, Castanheira et al.2021).

Ezy MIC strip test: Cefotaxime and Cefotaxime + clavulanic acid E strip were used and test was confirmed positive if MIC ratio e" 8 or deformed ellipse was present around Cefotaxime + clavulanic acid

Combination Disc Diffusion Test (CDDT): For each test, discs containing the cephalosporin alone (cefotaxime 30µgm) and in combination with clavulanic acid (30-10µgm) were applied. The inhibition zone diameter around the cephalosporin disc combined with clavulanic acid was 5mm larger to the zone around the disc with the cephalosporin alone indicated positive results (Garrec et al. 2011)

Inhibitory potential of on extended spectrum beta lactamase enzyme

Preparation of beta lactamase enzyme - Fresh overnight cultures of bacteria were inoculated into broth and grown for 2 h at 35°C in a rotary shaker. Inducer (penicillin-G 400 µg/ml) was added, and incubation was continued for an additional 4 h. The cell pellets were collected by centrifugation, resuspended, and washed with potassium phosphate buffer (0.05M, pH 7.0) at 4°C. The bacteria were recentrifuged and subsequently resuspended in the same buffer that is 10-fold concentrated. The bacteria were disrupted by sonic treatment for 5 minutes in an ice bath. Cellular debris was removed by centrifugation at 10000 rpm for 4 minutes at 4°C. The resulting supernatants containing beta lactamase enzyme were stored in portions at -20°C until required.

Preparation of spices

Spices were collected from local market and grinded. Powder was mixed with distill water to prepare concentration of 10 mg/ml which was further used for colorimetric assay.

Colorimetric assay – Beta lactamase inhibitory potential of seeds of dried powder of each spices *Trigonella foenum-graecum*,*Zingiber officinale*, *Trachyspermum ammi*, *Nigella sativa*, *Piper nigrum*,*Syzigium aromaticum* were analysed by the beta lactamase enzyme inhibitory assay using Chromogenic substrate Nitrocefin. Nitrocefin (98% pure) was dissolved in dimethyl sulfoxide at a final concentration of 0.4 mmol/L. Dried powder of each spices were taken respectively (10 mg/ml) along with the standard beta lactamase inhibitor Tazobactum (100 µM concentration). Briefly 8µl of enzyme was initially stabilized with the 72µl sodium phosphate buffer (100m Micro litre) with pH 7.0 for 10-15 minutes at 25°C. Later on standard beta lactamase inhibitors and powder of each spices were added into the respective wells and plate was again incubated for 20-25 minutes at 25°C.After 25 minutes substrate was added to the wells and again incubated for 20 minutes at 25°c.After desired incubation period plate was read using Lisa plus make Elisa reader – 96 well Micro titre plate. Colour development was analyzed at 405nm wavelength (Linscott and Brown 2005, Solanki and Selvanayagam 2013)

Minimum inhibitory Concentration detection of the Spices

MIC of the spices powder were also determined using serial tube dilution technique as per the method described by CLSI(2011). Spices powder aqueous solution were prepared and serially diluted in the range of 0.1mg/ml to 10 mg/ml. The tubes were inoculated with 100 µl of bacterial culture The density of selected bacteria was adjusted equal to that of the 0.5 McFarland standard (1.5 x 108 CFU/ml) by adding sterile distilled water. Tazobactum was used as the standard drug for comparison. The tubes were incubated at 37°C for 12-18 hours Broth along with inoculum without drug/spices were used as growth control. The growth of inoculum was decreased next tube was taken as MIC.

Statistical Analysis:

The data were analyzed using student's t test and ANOVA when appropriate. Results are presented as mean ± standard deviation. Values of pÂ0.05 were considered as statistically significant.

Results and Discussion

Isolation of ESBL producing Enterobacteriaceae group of bacteria

After initial screening of 100 samples 14 samples was found to be ESBL positive and 86 samples was negative giving a prevalence rate of only 14%. Present study included both pooled and mastitis milk samples. The prevalence rate in the mastitis milk was 0% as no samples turned out to be ESBL positive (Table 1).

Phenotypic Characterization

After initial screening of the 100 samples 14 positive samples were further confirmed by phenotypic methods. As per the CLSI and EUCAST three standard methods have been used for the phenotypic confirmation of ESBL producing isolates. Among the three methods CDDT method showed the maximum sensitivity than the Among the 14 samples all the samples were positive by CDDT method giving 100% sensitivity,10 samples were positive by DDST method giving71% of sensitivity and only 5 samples gave ellipsoidal shape in enzyme MIC strip method giving 35% sensitivity (Gutmann 1985, Taslima 2012).

Inhibitory potential of on extended spectrum beta lactamase enzyme

Inhibitory potential and per cent inhibition of beta lactamase enzyme were observed against dried powder of meethi seeds (*Trigonella foenum-graecum*), Dry Zinger (*Zingiber Officinale*), Ajwain seeds (*Trachyspermum ammi*), Kalonji seeds (*Nigella sativa*), Black pepper (*Piper nigrum*) , Clove buds (*Syzgium aromaticum*). Each of the spices was grinded and prepared and used in the final concentration of 10mg/ml by Colorimetric assay performed on the individual spices and in their different combinations. Inhibitory effect was observed on the basis of absorbent value. The absorbance level of each spices was observed using NITROCEFIN as a chromogenic substrate at 405nm wavelength. Minimum absorbance value was observed by Zingiber officinale (0.48 ± 0.009) and the maximum absorbance value of *Syzgium aromaticum* (1.698 ± 0.069). *T.ammi*, (1.172±0.125), *Piper nigrum* (1.047±0.103) *S.aromaticum* (1.698± 0.069) *Z.officinalis* (0.449± 0.009) showed significant difference

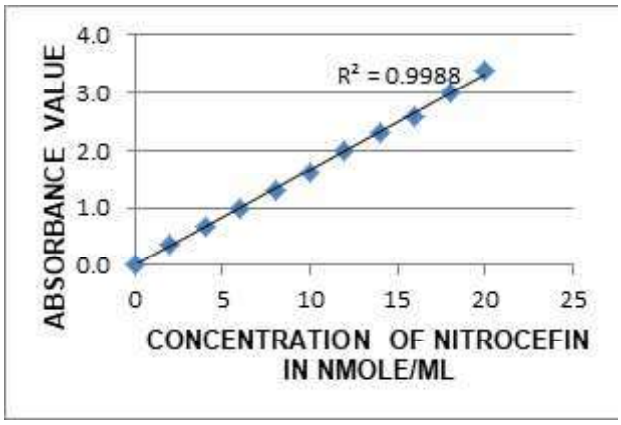

Fig. 1 Standard curve of Nitrocefin in Distill water

Table 1 Per cent Prevalence of ESBL isolates from Mastitis/Pooled milk

No. of Samples Pooled/Mastitis	Positive Samples	Negative Samples	Percent Prevalence
Pooled milk	14	79	14
Mastitis milk	0	7	0

Table 2: Inhibitory Effect of Individual Spices on ESBL enzyme by Colorimetric Method (Absorbance value)

Sample	*T .Ammi*		*P .Nigrum*		*S. Aromaticum*		*T. Fgraecum*		*Z.Officinale*		*N. Sativa*	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	1.229	0.066	1.110	0.065	1.712	0.133	1.30	0.034	0.444	0.007	0.932	0.013
2	1.347	0.228	1.216	0.077	1.625	0.222	1.18	0.250	0.476	0.016	1.157	0.063
3	1.253	0.067	1.171	0.298	1.942	0.041	1.59	0.330	0.469	0.004	0.892	0.066
4	1.342	0.250	1.038	0.039	2.007	0.121	1.82	0.044	0.44	0.011	1.052	0.046
5	1.046	0.030	0.985	0.050	1.806	0.034	1.27	0.260	0.465	0.011	1.152	0.031
6	1.181	0.041	1.102	0.008	1.795	0.044	0.92	0.078	0.451	0.014	0.926	0.037
7	0.971	0.021	0.999	0.041	1.829	0.032	0.90	0.177	0.439	0.004	0.970	0.031
8	1.229	0.331	1.068	0.036	1.725	0.025	1.49	0.210	0.447	0.003	1.000	0.019
9	1.001	0.173	0.853	0.135	1.676	0.032	1.03	0.022	0.450	0.014	0.996	0.021
10	1.131	0.050	1.193	0.077	1.627	0.040	1.33	0.061	0.438	0.008	0.993	0.024
11	1.217	0.124	0.917	0.189	1.579	0.048	1.23	0.117	0.441	0.013	0.990	0.026
12	1.233	0.142	1.132	0.038	1.530	0.056	1.21	0.097	0.444	0.010	0.987	0.029
13	1.159	0.166	1.037	0.099	1.481	0.064	1.21	0.064	0.437	0.004	0.983	0.032
14	1.073	0.068	0.840	0.298	1.432	0.072	1.16	0.078	0.437	0.009	0.980	0.036
Mean ± SE	1.172^{c}	0.125	1.047^{b}	0.103	1.698^{d}	0.069	1.26^{c}	0.130	0.449^{a}	0.009	1.001^{b}	0.034

Mean with different superscript differ significantly ($p < 0.05$)

in their absorbance value ($p<0.05$). Whereas *N.sativa* (1.001±0.034) and *Piper nigrum* (1.047±0.103); *T.ammi* (1.172±0.125), and *T.faenum graecum* (1.26±0.130) showed no significant difference in the absorbance value and inhibitory potential (Table 2 & Fig. 1).

The inhibitory effect of combination of the spices by colorimetric method showed maximum absorbance value (0.46 ± 0.06) by *Piper nigrum* and *Zingiber officinale*. Significant difference in the absorbance value was observed between *Z.officinalis*, T.faenum graecum (0.240±0.03); *Z.officinalis*, *Nigella sativa* (0.61±0.05), *Piper nigrum Z.officinalis*. (0.46±0.06). ($p<0.05$) .No significant difference was seen between the absorbance value of *Z.officinalis, N.sativa* (0.61± 0.05) and *P.nigrum T.faenum graecum* (0.62±0.03). Combination of *T.ammi Z.officinalis* (0.77±0.03); *N.sativa, T.faenum graecum* (0.93±0.04) and *T.ammi, T.faenum graecum* (0.99±0.03) showed significant difference in their absorbance value.($p<0.05$). Significant difference was also observed between P.nigrum, N.sativa (1.23±0.12); *S.aromaticum T.faenumgraecum* (1.38±0.07); *S.aromaticum, Z.officinalis* (1.54±0.02); *T.ammi, S.aromaticum* (2.00±0.14), *P.nigrum, S.aromaticum* (2.23±0.10); *S.aromaticum N.sativa* (2.34±0.03). In each of the above work conducted on the positive isolates Tazobactum was taken as the standard drug in the concentration of 8 µgm/ml. Tazobactum showed lowest absorbance value (0.213± 0.025) giving almost 98% of per cent inhibition against enzyme obtained from each isolate (Table 3-4 & Fig. 2-4).

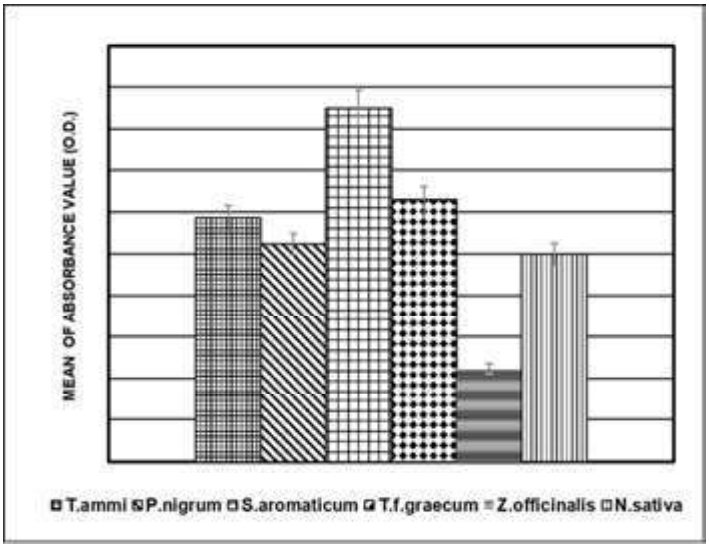

Fig. 2 Inhibitory Effect of Spices on ESBL enzyme by Colorimetric method

Extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae have become a matter of great concern in human and veterinary medicine as these pathogens pose a major challenge for the treatment of general infections and cause a problem with the extensive use of second- or third-generation cephalosporins for the treatment of bacterial infections (Serrano et al. 2009). Out of100 samples 14 samples were found to be ESBL producing Enterobacteriaceae group of bacteria in the initial screening giving a prevalence rate of 14%. Corelating with the study conducted by Batabyal *et al* (2018) in West Bengal where prevalence rate was 12% in healthy animals and another study conducted on healthy broilers of Jabalpur and its adjoining areas confirmed prevalence of 38% of ESBL *E.coli* (Shrivastav et al. 2016)

The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oil, and flavonoids (Keite et al. 2012). Spices, herbs, and their constituents are generally recognised as safe (GRAS) and approved by several regulatory agencies such as US Food and Drug Act, theEuropean Union standards, Codex Alimentarius, and Food Safety and

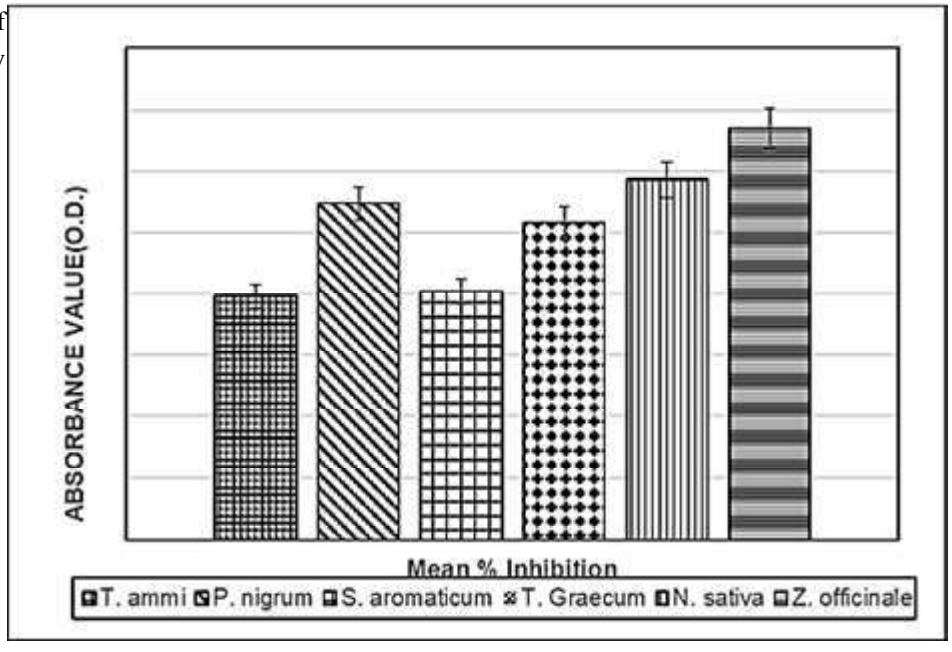

Fig. 3 Inhibitory Effect of Combination of two Spices on ESBL enzyme by Colorimetric method

Table3: Inhibitory Effect of Combination of Spices on ESBL enzyme by Colorimetric method (Absorbance value)

	ZT		***ZN***		***PZ***		***PT***		***TZ***		***NT***		***TT***	
Sample	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	0.30	0.06	0.61	0.02	0.47	0.04	0.63	0.03	0.77	0.02	0.94	0.03	1.09	0.00
2	0.24	0.02	0.58	0.02	0.46	0.06	0.66	0.05	0.81	0.03	0.89	0.02	1.00	0.02
3	0.21	0.04	0.58	0.03	0.45	0.05	0.63	0.03	0.77	0.04	0.84	0.03	0.93	0.07
4	0.26	0.08	0.55	0.08	0.44	0.06	0.59	0.02	0.79	0.02	0.95	0.02	1.01	0.04
5	0.33	0.04	0.57	0.03	0.45	0.08	0.62	0.01	0.80	0.01	0.91	0.04	0.96	0.04
6	0.27	0.03	0.55	0.06	0.44	0.07	0.62	0.01	0.78	0.02	0.95	0.02	0.99	0.03
7	0.27	0.03	0.54	0.07	0.42	0.09	0.61	0.01	0.76	0.01	0.97	0.04	1.03	0.03
8	0.17	0.03	0.61	0.08	0.47	0.05	0.63	0.04	0.79	0.02	0.97	0.04	1.02	0.04
9	0.22	0.04	0.60	0.04	0.44	0.08	0.60	0.03	0.80	0.03	0.95	0.03	0.92	0.03
10	0.22	0.01	0.59	0.05	0.45	0.07	0.61	0.04	0.74	0.02	0.97	0.05	0.97	0.04
11	0.25	0.02	0.58	0.06	0.47	0.06	0.60	0.05	0.79	0.00	0.86	0.07	0.96	0.03
12	0.20	0.03	0.59	0.05	0.46	0.07	0.64	0.02	0.69	0.05	0.92	0.04	0.96	0.01
13	0.20	0.03	0.60	0.05	0.49	0.04	0.64	0.02	0.74	0.02	0.94	0.06	0.99	0.05
14	0.24	0.02	0.62	0.05	0.48	0.04	0.61	0.04	0.73	0.06	0.92	0.08	0.97	0.05
Mean±SE	0.240^{a}	0.03	0.61^{c}	0.05	0.46^{b}	0.06	0.62^{c}	0.03	0.77^{d}	0.03	0.93^{e}	0.04	0.99^{f}	0.03

Mean with different superscript differ significantly ($p<0.05$).
ZT: Zingiber + T ammi; **ZN**- zingiber + Nigella sativa **PZ** – Piper nigrum + Zingiber **PT**-Piper nigrum+ T ammi; **TZ** T foenum-graecum+ Zingiber **NT**- Nigella sativa+ T ammi ; **TT**: T foenum-graecum+ T ammi

Table 4: Inhibitory Effect of Combination of Spices on ESBL enzyme by Colorimetric method (Absorbance value)

Sample	*PN*		*ST*		*TS*		*SZ*		*TS*		*PS*		*SN*		*TN*	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	1.07	0.03	1.27	0.03	1.41	0.02	1.56	0.01	2.18	0.03	2.34	0.01	2.42	0.02	2.43	0.05
2	1.07	0.03	1.23	0.01	1.44	0.02	1.56	0.02	2.05	0.04	2.28	0.10	2.38	0.02	2.40	0.11
3	1.36	0.27	1.29	0.04	1.43	0.04	1.57	0.01	2.06	0.11	2.34	0.07	2.36	0.03	2.34	0.08
4	1.33	0.24	1.38	0.05	1.44	0.03	1.53	0.03	2.15	0.05	2.28	0.10	2.33	0.02	2.33	0.12
5	1.09	0.00	1.33	0.07	1.48	0.01	1.55	0.01	2.04	0.04	2.21	0.10	2.34	0.05	2.42	0.06
6	1.05	0.03	1.36	0.06	1.40	0.08	1.55	0.03	1.89	0.15	2.18	0.06	2.35	0.03	2.28	0.14
7	1.39	0.29	1.46	0.02	1.47	0.02	1.55	0.02	1.99	0.07	2.15	0.07	2.34	0.01	2.54	0.16
8	1.13	0.03	1.40	0.08	1.47	0.01	1.50	0.01	2.09	0.21	2.02	0.01	2.36	0.03	2.40	0.03
9	1.23	0.10	1.33	0.10	1.47	0.02	1.55	0.03	2.07	0.23	2.09	0.10	2.37	0.05	2.36	0.05
10	1.17	0.03	1.36	0.03	1.48	0.01	1.58	0.00	1.96	0.19	2.28	0.13	2.26	0.06	2.39	0.08
11	1.03	0.03	1.46	0.15	1.46	0.01	1.49	0.03	1.90	0.19	2.11	0.12	2.23	0.07	2.30	0.04
12	1.12	0.03	1.41	0.08	1.46	0.03	1.57	0.01	1.88	0.22	2.35	0.27	2.34	0.05	2.33	0.07
13	1.69	0.29	1.50	0.09	1.47	0.03	1.53	0.04	1.88	0.25	2.28	0.12	2.32	0.01	2.34	0.09
14	1.50	0.22	1.50	0.11	1.46	0.04	1.53	0.04	1.80	0.24	2.33	0.15	2.30	0.02	2.36	0.13
Mean± S.E.	1.23^{g}	0.12	1.38^{h}	0.07	1.45^{h}	0.03	1.54^{j}	0.02	2.00^{k}	0.14	2.231	0.10	2.34^{m}	0.03	2.37^{m}	0.09

Mean with different superscript differ significantly ($p<0.05$).
PN- Piper nigrum+Nigella sativa ; ST Syzigium aromaticum + T ammi; TS T fenugraecum + S aromaticum; SZ Syzigium aromaticum+ Zingiber PS – Piper nigrum+ Sygizium aromaticum SN- S. aromaticum+ Nigella sativa; TN - T fenugraecum + Nigella sativa

Standards Authority of India (Dhiman et al. 2015). Colorimetric assay performed with Nitrocefin as the chromogenic substrate against beta lactamase enzyme for all the spices both individually as well as in combination. Nitrocefin, which has been used previously as a competing substrate to monitor enzyme-inhibitor interactions (Gutmann et al.1985; Hedges et al.1975; James 1983) was used in to calculate a relative substrate affinity index based on a 5-min reaction between enzyme, inhibitor, and nitrocefin.

Absorbance value/OD value determined inhibitory potential of spices against the ESBL enzyme. Highest inhibitory potential was observed by *Zingiber officinalis* with OD values of mean of triplicates (0.449±0.009). Lowest inhibitory potential was observed by *Syzygium aromaticum* (1.698± 0.069) correlating with the data reported earlier. No significant difference ($p>0.05$) was observed in the inhibitory effect of *T.ammi* and *T. foenum-graecum* (1.172±0.125; 1.26±0.130) and *Piper nigrum* and *Nigella sativa* (1.047± 0.103; 1.001±0.034) against ESBL enzyme. Inhibitory

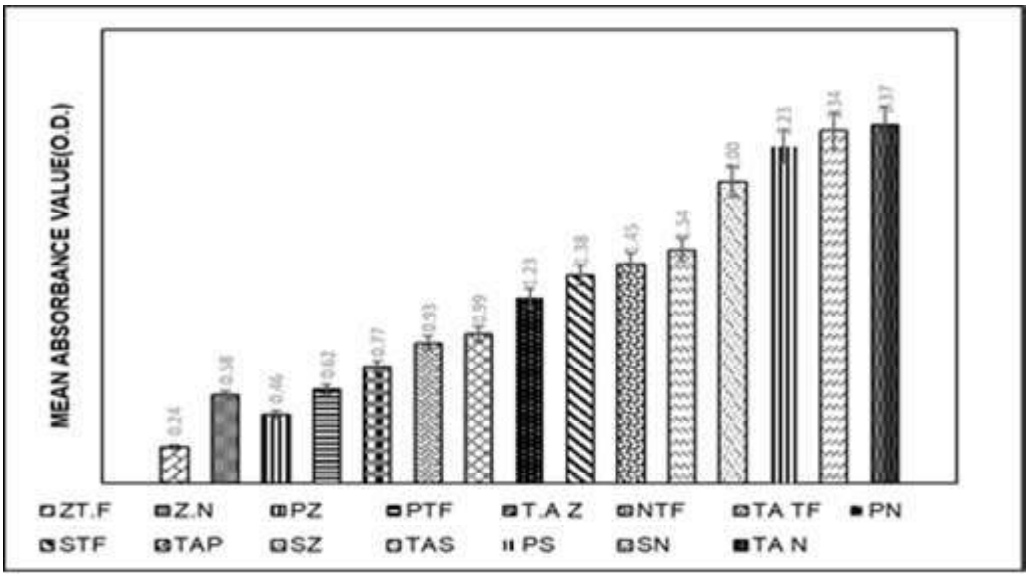

Fig. 4 Per cent Inhibition (Inhibitory Potential) of Spices by Colorimetric method

potential of *T.ammi* and *Piper nigrum* showed significant difference ($p<0.05$) with mean values (1.172±0.125, 1.047±0.103) corelating with the results obtained by Iodometric method (Yang et al.2004) for *Piper nigrum*, *T.ammi* ($p<0.05$). When two spices in equal concentration mixed together inhibitory potential was little different. Maximum inhibitory potential was shown by *Piper nigrum* and *Zingiber officinale* (0.46± 0.06). No significant difference ($p>0.05$) was observed with the combination of *Zingiber officinale* and *Nigella sativa* and *Piper nigrum* and *T.foenum-graecum* (0.61±0.05; 0.62±0.03). Significant difference in the mean values was observed with *Zingiber officinale T.foenum-graecum*; *Zingiber officinale T.ammi*; *Nigella sativa T.foenum-graecum; T.ammi T.foenum-graecum; Nigella sativa T.ammi; Zingiber officinale Piper nigrum Zingiber officinale T.ammi; Zingiber officinale T.foenum-graecum* .Tazobactum taken as standard control gave inhibitory effect as absorbance value of 0.213±0.025 showing almost 95% of inhibition of beta lactamase activity. MIC values for the aqueous solution of the spices were observed by tube dilution method. The MIC of *Zingiber Officinale* was found to be 0.7mg/ml, *Nigella sativa* showed minimum inhibitory concentration at 0.8mg/ml, MIC value of *T.foenum-graecum P.nigrum* and *T.ammi* seed powder in water was observed as 0.9mg/ml,1mg/ml and 0.9mg/ml and highest MIC value was observed for *S.aromaticum* (2mg/ml). Correlating with the data reported earlier with the nitrocefin competition assay. The antimicrobial activity of *C. longa* and *Z. officinalis* was also observed in water and other solvents against food borne pathogens. The solvent extracts of *C. longa* and *Zingiber officinalis* displayed antibacterial and anti-yeast activity. (Sunilson et al. 2009). Similar findings of *Zingiber officinalis* were observed by Lakshmi *et al* (2015) against ESBL isolates. Perusal of their data revealed commonly used herbs and spices such as garlic, black cumin, cloves, cinnamon, thyme, all spices, bay leaves, mustard, and rosemary, possess antimicrobial properties that, in some cases, can be used therapeutically (Kaveri 2019; Lai and Roy, 2004). β-lactamase inhibitors screened from the extracts of traditional Chinese medicines and concluded that the solution of the extracts is often brown or yellow and hinders the reasonable judgment of screening experiments. Tazobactum used as the standard drug showed minimum inhibitory concentration of 8µg/ml. Higher values in many species indicate only a very limited antibacterial efficacy. The drug-resistant ESBL gene is significantly present in approximately 14% of the bacterial strains isolated from pooled milk samples which may be of great health concern for human beings. This drug resistance can easily be transferred between closely related pathogens *in vivo* which may result in risky and fatal health hazards due to unsuccessful treatment with common antimicrobials.

Conclusion

The results of the present study warn the need for stricter preventive measures. For this, regular sterilization of dairy equipment, washing of utensils, milker's hands, udders, eradication of diseased animals, pasteurization/boiling of milk is required before collection and distribution for consumption and product making. The inhibitory effect of spices on the activity of beta lactamase enzyme further states the utility of herbs in the dairy management and could help in limiting the use of antimicrobial agents.

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RESEARCH ARTICLE

Quality assessment of buffalo milk *Chakka* prepared from different starter culture

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Abstract: *Chakka* is a soured dairy product that is produced by removing the whey from fermented milk (dahi, known as Indian yogurt). The starter culture plays a significant role in determining the overall elemental quality of fermented dairy products. The present study was carried to find out the suitability of three different cultures for development of *chakka* viz. T1 (1.5% Mixed microbial culture), T2 (1.5% bacterial culture, STI-13 freeze-dried Lactic culture), and T3 (1.5% yeast cultures). Among the different culture various quality characteristics, physico-chemical parameters and sensory characteristics were assessed for their ability to form *chakka*. Curd prepared from the *Lactobacillus* culture (T2) was found most suitable for preparation of *chakka* as T2 delivered highest yield of curd mass, protein, least pH value and moisture content compared to microbial culture and yeast culture. Sensory attributes of *chakka* also varied significantly (P≤ 0.05) among cultures. All sensory attributes rated higher for T2 (*Lactobacillus* cultures). Therefore, result of the study concluded that T2 (1.5% *Lactobacillus*) culture was most appropriate for formulation of *chakka*.

Keywords: *Chakka*, Cultures, pH, Proximate composition, Sensory

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Introduction

Milk is highly nourishing and perhaps a fundamental food for human diet. With a production of 209.96 million tonnes in 2020–21, India is presently a leading producer of milk. Approximately 65% to 70% of the total amount of milk produced is sold as liquid milk, and 14% is used to make dairy products (ICFA, 2019). Fermented products comprise on an average 30% of the dietary worldwide (Borresen et al. 2012). In India, according to estimates 6.9% of all the milk produced is used to make dahi (Joshna et al. 2021). Among the fermented dairy products, traditional to India; curd/dahi, srikhand, lassi, mishti dahi and butter milk are major products.

Curd, also known as Dahi is the well liked traditional fermented milk product, which is developed from lactic acid fermentation of milk. Similar to milk, curd is very nutrient-dense and a crucial component of a balanced diet. Curd is a super-food that promotes healthy digestion, acts as a natural laxative, and is packed with essential nutrients like calcium, vitamin B-2, vitamin B-12, potassium, and magnesium. Among different types of milk, buffalo milk is more readily used in curd production and contributes 35% of total milk production (BAHS, 2019). Buffalo milk produces higher yield of curd because of higher total solids content especially fat and protein. Buffalo curd complements the required curd traits with its firm solid texture and white color. It also has lower percent of syneresis because of its higher fat and total solid content. Naturally occurring constituent act as stabilizer in curd that lower the exclusion of whey from curd mass. Therefore, curd prepared from buffalo milk is more superior in texture and body.

In order to better serve consumers, dahi varieties have also been developed. Other products, like *chakka*, are also prepared using it. By draining the whey from curd, a white to pale yellow semi-solid product with good texture and consistency is known as *chakka*. *Chakka* has an acidity of 0.7 to 1% and has a sour flavour (lactic acid). It has superior nutritional qualities and is a good source of proteins, vitamins, minerals, and calcium. It also has all the same health advantages as dahi (Sarkar, 2008). According to reports, *chakka* had a higher protein efficiency ratio (PER) than

pure casein (Joshna et al. 2021).

The present investigation was carried out to find out the suitability of various cultures for development of *chakka*. Mixed culture, bacterial culture (STI-13 freeze-dried lactic culture), and yeast cultures were assessed for their ability to form desirable curd and curd mass on the basis of physico-chemical parameters and sensory characteristics.

Materials and Methods

Raw buffalo's milk was purchased from nearby village dairy plant, featuring properties like**:** pH = 6.7, titratable acidity = 0.14%, moisture = 84.1%, ash = 0.69%, fat = 5.37% determined with help of milk lab equipment's. All chemicals and reagents used in the study were of analytical grade and were obtained from standard firms (Himedia, SRL and CDH etc.). For packaging of samples Low density polyethylene (LDPE) of 200 gauge was purchased locally from Meerut market.

Culture: In the study three types of cultures were used for inoculation and were encoded as:

T1: milk + 1.5% mixed culture;

T2: milk + 1.5% freeze dried bacterial culture

T3: milk + 1.5% yeast culture.

The mixed culture produced by back sloping method was utilized. Freeze-dried lactic culture was bought from CHR Hensen, Denmark. In 50 ml of distilled water, 0.4 g of STI-13 freeze-dried lactic culture was dissolved to create the active culture. The mixture was then placed in a bottle, sealed, and refrigerate until use. While for yeast culture commercially available dry yeast culture was obtained from local market. The yeast culture was activated by mixing measured amount of dry yeast @1.5% with ½ cup (app. 125 ml) of water (at a temperature range between 37 to 43°C). Stirred till dissolved and then used.

Preparation of *Chakka*

Chakka was prepared using the techniques outlined by Ronak et al. (2016) with a few minor modifications. In brief, 1500 ml of buffalo's raw milk was heated to 85°C±5 for 20 minutes, followed by cooling to 43°C±2, followed by distribution in three portions (500 ml milk per portion): the first portion was inoculated with 1.5% traditional culture and named T1, the second portion was inoculated with 1.5% freeze-dried lactic culture (bacterial culture) and named T2 and the third portion was inoculated with 1.5% yeast culture and named T3. Then, incubation was allowed to be carried out at 37±2°C for 5-6 hours. For each curd type, the obtained curd was then hanged in muslin cloth at 4°C overnight and whey was allowed to drain (Fig. 1 & 2).

Analytical procedure

Curd yield (%)

Percent curd yield of the chakka was estimated as per Murphy et al. (1975) by the following formula:

$$\text{Curd yield} = \frac{\text{weight of curd obtained g}}{\text{weight of milk g}} \times 100$$

pH and Titratable acidity

Using a pestle and mortar, ten grams of samples were mixed for one minute with 50 ml of distilled water. By dipping an aliquot of the sample into the combined glass electrode of a digital pH metre (Esico (Model-1012), Microprocessor based pH system), the pH of the sample was determined (Trout et al. 1992). While, the titratable acidity in terms of percent lactic acid was determined by method as described by Shelef and Jay (1970).

Spontaneous whey separation (%)

The spontaneous whey separation (SWS) was assessed by the drainage method under refrigeration temperature as per Isanga and Zhang (2009) by employing the following equation

$$SWS\ (\%) = V1/V2 \times 100$$

Where,

V1- volume of expelled whey collected after draining

V2- volume of curd sample

Chakka mass yield:

Percent chakka mass yield was estimated as per Murphy et al. (1975) by the following formula:

$$\text{Chakka mass yield} = \frac{\text{weight of chakka obtained g}}{\text{weight of curd g}} \times 100$$

Proximate analysis

The proximate analysis of chakka was done following methods described by AOAC (1995) for moisture using hot air oven (Meta-Lab Scientific Industries), fat with SOCS PLUS (SCS-06-AS DLS TS, Pelican Industries, Chennai) and ash (muffle furnace) while automatic digestion and distillation unit (KEL PLUS-KES 12L R TS, Pelican Industries, Chennai) was used for protein estimation following methodology given in AOAC (2000). Total carbohydrates percent was calculated using Atwater values by difference as follows using Atwater values:

$$TC\ \% = [100 - (\text{moisture} + \text{protein} + \text{fat} + \text{ash})]$$

Sensory evaluation

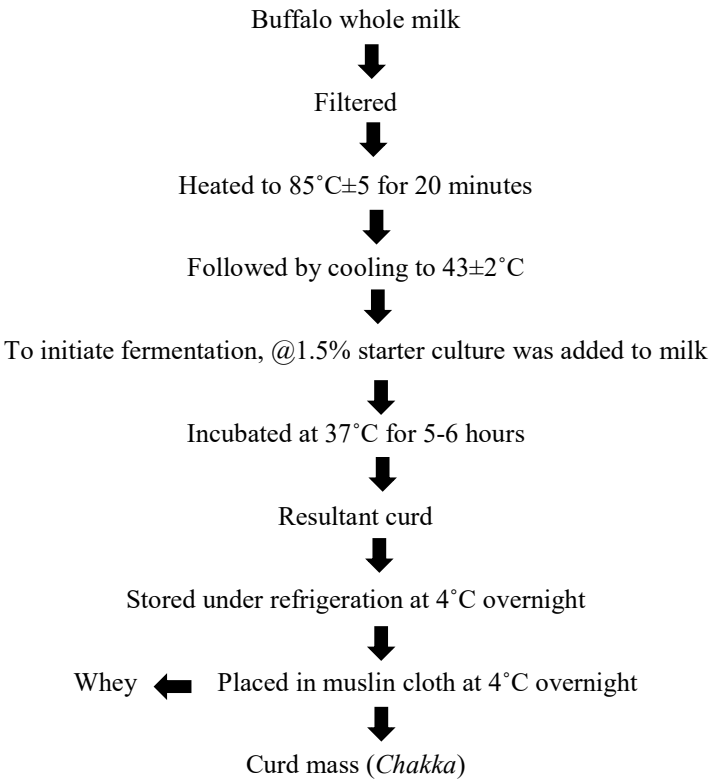

Fig. 1 Flow chart for preparation of *chakka*

The sensory evaluation of chakka prepared were analyzed by experienced panelists from the Department of Livestock Products Technology and College of Veterinary and Animal Sciences, SVPUAT- Meerut, Uttar Pradesh. A nine-point descriptive scale was used for analysis of taste, aroma, texture and overall acceptability of chakka, where 9 was extremely desirable, 5 was neither like nor dislike and 1 was for extremely undesirable.

Statistical analysis

The whole experiment was repeated thrice with duplicate sampling (six observations) for all studied parameters except sensory evaluation where number of observations were 21 (7x3). The data obtained was analyzed for ANOVA using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). While, level of significance was evaluated by Duncan's multiple range test and homogeneity test at ($P \leq 0.05$) level.

Results and Discussion

Quality characteristics of curd

Curd yield

The microorganisms of starter culture play a very crucial role in production of curd with characteristic flavour and acidity. The three starter cultures in the present study mixed, bacterial and yeast culture did not show significant ($P \geq 0.05$) difference in the curd yield (Table 1). The mean ± S.E. values for curd yield of T1, T2 and T3 were recorded as 90.04±1.09 (T1), 92.49±1.05 (T2) and 91.48±1.45 (T3), respectively.

pH and acidity of curd

Addition of microorganisms in milk lead to aggregation of milk proteins due to acidification. The inoculation of different cultures produced curd with varying pH (Table 1). The pH values of mixed and bacterial culture did not differ significantly ($P \geq 0.05$) which might be attributed to the presence of *Lactobacillus*. The mixed culture is primarily cocktail of *Streptococcus* and *Lactoba-*

Table 1: Quality characteristics of curd made from different cultures

Parameters	T1	T2	T3
Curd yield (%)	90.04±1.09	92.49±1.05	91.48±1.45
pH	4.83^{a}±0.02	4.84^{a}±0.03	5.88^{b}±0.03
Acidity (% lactic acid)	0.875^{c}±0.026	0.748^{b}±0.033	0.478^{a}±0.014

Means values bearing small letters (a, b, c) groups wise indicate differ significantly ($P \leq 0.05$) n=6; T1: curd made with mixed microbial culture; T2: curd made with lactobacillus culture; T3: curd made with yeast culture.

Table 2: Quality characteristics of whey made from different cultures

Parameters	T1	T2	T3
Volume of whey (ml)	20.49^{a}±0.57	18.35^{a}±0.46	36.24^{b}±1.39
pH	4.80^{a}±0.02	4.92^{a}±0.01	5.62^{b}±0.10
Acidity (% lactic acid)	0.915^{c}±0.020	0.760^{b}±0.019	0.395^{a}±0.018
SWS (%)	22.78^{b}±0.75	19.82^{a}±0.35	39.57^{c}±1.10

Means values bearing small letters (a, b, c) groups wise indicate differ significantly ($P \leq 0.05$) n=6; T1: curd made with mixed microbial culture; T2: curd made with lactobacillus culture; T3: curd made with yeast culture

Fig. 2 Curd mass (*chaka*) prepared by using various culture

Mixed culture curd mass

Bacterial culture curd mass

Yeast culture curd mass

cillus both of which act on lactose to produce lactic acid. The T3 evinced significantly (P≤ 0.05) highest pH values among all.

Acidity of curd plays very crucial role in its structure and firmness which is a desirable characteristic of good quality curd (Table 1). All the samples exhibited significant differences (P≤ 0.05) in acidity values of which T3 recorded the lowest value and T1, the highest. The difference in acidity might be attributed to fermentation process in which mixed and bacterial cultures utilise lactose in milk while *Saccharomyces cerevisiae* does not utilises lactose (Roostita and Fleet, 1996). The titratable acidity for a good product is around 0.85-0.9% (Jay et al. 2008).

Quality characteristics of whey made from different cultures

Volume of whey (ml)

Volume of whey produced during curd formation depends on factors such as composition of milk, starter culture and processing conditions (Gyawali and Ibrahim, 2016). T1 and T2 showed insignificant (P≥0.05) difference in the volume of whey produced while, T3 recorded significantly (P≤ 0.05) highest volume (Table 2). This might be related to our previous results of curd acidity where it is evident that due to low acidity the structure of curd was not firm. Firm structure has higher water holding capacity due to the branched protein network which entraps water molecules. Due to loose structure, higher whey off was obtained in T3. Weak yoghurt gel showed higher permeability and tend to release higher whey volumes (Lee and Lucey, 2003).

pH and acidity of whey

The pH of whey obtained from T3 recorded significantly (P≤ 0.05) higher values than T1 and T2 whereas, pH of T1 and T2 differed non-significantly (P≥0.05) (Table 2). Lower pH value in T1 and T2 might be implicated to higher lactose degradation by bacterial cultures present in mixed and bacterial culture leading to lactic acid accumulation. The acidity of whey of all samples were recorded and expressed as percent lactic acid. All the samples evinced significantly (P≤ 0.05) different acidity that were recorded as 0.915±0.02, 0.760±0.019 and 0.395±0.018 for T1, T2 and T3, respectively. Overall, T1 unveiled highest acidity, followed by T2 and T3 recorded least percent acidity. T1 was incubated with mixed culture which contained various bacterial population of which *Streptococcus* grows rapidly during initial phase and is largely responsible for acid production (Jay et al. 2008). This acid production occurs at higher rate than that produced later by *Lactobacillus* sp. this might be implicated to the lower pH of T1 followed by T2.

Spontaneous whey separation (SWS)

The spontaneous appearance of serum from milk gel due to acidification is known as Whey off or spontaneous whey separation. It is a very important aspect of yoghurt or dahi production which is vital for its consumer appeal and quality. In the present experiment spontaneous whey separation from all three samples were significantly (P≤ 0.05) different (Table 2). T3 recorded the highest whey off followed by T1. T2 evinced the least whey off. A strong and firm gel network prevents wheying off. Dahi or yoghurt gels are formed due to interaction of denatured whey protein with the casein micelles forming a cross link network. As the pH of milk falls and reaches casein isoelectric point (4.6) the structure gets firm. The high whey off in T3 might be attributed to its weak structure due to low acidity and high pH. Lee and Lucey (2004) have reported that unstable network causes greater spontaneous whey separation due to gel network.

Physicochemical characteristics and proximate composition of curd mass (*chakka*) prepared from different cultures

Curd mass yield

The curd mass yield made from different cultures evinced significant (P≤ 0.05) differences (Table 3). Overall, lactic culture (T2) unveiled the highest yield of curd mass which might be due to the least whey off, followed by mixed culture curd (T1). The least yield by yeast culture curd (T3) might be due to lower coagulation of milk proteins and high whey off.

pH and acidity of chakka

Two concepts that relate to acidity in food analysis are titratable acidity and pH. Each of these quantities has been analytically

determined in a unique manner, and each offers a unique perspective on the quality of the food. The pH values for T1, T2 and T3 showed significant (P≤ 0.05) difference (Table 3). T2 recorded the lowest (P≤ 0.05) pH values followed by T1. The low value of T1 and T2 might be due to the formation of lactic acid when milk changes to curd. The findings were supported by the reports of Daeschel (1993) who noted the ability of lactic acid bacteria to form lactic acid, thereby declining the fermenting medium pH value. Thus, decline in pH makes the medium acidic which is not favorable for the endurance of spoilage organism. Akabanda et al. (2014) also reported that low pH is essential for coagulation and prohibition or depletion of growth of unpremeditated microflora in curd/ yoghurt production. Yeast cultured curd mass T3 reported highest (Pd"0.05) pH which might be attributed to the ability of yeast culture to exploit other organic acids, enhancing the pH of the medium (Rekha and Vijayalakshmi, 2008).

While pH is important for determining a microorganism's ability to grow in a specific food, titratable acidity is a better predictor of how organic acids in the food impact flavour than pH. The difference in acidity was significantly (P≤ 0.05) evident in all samples (Table 3). T1 presented the highest acidity and T3 the least. Treatment T3 evinced lower percent lactic acid which might be due to utilization of more lactose as their ability to produce carbon dioxide and ethanol (Gadaga et al. 2007) along with lactic acid production. Fast acidification is a pre-eminence of starter cultures for development of fermented milk products. For dairy fermentation process, the rapid acidifying culture are therefore good agents as prime starter culture, while culture with poor acidification utilized as adjunct cultures relying on other characteristics (Ayad et al. 2004). T1 evinced more acidity followed by T2 as lactic acid bacteria (LAB) present in culture ferment the carbon source available in raw products into lactic acid with concomitant pH depletion (Kandasamy et al. 2018). The findings are also very well supported by the report of Rekha and Vijayalakshmi (2008) who reported increase in titratable acidity of fermented soy milk during fermentation is due to production of acid.

Proximate composition

The T3 showed significantly highest moisture (%) among all, whereas T2 recorded least moisture value (Table 3). The moisture content of T1 was non-significantly higher than T2. The present study was in agreement with the study of Everard et al. (2011) which stated that curd moisture was affected by total solids of milk. In the study, it was observed that decreased Protein: Fat ratio was associated with improved total solids in milk which led to reduced curd moisture and increased curd yield. The result also concorded with the finding of Ozer (2006) that reported higher total solids in strained yogurt.

Casein, the reserve protein of milk that gives curd its white colour, is the main ingredient in curd. The curd's primary biological value is its high protein content (typically 10-12%), which varies slightly depending on the curd variety. All samples recorded significantly (P≤ 0.05) different protein percent, where T2 showed the highest protein content followed by T1 and T3 reported the lowest protein content (Table 3). The higher protein content of T2 and T1

Table 3. Physicochemical characteristics and proximate composition of curd mass (*chakka*) prepared from different cultures

Parameters	T1	T2	T3
Curd mass yield (gm)	73.77^{b}±0.65	77.05^{c}±0.78	52.02^{a}±1.41
pH	4.74^{b}±0.02	4.46^{a}±0.03	5.63^{c}±0.04
Acidity (% lactic acid)	1.063^{c}±0.025	0.836^{b}±0.016	0.51^{a}±0.017
Moisture (%)	61.46^{a}±1.55	59.05^{a}±0.76	69.66^{b}±0.33
Protein (%)	16.10^{b}±0.38	17.72^{c}±0.55	14.32^{a}±0.30
Fat (%)	13.57^{b}±0.34	14.06^{b}±0.44	12.46^{a}±0.30
Ash (%)	1.08^{b}±0.02	1.12^{b}±0.01	1.00^{a}±0.02
Carbohydrates (%)	5.79^{b}±1.37	5.57^{b}±0.75	2.58^{a}±0.23

Means values bearing small letters (a, b, c) groups wise indicate differ significantly (P≤0.05) n=6; T1: curd made with mixed microbial culture; T2: curd made with lactobacillus culture; T3: curd made with yeast culture.

Table 4: Sensory evaluation of curd mass (*chakka*) prepared from different cultures

Parameters	T1	T2	T3
Colour and appearance	7.93^{b}±0.20	8.29^{b}±0.18	6.29^{a}±0.15
Taste	7.71^{b}±0.14	8.32^{c}±0.09	5.50^{a}±0.18
Aroma	7.57^{b}±0.44	8.36^{b}±0.21	5.71^{a}±0.41
Texture	6.93^{ab}±1.16	8.50^{b}±0.12	6.04^{a}±0.15
Overall acceptability	7.64^{b}±0.18	8.25^{c}±0.09	5.54^{a}±0.23

Means values bearing small letters (a, b, c) groups wise indicate differ significantly (P≤0.05) n=21; T1: curd made with mixed microbial culture; T2: curd made with lactobacillus culture; T3: curd made with yeast culture.

might be implicated to the presence of *Lactobacillus* species which were present in mixed and bacterial cultures. The protein content in fermented milks is increased due to protein arising from *Lactobacillus helvictus* (Rekha and Vijayalakshmi, 2008). Similarly, Hou et al. (2000) also observed increased protein content in fermented milk with Bifidobacteria.

Overall, T2 showed the highest Fat (%) that differed non significantly (P≥0.05) from T1 while T3 showed significantly the lowest fat (%) (Table 3). Lower fat content in T3 than T1 and T2 might be due to more loss of fat content in whey off as T3 evinced highest spontaneous whey separation value. Samanta, et al. (2015) stated that curd have more nutritional values than milk. Although there is no increase in fat content of milk during the process of fermentation.

The estimation of ash content of *chakka* revealed that T2 showed the highest ash (%) that differed non significantly (P≥0.05) from T1, whereas T3 exhibited significantly (P≤ 0.05) the lowest ash (%) (Table 3). The carbohydrates (%) of *chakka* recorded the highest values in T1 which was comparable to T2 and T3 showed the lowest carbohydrate (%) value. No significant (Pe"0.05) differences were observed in carbohydrate content between T1 and T2, the findings were supported by the reports of Samanta et al. (2015) that reported no increase in carbohydrates during fermentation of milk. The significantly lower content of carbohydrates in yeast culture curd (T3) might be due to their capability to amend carbohydrates into ethanol and carbon dioxide (CO_2), is determined by Louis Pasteur in the 1860s.

Sensory evaluation of curd mass (*chakka*) prepared from different cultures

The data presented in Table-4 revealed significant difference in sensory scores of colour and appearance, taste, aroma, texture and overall acceptability among cultures. Among the three cultures, T2 showed fairly high sensory scores of 8.25 and above, followed by T1 sensory scores in the range of 6.93 to 7.93 indicating acceptability by the sensory panelists and T3 showed lowest sensory score ranging from 5.5 to 6.29 indicating non acceptability. Granata and Morr (1996) reported that the physical stability, texture, aroma and taste of yogurt are related to pH changes. T2 exhibited highest colour and appearance score as compared to T1, however the difference was not significant (P≤ 0.05). T3 exhibited the lowest appearance score among all cultures. The lower appearance score for T3 might be due to the appearance of large pores and runny structure.

Significant difference (P≤ 0.05) was observed in sensory taste scores among cultures. T2 exhibited highest taste score at 8.32 followed by T1 whereas, T3 exhibited the significant (P≤ 0.05) lowest taste score. Fermentation of milk, yeast culture breaks down the native protein of milk and generated some bitter peptides molecules, alcohol as well as carbon dioxide which leads to decreased sensory attributes than the *Lactobacillus* and mixed culture. Sharma et al. (2020) also reported that during the fermentation yeast culture produce alcohol as well as carbon dioxide.

Aroma is one of the most important sensory parameters for dairy products. Dairy products are rich in small chain fatty acids which are responsible for its characteristic flavour. The flavour of fermented dairy products depends largely on the fast acidification of raw materials that prevents the growth of harmful microorganisms, and imparts desired characteristic aroma, texture and taste of the final product (Akabanda et al. 2014). Bacterial culture (T2) exhibited highest flavor score followed by mixed culture (T1) although the difference was not significant. Lactic acid bacteria improve the nutritional value, taste, aroma and texture of fermented foods, including dairy products (yogurt, cheese buttermilk and fermented milk), sour dough bread, fermented beverages, fermented vegetables and meat (Leroy and De Vuyst, 2004; Landete, 2017). Yeast culture (T3) exhibited lowest sensory score showed significant difference (P≤ 0.05) as compared to T1 and T2. Texture score was found to be highest in T2. T3 exhibited lower texture scores as compared to T1 and T2. Acidity can also cause souring in the final product, which is a characteristic of fermented foods. Mostly, lactic acid bacteria ferment the available carbon sources in raw food into lactic acid and at the same time lower the pH value, resulting in significant effects, such as removing undesirable organisms and improving sensory properties and texture, as well as imparting health benefits. The overall acceptability score differed significantly (P≤ 0.05) among different cultures. T2 recorded highest overall acceptability followed by T1 and T3 exhibited lower scores. The higher acceptability of T2 might be attributed to the overall good appearance, well developed flavor, firm structure and appealing taste due to proper fermentation.

Conclusion

Utilizing mixed culture, bacterial culture and yeast culture for developing chakka evinced significant changes in quality characteristics. Inoculating milk with 1.5 % of mixed, bacterial and yeast culture respectively produced curd, whey and chakka of varying quality. The results indicate that the highest curd yield, chakka yield, protein content as well as overall acceptability was observed in T2 (bacterial culture inoculation).

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RESEARCH ARTICLE

Energy saving through partial homogenization of milk over conventional milk homogenization

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Abstract: The present study was conducted to check the feasibility of partial homogenization of market milk used for the preparation of tea (commonly known as '*chai*' in India) and thereby estimate the energy-saving potential of the process. In the case of partial homogenization of milk, cream with different fat content (15%, 20%, and 25%) was homogenized at three first-stage pressures viz., 1000 psi, 1200 psi, and 1500 psi while keeping the second-stage pressure constant at 500 psi. The homogenization of milk was conducted at 65°C and 70°C temperatures. The homogenized cream was used to prepare standardized milk having 4.5% fat and 8.5% solids-not-fat. The temperature and pressure for homogenization were optimized based on the desirable least creaming index of samples of standardized milk at 48 h of refrigerated storage (4±2°C). In the case of conventional homogenization of milk, milk was first standardized to 4.5% fat and 8.5% solids-not-fat and then homogenized at 2000 psi and 500 psi for the first and second stages respectively. Tea was prepared from milk obtained from both the homogenization processes and they were subjected to sensory evaluation. The energy consumption in both cases was measured from the energy meter installed at the homogenizer motor. The study revealed that, without affecting the quality of homogenized milk, about 68% of energy could be saved in partial homogenization of milk as compared to conventional homogenization of milk.

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Introduction

Homogenization refers to the process of forcing the milk through a homogenizer with the object of sub-dividing the fat globules (De, 2001). The greater part of the fat volume in milk consists of globules with a diameter ranging from 2 to 6 µm (Ahmad, 2012). Homogenization of milk has become a standard industrial process, universally practiced as a means of stabilizing the fat emulsion against gravity separation (Huppertz, 2022, Bylund, 2003). Using pressures between 20 and 100 MPa, the dairy industry has been securing the quality and stability of its products for decades (Dos Santos, 2022). The aim of homogenization is to prevent the unsolicited fat separation occurring in milk, destined for long storage and also to increase viscosity of milk to prepare a greater number of tea cups from a given quantity. The standardized pasteurized milk is not homogenized except tea-special milk (AMUL Brand) intended to be used for preparation of tea. Complete homogenization is the most commonly used form of homogenization of milk wherein the entire quantity of milk is passed through the homogenizer. In partial homogenization, only cream portion is subjected to homogenization; skim milk used to standardize the fat content of cream remains unhomogenized. Partial homogenization of milk can be used to reduce energy and operating costs (Bylund, 2003). The effect of partial homogenization on quality of dairy products have been studied by a few researchers (Jana et al. 2016), however, no study has been conducted to evaluate the effect of partial homogenization of milk on quality of milk and tea (commonly known as '*chai*' in India) prepared from such milk. The homogenization of milk is one of the energy intensive processes in the dairy industry (Samuelsson and Lindberg, 2018). Partial homogenization can be adopted to reduce energy consumption and processing cost in dairy industry. According to the International Energy Agency (2019), improved energy efficiency in industrial processes could reduce the world's energy needs in 2050 by one third, and help control global emissions of greenhouse gases. Hence, the present study was conducted to evaluate energy saving potential of partial homogenization of milk over conventional homogenization of milk. The sensory evaluation of tea prepared from the milks

obtained from both the homogenization processes were carried out to check the feasibility of partial homogenization of milk over conventional homogenization of milk.

Material and Methods

Optimizing temperature of partial homogenization

Required quantity of skimmed milk (0.05% fat) and pasteurized cream (40% fat) were procured from milk co-operative society of Anand city of Gujarat. They were used to prepare standardized cream with varying fat content i.e. 15%, 20% and 25%. Standardized creams were subjected to homogenization at 65°Cand 70°C by varying first stage pressure i.e. 1000 psi, 1200 psi and 1500 psi; second stage pressure was 500 psi. These creams were used to prepare standardized milk of 4.5% fat which was then subjected to pasteurization at 78°C for 1 min and immediately cooled to 4°C. Milk samples were evaluated for cream line formation through visual observation by storing them in one liter beakers at 4°C for 48 h. Beakers used for the storage of samples had same dimensions. Based on visual observation of cream line formation in milk samples, temperature of cream homogenization was optimized.

Optimizing the cream fat and the pressure of partial homogenization

Required quantity of skimmed milk (0.05 % fat) and pasteurized cream (40% fat) were used to prepare standardized cream of different fat percentages i.e. 15%, 20% and 25%. Standardized creams were then subjected to homogenization at optimized temperature by varying first stage pressure i.e. 1000 psi, 1200 psi and 1500 psi; second stage pressure was 500 psi. These creams were used to prepare standardized milk of 4.5% fat which was subjected to pasteurization at 78°C for 1 min. and immediately cooled to 4°C. Milk samples were evaluated for Creaming Index (CI) and viscosity (Centipoise) by quiescently storing them in one liter beakers at 4°C for 48 h. Beakers used for the storage of samples had same dimensions.

CI of milk samples was performed by USPHS method (Bylund, 2003). According to the method, a sample of 1000 ml milk is stored for 48 h at 4°C, after which the fat content of the top 100 ml (sample A) and remaining 900 ml (sample B) were determined by MilcoScreen™ (Manufacturer: Foss, Denmark). CI of milk was calculated as follows

$$CI = \frac{A-B}{B} \times 100$$

Where, A: fat (%) of top 100 ml of sample at 48 h of storage at 4°C

B: fat (%) of remaining 900 ml of sample at 48 h of storage at 4°C

Viscometer (Make: Brookfield, Model: DV) was used to measure the viscosity of milk samples using S61 spindle at 100 rpm and 25°C. One litre milk was taken for the analysis. Based on CI and viscosity of milk samples, the level of cream fat and first stage homogenization pressure for cream were optimized.

Comparison of CI and viscosity of optimized partially homogenized milk with the conventionally homogenized milk

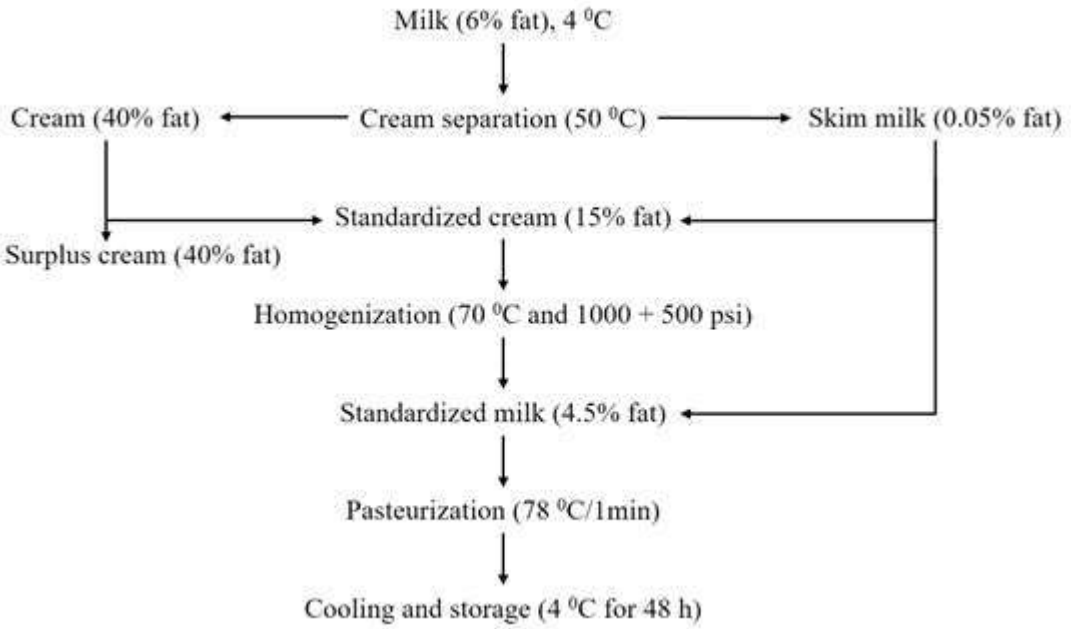

Fig. 1 Optimized process flow chart for partial homogenization of milk

***Preparation of partially homogenized milk*:** Required quantity of skimmed milk (0.05 % fat) and pasteurized cream (40% fat) were used to prepare standardized cream of optimized fat contents. Standardized creams were then subjected to homogenization at optimized temperature and pressure. These creams were used to prepare standardized milk of 4.5% fat. The further steps followed is indicated in figure 1.

***Preparation of conventionally homogenized milk*:** Required quantity of skimmed milk (0.05 % fat) and full cream milk (6.00% fat) were used to prepare standardized milk with 4.5% fat.

Both the milks were then subjected to pasteurization at 78°C for one minute and immediately cooled to 4°C. The further steps followed is indicated in figure 2. Milk samples were evaluated for CI and viscosity by storing them quiescently in one-liter beakers at 4°C for 48 h. Beakers used for the storage of samples were having the same dimensions. The same methods were used for analysis of milk samples for the CI and viscosity as mentioned below.

Comparison of sensory scores of tea prepared using optimized partially homogenized milk and conventionally homogenized milk

Tea was prepared from 'partially' and 'conventionally' homogenized milk and subjected to sensory analysis using nine-point hedonic scale (Nicolas et al. 2010).

Measurement of energy consumption during partial homogenization and conventional homogenization of milk

Energy consumption of homogenizer during partial and conventional homogenization of milk were measured using three phase energy meter (Debastiani et al. 2014).

Calculation of energy savings through partial homogenization of milk vis-a-vis conventional homogenization

Total energy savings during partial vis-a-vis conventional homogenization of milk was calculated using the following formula

$$\% \text{ Energy saving} = \frac{C-P}{C} \times 100$$

Where, C = Energy consumed during conventional homogenization (kWh)

P = Energy consumed during partial homogenization (kWh)

Results & Discussion

Optimizing temperature of partial homogenization through cream line formation in standardized milk

Visual observation of cream line formation at the surface of milk samples was done (Table 1). Beakers containing milk were tilted to observe the degree of cream line. Sign '+' shown in Table 1 indicates the thickness of cream layer at the top of milk samples. Highest cream line was observed in milks prepared from 25% fat cream followed by 20% fat and 15% fat when homogenized at 65°C and 1500 psi and 1200 psi pressure. This may be due to high fat content in cream. The lowest cream line was observed at 70°C for all the milk samples prepared from cream of different fat content, as high temperature leads to efficient homogenization. Hence, 70°C temperature was optimized for the partial homogenization.

Optimizing the cream fat and the pressure of partial homogenization through CI of standardized milk

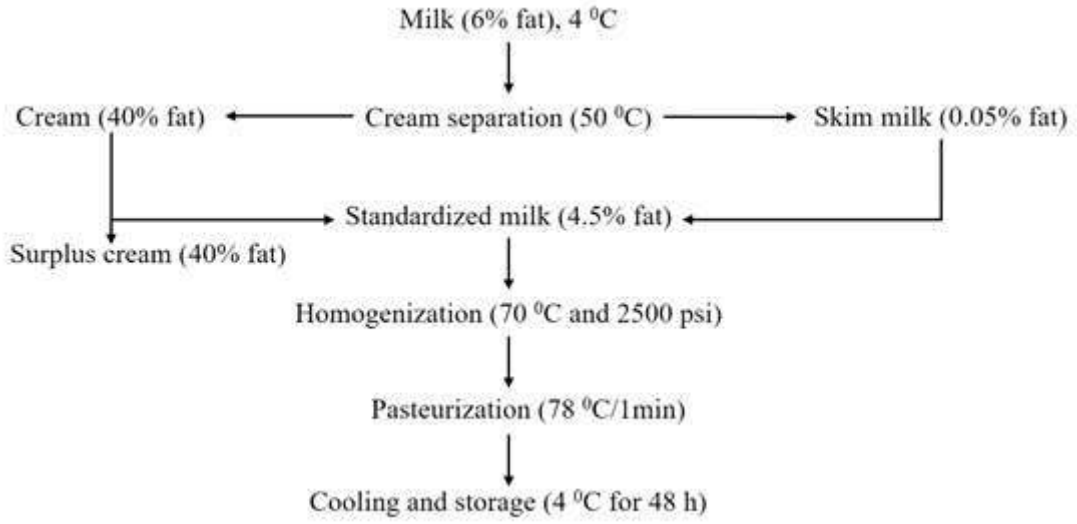

Fig. 2 Process flow chart for conventional homogenization of milk

Standardized milk of 4.5% fat was prepared from cream of different fat content homogenized at 70°C with varying first stage pressure. CI of the milk samples stored at 4°C for 48 h were estimated by determining the fat content of top 100 ml and remaining 900 ml volume separately (Table 2). Varying fat (%), first stage homogenization pressure and interaction effect showed significant difference ($p<0.05$) in CI milk samples. Low CI (d" 10) is desirable for efficient homogenization. CI of milk prepared from cream of 15% fat content and homogenized at 1000 psi first stage pressure was 5.23 which was lowest compared to all other treatments; such effect was significant ($p<0.05$).

Table 3 shows the viscosity of standardized milk prepared from cream of varying fat content homogenized at varying homogenization pressures. Varying fat (%) and first stage homogenization pressure showed significant effect ($p<0.05$) on the viscosity of standardized milk whereas their interaction effect was found to be non-significant. Based on CI and viscosity values, both 15% and 20% creams can be selected. However, due to good sensory score obtained for tea prepared from 15% fat cream, the standardized milk prepared from 15% fat cream homogenized at 1000 psi pressure was selected as optimized parameters.

Process flow chart for standardized homogenized milk prepared by optimized partial homogenization method is shown in Figure 1 while similar aspect for conventional homogenization of milk is shown in Figure 2. The experimental setup for homogenization is shown in Figure 3.

Comparison of CI and viscosity of optimized partially homogenized milk vis-a-vis conventionally homogenized milk

Table 4 shows the CI and viscosity values of partially and conventionally homogenized milks. As per Bureau of Indian Standard (1967), low creaming index (< 10) is desirable i.e. efficient homogenization. CI of partially and conventionally homogenized milks were 5.23 and 3.90 respectively which satisfied the standard. Viscosity of partially and conventionally homogenized milks were 0.98 and 1.01 cP respectively. The difference between these values are non-significant. Based on these observations, it can be concluded that partially homogenized milk is at par with conventionally homogenized milk in terms of CI and viscosity. Trout et al. (1935) studied the effect of homogenization on the viscosity of pasteurized whole milk. Bateman and Sharp (1928) reported that the viscosity of whole milk increases marginally

Table 1: Cream line formation in standardized milk (4.5% fat) as affected by cream fat and temperature & first stage pressure of homogenization

Temperature and pressure of homogenization	Cream fat (%)		
	15	20	25
65°C, 1000 psi	++	++	+++
65°C, 1200 psi	++	++	++++
65°C, 1500 psi	++	++	++++
70°C, 1000 psi	+	+	+++
70°C, 1200 psi	+	+	+++
70°C, 1500 psi	+	+	+++

+: No visible cream line (Acceptable); ++: Slight cream line (Unacceptable);
+++: Thick cream line (Unacceptable); ++++: Too thick cream line (Unacceptable)

Fig. 3 Experimental setup for homogenization

upon homogenization. Lisk (1924) reported that homogenization of whole milk at 3500 psi, increases considerably the viscosity of milk. At 1200 psi, the increase in viscosity was to a small extent only.

Comparison of sensory scores of tea prepared using optimized partially homogenized milk and conventionally homogenized milk

Sensory evaluation of tea prepared from partially and conventionally homogenized milk was carried out using 9-point hedonic scale. Sensory scores for all the parameters were found to be non-significant ($p>0.05$) for tea(s) made from partially and conventionally homogenized milks (Table 5).

The purpose of homogenization varies with the application. Consequently, the methods of measuring efficiency also vary. Creaming index and homogenization efficiency are inversely related. This implies that higher homogenization efficiency and lower creaming index are desirable to have efficient homogenization and prevent fat separation in product upon storage. Samuelsson and Lindberg (2018) reported that no effect of homogenization pressure on the proteins and the quality of the yoghurt could be found. The higher homogenization temperature led to a decrease in the fat globule size but none of the quality parameters nor the sensory analysis showed to be affected by the temperature change. This would mean that partial homogenization of yoghurt milk would be possible without compromising the quality.

Table 2: Effect on CI of standardized milk (4.5% fat) as affected by cream fat and first stage pressure of homogenization

Homogenization pressure (psi)	Cream fat (%)			
	15	20	25	Average
1000 + 500	5.23±0.55	6.31±0.20	16.46±0.37	9.33
1200 + 500	5.42±0.55	6.67±0.56	30.54±0.61	14.21
1500 + 500	6.02±0.74	7.33±0.34	75.67±0.58	29.67
Average	5.56	6.77	40.89	
	SEm		CD (0.05)	CV%
Fat	0.174		0.52	
Pressure	0.174		0.52	2.95
Fat× Pressure	0.302		0.90	

All values are mean of three replicates ± Standard Deviation (SD)

Table 3: Effect on viscosity (cP) of standardized milk (4.5% fat) at 25°C as affected by cream fat and pressure of homogenization

Homogenization pressure (psi)	Cream fat (%)			
	15	20	25	Average
1000 + 500	1.05±0.04	1.01±0.02	0.97±0.02	1.01
1200 + 500	0.99±0.04	1.00±0.03	0.98±0.03	0.99
1500 + 500	0.99±0.02	0.99±0.02	0.95±0.03	0.98
Average	1.01	1.00	0.97	
	SEm		CD (0.05)	CV%
Fat	0.009		0.03	
Pressure	0.009		0.03	2.73
Fat × Pressure	0.016		NS	

All values are mean of three replicates ± SD, cP: centi poise; NS= Non-significant

Table 4: CI and viscosity of partially homogenized and conventionally homogenized milks

Parameters	Partially homogenized milk#	Conventionally homogenized milk#	Recommended standard (BIS)	Remark
Creaming index	5.23 ±0.55	3.9 ±0.67	<10	Acceptable
Viscosity (cP) at 25^0C	1.03±0.06	1.01±0.03	NA	Acceptable

values are mean of three trials ± SD; NA = Not Applicable

Table 5: Sensory score (9-point hedonic scale) of tea prepared using optimized partially homogenized milk and conventionally homogenized milk

Parameters	Partially homogenized milk#	Conventionally homogenized milk#	Cal. t-value	p-value	Significance
Flavour	8.44±0.50	7.88±0.83	1.64	0.12	NS
Consistency	8.47±0.71	7.98±0.72	1.38	0.19	NS
Colour and Appearance	8.44±0.50	8.38±0.74	0.20	0.85	NS
Overall Acceptability	8.45±0.45	8.00±0.77	1.41	0.18	NS

Values are average of eight trials; NS = Non Significant; Tabulated t-value: 2.14 (cal. t-value less than tabulated value = NS); 5% level of significance

Table 6: Energy consumption during partial homogenization and conventional homogenization of milk

Parameters	Partial homogenization	Conventional homogenization
Total energy (kWh)*	1.40±0.35	4.47±0.28

Table 7: Energy saving in partial homogenization of milk over conventional homogenization at 500 L/h milk flow rate

Energy consumption (kWh)		Energy saving
Partial homogenization	Conventional homogenization	
1.40±0.35	4.47±0.28	[(4.47-1.40)/4.47] x 100 = 68.80%

Measurement of energy consumption during partial homogenization and conventional homogenization of milk

Energy consumed during cream separation and homogenization processes were measured using energy meter while preparing partially and conventionally homogenized milk (Table 6). It was observed that considerably less energy consumed in partial homogenization (1.40±0.35 kWh) compared to conventional homogenization (4.47±0.28 kWh) when same quantity of milks was prepared. This was due to lower operating pressure (1000 psi) of homogenization and less time (0.3 h) of operation of homogenizer during partial homogenization over conventional homogenization. Though energy consumption in case of cream separation during milk prepared by partial homogenization was little higher than conventional process, the total energy required to prepare same quantity of standardized homogenized milk was found to be less. Deynichenko et al. (2018) reported that the specific energy consumption of 3400 J/kg for milk emulsion.

Calculation of energy savings through partial homogenization of milk vis-a-vis conventional homogenization at 500 litre per hour milk flow rate

Difference in the energy consumed between partial and conventional homogenization was divided by the energy consumed during conventional homogenization to arrive at the energy saving (Table 7).

Conclusions

Homogenization efficiency was superior at 70°C compared to at 65°C based on cream line formation in standardized milk stored at 4°C for 48 h. The CI value was 5.23±0.55 at 1000+500 psi pressure of homogenization at 70°C temperature when cream of 15% fat was homogenized (partial homogenization). This is lowest and desirable among all the treatments. A non-significant difference in viscosity was observed for milks homogenized at optimized fat content of cream, temperature and pressure of homogenization as compared to milk subjected to conventional homogenization. In addition to this, tea prepared from these two milks were statistically alike ($p<0.05$) with regard to the sensory scores. It was found that partial homogenization of milk requires about 68.80% less energy compared to the energy required for conventional milk homogenization.

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RESEARCH ARTICLE

Genetic parameters of fertility traits in Murrah buffaloes

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Abstract: The data pertaining to 662 Murrah buffaloes of 24 years (1996-2019) collected from history cum pedigree sheets maintained at Buffalo Farm, department of LPM, LUVAS, Hisar studied to determine the influence of non-genetic factors *viz.* period, season and parity and to estimate the genetic parameter of fertility performance traits using mixed linear model. The traits studied were: age at first calving (AFC), service period (SP), conception rate (CR), calving interval (CI), number of services per conception (NSC) and pregnancy rate (PR). Period of calving had non-significant effect on all fertility traits viz. SP, CR, CI, NSC and PR, except AFC. Season of calving reported to have highly significant (P<0.01) effect on SP, NSC and PR and significant (P<0.05) effect of season of calving was obtained on CR and CI. Highly (P<0.01) significant effect of parity was reported on SP and NSC and significant effect of parity at P<0.05 was also seen on CR, CI and PR. The least squares means of AFC, SP, CR, CI, NSC and PR were 1345.75±13.88 days, 153.87±4.34 days, 67.08±1.18 %, 459.53±4.50 days, 2.01±0.05 and 0.27±0.01 %, respectively. The heritability estimates among traits in overall lactation fertility traits viz. AFC, SP, CR, CI, NSC and PR were 0.36±0.21, 0.11±0.03, 0.09±0.02, 0.23±0.10, 0.04±0.01 and 0.07±0.01, respectively. PR had negative genetic and phenotypic correlations with SP, CI and NSC whereas positive genetic and phenotypic correlation with CR. It was concluded that selective breeding and better management can lead to enhancement in performance of buffaloes.

Keywords: Fertility traits, Heritability, Pregnancy rate, Murrah buffalo

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Introduction

India is the one of the leading producers of milk in the world having a compound annual growth rate in milk production of about 6.2 percent to reach 209.96 million tonnes in 2020-21 from 187 million tonnes in 2019. Livestock sector contributes 4.11% GDP and 25.6% of total Agriculture GDP (BAHS, 2019). Murrah is one of the best recognized breeds of buffalo and is the most efficient producer of milk, not only in the India but also in the Asia. The home tract of this breed is in Haryana and adjoining states of Punjab, UP and Delhi. Total buffalo population in the country is 109.85 million during 2019 (20th Livestock census).

The economic worth of buffalo is primarily determined by these reproductive performances. Fertility is defined as the ability of the female animals to produce offsprings. These traits determine the lifetime production ability of the animals. Fertility traits add value to animal overall performance as they decide the economic life of the animal. Evaluation on fertility traits reduces the culling rate of farm as well as it increases the overall farm profit. Many researchers *viz.* Dev et al. 2015; Dash et al. 2015; Jamuna et al. 2015 and Patil et al. (2018) also reported varying degree of non-genetic effect on performance traits *viz.* AFC, CI, SP and CR in Murrah buffalo. The production of a dairy animal is an indication of its utility and is influenced by key fertility parameters such as calving intervals, length of each lactation and probability of surviving from one lactation period to the next (Zadeh, 2016). Likewise, the economic return of buffalo milk depends on the milk production and reproductive efficiency of animals, the latter being particularly affected by calving interval (Ramos et al. 2006). Therefore, fertility performance traits like AFC, SP, CR, CI, NSC and PR of Murrah buffalo require immediate attention of breeders for their evaluation.

Materials and Methods

The data of 662 Murrah buffaloes related to fertility traits over a period of 24 years from 1996 to 2019 was collected from history cum pedigree sheets maintained at Buffalo Farm, Department of Livestock Production Management, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. Climatic condition of Hisar is sub-tropical in nature and is situated in semi-arid region.

Geographically, Hisar is situated at 29°10' N latitude, 75°40' E longitude and 215.20 meters altitude.

Assuming that there was not much variation in adjacent years, entire period of 24 years was divided into six periods, each consisting of four consecutive years viz. 1996-1999 (Period 1), 2000-2003 (Period 2), 2004-2007 (Period 3), 2008-2011 (Period 4), 2012-2015 (Period 5) and 2016-2019 (Period 6). Each year was further delineated into four seasons of calving according to the geo-climatic conditions in the area viz.; Summer (April to June), Monsoon (July to September), Autumn (October to November) and Winter (December to March). Data up to third parity was included in the present study.

Animals having lactation shorter than 150 days, suspected outliers on the basis of histograms and abnormal records like abortion, mastitis and chronic illness were excluded from present study. Completion of minimum one lactation in the herd for studying fertility traits were considered for those animals that have the information of their date of birth, date of first calving, date of disposal and subsequent calving.

The performance traits included under this study were age at first calving (AFC), service period (SP), conception rate (CR), calving interval (CI), number of services per conception (NSC) and pregnancy rate (PR) up to 3 calving. Pregnancy rate (PR) measures the per cent of eligible buffaloes that become pregnant during each oestrous cycle and was estimated as: PR = 21 / (Service Period – Voluntary Waiting Period + 11), suggested by USDA, (2003). The constant factors 11 centralize the measure of possible conception within each 21 days' time period. The Voluntary Waiting Period (VWP) is the period after calving during which no inseminations occur, voluntarily left by the management for better pregnancy rate. The standardized voluntary waiting period of Murrah buffaloes was taken as 63 days (Patil et al. 2014). All the reproductive traits of Murrah buffalo under the present study served as standard and hence can be used as a reference or standard at a glance to compare the performances of Murrah buffalo reared under different agro-climatic zones of India.

In order to overcome non-orthogonality of the data due to unequal subclass frequencies, least squares and maximum likelihood computer program (Harvey, 1990) using Henderson's method III (Henderson, 1953) was utilized to estimate the effect of various tangible factors on performance traits and to estimate genetic and phenotypic parameters. The following statistical model was used to explain the underlying biology of the traits included in the study:

$$Y_{ijklm} = \mu + S_i + P_j + N_k + A_l + e_{ijklm}$$

Where Y_{ijklm} = is the m^{th} record of the individual belonging to i^{th} sire, j^{th} period, k^{th} season and l^{th} parity; μ = is the overall population mean; S_i= is the random effect of i^{th} sire (1 to n); P_j= is the fixed effect of j^{th} period of calving (1 to 6); N_k= is the fixed effect of i^{th} season of calving(1 to 4); A_l = is the fixed effect of l^{th} parity (1, 2 and 3); e_{ijklm}= is the random error associated with each observation, assumed to be normally and independently distributed with mean zero and variance $ó^2_e$.

Results and Discussion

The analysis of variance and the least squares means of fertility traits *viz*. AFC, SP, CR, CI, NSC and PR have been given in table 1 and table 2, respectively. The least squares mean of AFC was 1345.75±13.88 days which was in accordance with Wakchaure et al. (2008). Lower value was estimated by Jamuna et al. (2015) and higher values were estimated by Patil et al. (2018) and Jamal et al. (2018). This difference in age at first calving might be due to the difference in core herd, management practices and environmental conditions in various regions and within the same herd and region, management practices of different period may vary due to number

Table 1: Mean sum of squares of fertility traits

Source/Traits	Mean Sum of Squares					
	AFC	SP	CR	CI	NSC	PR
Sire	73832.75 (179)	11718.36 (152)	904.16 (152)	12299.78 (152)	1.58 (152)	0.05 (152)
Period	409816.09** (5)	12614.43 (5)	774.18 (5)	13398.25 (5)	1.04 (5)	0.04 (5)
Season	49741.07 (3)	69702.55** (3)	10451.01* (3)	75182.66* (3)	15.14** (3)	0.49** (3)
Parity	-	307019.66** (2)	14860.65* (2)	327424.09* (2)	17.82** (2)	0.58** (2)
Error	34941.98 (474)	9745.31 (809)	881.64 (809)	9840.86 (809)	1.47 (809)	0.039 (809)

Where AFC: age at first calving, SP: service period, CR: conception rate, CI: calving interval, NSC: number of services per conception and PR: pregnancy rate; *P<0.05, **P<0.01; figures in parenthesis denotes degree of freedom

Table 2: Estimates of least-squares means ± standard errors and effect of non-genetic factors on fertility traits

Ind. Var.	AFC (days)	SP (days)	CR (%)	CI (days)	NSC	PR (%)
OVERALL	1345.75±13.88 (662)	153.87±4.34 (972)	67.08±1.18 (972)	459.53±4.50 (972)	2.01±0.05 (972)	0.27±0.01 (972)
			Period of calving			
1996-1999	1200.88^{a}±44.36 (96)	143.66±19.40 (95)	71.03±5.98 (95)	448.01±19.53 (95)	1.86±0.24 (95)	0.21±0.04 (95)
2000-2003	1442.20^{bc}±33.58 (113)	143.75±11.64 (172)	72.88±3.57 (172)	447.17±11.76 (172)	1.84±0.15 (172)	0.26±0.03 (172)
2004-2007	1175.00^{a}±33.36 (86)	173.35±10.68 (177)	64.59±3.23 (177)	477.91±10.80 (177)	2.16±0.13 (177)	0.24±0.03 (177)
2008-2011	1372.71^{b}±33.95 (121)	145.94±11.77 (158)	64.85±3.63 (158)	450.45±11.88 (158)	2.08±0.15 (158)	0.28±0.03 (158)
2012-2015	1384.81^{bc}±33.46 (117)	157.73±12.14 (211)	65.31±3.69 (211)	464.24±12.25 (211)	2.05±0.15 (211)	0.31±0.03 (211)
2016-2019	1498.93^{c}±52.06 (129)	158.77±18.14 (159)	63.79±5.60 (159)	469.37±18.27 (159)	2.07±0.23 (159)	0.31±0.04 (159)
			Season of calving			
Summer (April to June)	1318.58±19.63 (186)	156.5^{ab}±8.15 (205)	65.01a^{b}±2.41 (205)	463.02^{ab}±8.26 (205)	1.96^{b}±0.10 (205)	0.26^{a}±0.02 (205)
Monsoon (July to September)	1363.15±19.02 (214)	133.1^{a}±6.21 (370)	75.5^{b}±1.81 (370)	437.89^{a}±6.34 (370)	1.7^{a}±0.08 (370)	0.34^{b}±0.02 (370)
Autumn (October to November)	1349.01±22.55 (122)	149.41^{ab}±8.05 (197)	69.20^{b}±2.51 (197)	454.45^{ab}±8.16 (197)	2.02^{b}±0.10 (197)	0.26^{a}±0.02 (197)
Winter (December to March)	1352.29±21.61 (140)	176.46^{b}±8.05 (200)	58.59^{a}±2.40 (200)	482.73^{b}±8.17 (200)	2.36^{c}±0.10 (200)	0.22^{a}±0.02 (200)
			Parity of calving			
First	-	191.39^{c}±5.56 (479)	58.49^{a}±1.58 (479)	498.32^{c}±5.71 (479)	2.29^{c}±0.07 (479)	0.21^{a}±0.01 (479)
Second	-	152.76^{b}±6.42 (304)	67.43^{b}±1.91 (304)	458.23^{b}±6.54 (304)	2.04^{b}±0.08 (304)	0.26^{b}±0.02 (304)
Third	-	117.45^{a}±8.10 (189)	75.30^{c}±2.45 (189)	422.03^{a}±8.21 (189)	1.69^{a}±0.10 (189)	0.34^{c}±0.02 (189)

Where AFC: age at first calving, SP: service period, CR: conception rate, CI: calving interval, NSC: number of services per conception and PR: pregnancy rate; Means superscripted by different letters differ significantly among themselves; figures in parenthesis are number of observations

of animals replaced in different periods and selection criteria used during the period. Estimated least squares means value of SP in present study was 153.87±4.34 days which was near to the estimated values of Wakchaure et al. (2008) and Dev et al. (2015). The difference in values of service period mainly occurred due to heat detection efficiency in various herds as effective heat detection was the sole reason for better service period in this herd. Least squares mean value of CR came out to be 67.08±1.18%, similar to the value of Dash et al. (2015) which was 68.80±1.18%. Conception rate vary from individual to individual and the sire used, therefore the overall result was the outcome of summation of individual performance. The least squares mean of CI was 459.53±4.50 days which lied around the values of Seno et al. (2010) and Jamal et al. (2018). Calving interval depends solely on service period, improvement in service period would lead to improvement in calving interval. Least squares mean of NSC was estimated out as 2.01±0.05. Lower values were reported by Patil et al. (2018) and higher value was obtained by Thiruvenkadan et al. (2014). This was due to use of various sires on different dams randomly over the periods. The estimated least squares mean value of PR was 0.27±0.01%. Higher values were obtained by Dash et al. (2015) and Jamuna et al. (2015). This was due to intensive use and skill in practicing artificial insemination.

Effect of non-genetic factors on fertility traits

Period of calving (POC) had no effect on all fertility traits viz. SP, CR, CI, NSC and PR, except AFC. Significant ($P<0.01$) effect of POC was obtained on AFC which was in same plane with Charlini

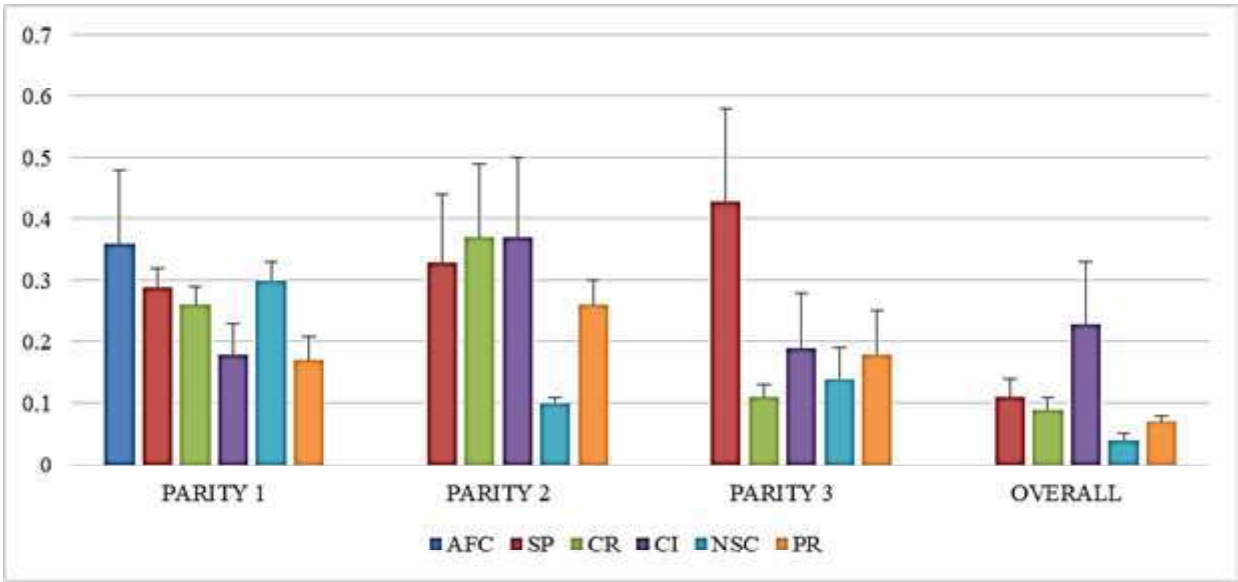

Fig.1 Diagram showing heritability of fertility traits in all three lactations and overall lactation along with their standard error

and Sinniah (2015) whereas non-significant effect of POC was reported by Naqvi and Shami (1999). Age at first calving was minimum in third period calvers (2004-2007) valued as 1175±33.36 days and maximum in sixth period calvers came out to be 1498.93±52.06 days. The overall AFC increased over several periods, is not a desirable factor for selection, as if, we are much concerned with early AFC for breeding. AFC increased from 1200.88 days in first period (1996-1999) to 1498.93 days in sixth period (2016-2019) indicating that this fertility trait needs to address in order to have more calf crop in the lifetime of buffaloes.

Service period showed irregular change over the period being the lowest in first period calvers as 143.66±19.40 days and highest in third period calvers as 173.35±10.68 days and went on decreasing without any trend till sixth period. Conception rate decreased with period from 71.03±5.98% to 63.79±5.60%. Chaudhari (2015) and Jamal et al. (2018) reported non-significant effect of POC on SP and CI whereas Jamuna et al. (2015) reported significant effect of POC on SP. Moreover, Sarkar et al. (2006) reported that POC had significant effect on conception rate in Murrah buffaloes. Calving interval showed irregular change, it was highest in third period calvers valued 477.91±10.80 days and lowest in second period calvers valued as 447.17±11.76 days. Patil et al. (2014) and Thiruvenkadan et al. (2014) reported that POC had significant effect on NSC while Kumar et al. (2003) obtained contrary results and reported non-significant effect of POC on NSC. Number of services per conception also had the irregular pattern of change being the lowest in second period calvers valued 1.84±0.15 and highest in third period calvers valued as 2.16±0.13. Overall PR gets increased from 0.21±0.04% to 0.31±0.04% with period (Table 2). All the fertility traits showed irregular pattern over the periods depending on the management and environmental conditions prevailed at that time.

Season of calving (SOC) reported to have highly significant ($P<0.01$) effect on SP, NSC and PR and significant ($P<0.05$) effect of SOC was obtained on CR and CI whereas non-significant effect of SOC was reported on AFC. Chaudhari (2015) also found non-significant effect of SOC on AFC in Murrah buffalo whereas Jamal et al. (2018) obtained significant effect of SOC on AFC. Age at first calving was the highest in monsoon season calvers estimated as 1363.15 days and the lowest in summer season calvers as 1318.58 days. Jamuna et al. (2015); Chaudhari (2015); Jakhar et al. (2016) and Jamal et al. (2018) found the significant effect of SOC on SP and CI. Service period was the highest in winter season calvers valued as 176.46 days and lowest in monsoon season calvers as 133.10 days. CR valued as 75.5±1.81%, highest in monsoon season calvers and lowest value in summer season calvers as 58.59±2.40%. Pasha et al. (1986) reported conception rate as 47.07%, 41.51%, 39.81%, and 51.96% in winter, spring, summer and autumn, respectively in Nili-Ravi buffaloes with significant effect of season on conception rate. Patil et al. (2014) and Thiruvenkadan et al. (2014) reported significant effect of SOC on NSC. Calving interval and number of services per conception both were highest and lowest in winter and monsoon season calvers valued as 482.73±8.17 days and 437.89±6.34 days and 2.36±0.10 and 1.7±0.08, respectively. PR had its highest value in monsoon season calvers as 0.34±0.02% and lowest in winter season calvers as 0.22±0.02%. Critical appraisal of the results indicated that monsoon season calvers (July to September) excelled in performance for all fertility traits except AFC and the better performance of monsoon season calvers could be attributed to plenty of green fodders availability to these animals at the time of calving.

Highly ($P<0.01$) significant effect of parity was reported on SP and NSC and significant effect of parity at $P<0.05$ was also seen on CR, CI and PR. Likewise, Chaudhari (2015), Jamuna et al. (2015);

Jakhar et al. (2016) and Jamal et al. (2018) obtained significant effect of parity on SP. Service period decreased with lactation order from 191.39±5.56 days to 117.45±8.10 days which is a required character for reproductive worth of the animal. CR increased with parity from 58.49±1.58% to 75.30±2.45% indicates improvement of trait with parity order. Catillo et al. (2001) reported non-significant effect of parity on calving interval whereas Chaudhari (2015), Jakhar et al. (2016) and Jamal et al. (2018) recorded significant effect of parity on CI. Calving interval and number of services per conception had the right direction of selection as these traits went down from 498.32±5.71 days to 422.03±8.21 days and 2.29±0.07 to 1.69±0.10 with increasing lactation order. Pregnancy rate increased with parity from 0.21±0.01% to 0.34±0.02% indicated towards the effective fertility performance. Better performance of all fertility traits in third lactation is a matter of attainment of physiological maturity and increased efficiency of buffaloes to convert dietary nutrients into milk due to attainment of adult weight of buffaloes.

Heritability estimates of fertility traits

Heritability estimates for first lactation performance traits ranged from 0.17±0.04 (PR) to 0.36±0.12 (AFC), for second lactation 0.10±0.01(NSC) to 0.37±0.12 (CR), for third lactation 0.11±0.02 (CR) to 0.43±0.15 (SP) and for overall production performance traits ranged from low (0.04±0.01) of NSC to high (0.23±0.10) of CI (Table 3). Maximum estimates of heritability were seen in second lactation and minimum heritability estimates were obtained in overall lactations. Heritability of fertility traits of all three lactations and overall lactation along with their standard error has been shown in figure 1. However, NSC exhibited maximum heritability in first lactation. Age at first calving was found to be moderately heritable (0.36±0.21) and service period was reported to be low (0.11±0.03) heritable in overall lactation which lied same with the results of Patil et al. (2018). CR was reported low (0.09±0.02) heritable, similar to Dash et al. (2015). CI was found moderately (0.23±0.10) heritable trait which was similar to Jakhar et al. (2016). Heritability estimate of NSC was found very low (0.04±0.01), similar to Patil et al. (2018). PR had low (0.07±0.01) heritability estimates as reported by Dash et al. (2015) and Jamuna et al. (2015). Fertility traits exhibited low to moderate heritability in first, second, third and overall lactations.

The differences in heritability estimates of various research studies and their standard errors may be due to the changes in recording procedure, the correction for different non-genetic parameters and the methodology or model used for the estimates of heritability for the trait. In addition to this, herd size, feeding management of the different farm and climatic condition of the country may also be cause of such variations. The estimates for the fertility traits with low heritability indicated a good scope for refinement in these traits by providing uniform environment. Further improvement in these traits would require information from other relatives and enhancement in managemental practices. In general, fertility traits were influenced by environment and exhibited low heritability indicated that improvement could be possible through selective breeding, adjusting management practices and nutrient intake.

Correlation between the fertility traits

Table 3: Heritability of fertility traits in three lactations and in overall lactation

TRAITS	PARITY 1	PARITY 2	PARITY 3	OVERALL
AFC	0.36 ± 0.12			
SP	0.29 ± 0.03	0.33 ± 0.11	0.43 ± 0.15	0.11 ± 0.03
CR	0.26 ± 0.03	0.37 ± 0.12	0.11 ± 0.02	0.09 ± 0.02
CI	0.18 ± 0.05	0.37 ± 0.13	0.19 ± 0.09	0.23 ± 0.10
NSC	0.30 ± 0.03	0.10 ± 0.01	0.14 ± 0.05	0.04 ± 0.01
PR	0.17± 0.04	0.26 ± 0.04	0.18 ± 0.07	0.07 ± 0.01

Where AFC: age at first calving, SP: service period, CR: conception rate, CI: calving interval, NSC: number of services per conception and PR: pregnancy rate

Table 4: Heritability, genetic (below diagonal) and phenotypic correlation (above diagonal) among overall lactation fertility traits

Traits	AFC#	SP	CR	CI	NSC	PR
AFC	0.36±0.21	-0.07±0.02	0.07±0.02	-0.06±0.05	-0.05±0.01	0.04±0.001
SP	0.02±0.07	0.11±0.03	-0.65**±0.17	0.95**±0.25	0.68**±0.18	-0.27**±0.11
CR	0.08±0.09	-0.64±0.17	0.09±0.02	-0.62**±0.19	-0.69**±0.24	0.28**±0.12
CI	0.04±0.08	0.47±0.08	-0.52±0.15	0.23±0.10	0.64**±0.25	-0.26**±0.11
NSC	-0.03±0.07	0.81±0.06	-0.20±0.13	0.70±0.07	0.04±0.01	-0.21**±0.11
PR	0.05±0.09	-0.28±0.14	0.22±0.14	-0.18±0.14	-0.69±0.18	0.07±0.01

Where AFC: age at first calving, SP: service period, CR: conception rate, CI: calving interval, NSC: number of services per conception and PR: pregnancy rate: *P<0.05, **P<0.01; #first lactation only

In overall lactations, the minimum and maximum genetic correlation was found between NSC/PR and SP/NSC, valued as -0.69±0.18 and 0.81±0.06, respectively. The range of phenotypic correlation varied from -0.69±0.24 (CR/NSC) to 0.95±0.25 (SP/CI), respectively which was highly significant as given in table 4.

Service period exhibited negative genetic and phenotypic correlation with CR valued as -0.64±0.17 and -0.65±0.17. Similarly, CR was reported to have negative genetic and phenotypic correlation with CI valued as -0.52±0.15 and -0.62±0.19 and with NSC valued as -0.20±0.13 and -0.69±0.24, respectively. Moreover, it was found that PR had negative genetic and phenotypic correlation with SP valued as -0.28±0.14 and -0.27±0.11, with CI had the values as -0.18±0.14 and -0.26±0.11 and with NSC values reported were -0.69±0.18 and -0.21±0.11 which were highly significant.

Chakraborty et al. (2010) and Dev et al. (2015) reported the high genetic and phenotypic correlation between FSP and FCI but in this study, moderate to highly positive genetic and phenotypic correlation between SP and CI. Pregnancy rate showed moderately positive genetic and phenotypic correlation with CR which was 0.22±0.14 and 0.28±0.12, respectively. CR had highly negative genetic correlation with SP (-0.64±0.17), CI (-0.52±0.15) and NSC (-0.20±0.13).

Conclusion

It is concluded that conception rate and pregnancy rate had negative association with all other fertility traits but these traits are positively correlated with each other. Fertility traits showed low to moderate genetic and phenotypic correlation with each other except with AFC. Their association with each other indicated that improvement in one trait would result in enhancement of other traits as well.

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RESEARCH ARTICLE

Trend and future perspective of milk production in Karnataka

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Abstract: The present study was conducted to understand the present and future perspective of milk production in Karnataka and to develop a suitable model for prediction of milk production in the state. The present study is having advantage as the past trends of milk production in the state have been impressive with growth, contributing significantly to overall milk production in the country. The Auto-Regressive Integrated Moving Average (ARIMA) and Artificial Neural Network (ANN) models were applied to the data sets of different dairy units for modeling and forecasting milk production in the state. The milk production of different dairy units has indicated a significant and positive trend over the years (Mann Kendal and Sen's estimator). The purpose of the study was based on the accuracy criteria to identify the best ARIMA and ANN models for forecasting. It was found that the ARIMA model predicted cows, goats, and total milk production with better R-Square, MAPE and percentage prediction error compared to ANN models. ANN model performed better in predicting the buffalo's milk yield compared to the ARIMA model. The best-identified models were used for out-sample forecast.

Keywords: ARIMA, ANN, Milk Production, Statistical Modeling, Trend Estimation,

Introduction

Milk is a complete source of nutrients for overall growth and development of the human body. In the world, total protein intake through milk and other dairy products is around 10.3 %. India is a leading country in milk production with an annual production of 187.7 million ton in 2018-19 with a per capita availability of 394 gm/day of milk. Indian dairy sector contributes up to Rs. 3.6 lakh crores through milk and milk products (DAHD & F, 2014) and has a recorded growth of 6.7 percent during 2017-18 (Anon, 2018). Despite the significant growth in export of milk and other dairy products during 2019-20 (US $ 280 million), it was lesser as compared to the export of 2018-19 (US$ 483 million). The countries *viz*., China, Algeria, Indonesia, Brazil, and Russia are the leading importer of milk and milk products (FICCI, 2020). India is at the top position in the production of buffalo's milk (66 million ton) as well as goat milk (5 million tons) production while second in cow milk production (54 million tons) next to USA. Even though at present, India is self-sufficient in dairy sector, but it may not be so soon due to the rapid growth rate in human population which may lead to substantial increase in demand for milk and milk products.

Karnataka is an agrarian state where most of its population is dependent on Agriculture and allied activities. The dairy sector is practiced and having capacity of generating 8 billion liters of milk. Karnataka state is the seventh largest dairy market in India which provides enough opportunity for those practicing dairy. The cow and buffalo milk account for most of the total milk production in the state.

Forecasting plays a vital role in many fields of science *viz*., plant production (Dahikar and Rode, 2014), animal husbandry (Mgaya, 2019; Suresh et al. 2019), economics and engineering etc. Time series models are used for forecasting purposes and these models are widely used by the researchers and policy makers. The ARIMA (Auto-Regressive Integrated Moving Average) is a forecast engine used to analyze the milk yield through understanding the current and predicted behaviors. Therefore, forecast from the developed models would be extremely helpful for the decision makers in the dairy sector for policy implementation related to price support to farmers during excess milk yield, expanding the export capability to the new destination, to maintain the quality of the products and to expand the storage capacity. Hence, the present study will be helpful to the policy makers to plan and strategize future milk production in the state through the implementation of developed Autoregressive Integrated Moving

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For figures kindly visit website (Supplementary file)

Average (ARIMA) and Neural Network Autoregressive models (NNAR).

Materials and Methods

The analysis has been carried out using 33-year (1986-2017) time series data for cow, buffalo, goats and total milk production in the Karnataka state of India. The secondary data was collected from the publicly available database maintained by the state veterinary department (Commissionerate Animal Husbandry and Veterinary Services, Karnataka, 2017-18). The above data has been utilized for model development, whereas 2018-19 data on total milk production (https://www.nddb.coop/information/stats/milkprodstate) has been used for model validation.

Trend analysis was performed using non-parametric techniques such as Mann-Kendal's Z (Kendal, 1975) and Sen's slope (Sen, 1968). The data has been tested for white noise, subsequently analyzed for ARIMA (Auto regressive Integrated Moving Average) and NN (neural network) model. The prediction efficiency of NN and ARIMA models have been analyzed using accuracy criteria like root mean square error (RMSE), MAPE (Mean Absolute Percentage Error), Akaike Information criteria (AIC) (Akaike, 1974), Schwartz Information Criteria/ Bayesian Information criteria (SBC/BIC) and R-Square.

Choosing the best models

The data was analyzed using R-Studio (R-Core Team, 2020) and SAS (SAS 9.3 ver.) environment. The R-packages like forecast, t-series were utilized to build forecast models. The models were automatically built using auto.arima function which gives the best models tested among different combination of p, d, q parameters based on the least AIC and SBC values. The feed forward neural network i.e.., NNAR was analyzed using 'forecast package' function called 'nnetar' which works similar to an auto.arima function. The estimated coefficients of the ARIMA models were tested at 5% level of significance.

Trend Analysis

The data series of cow, buffalo, goat, and total milk production has been analyzed using nonparametric Mann Kendal's Z and Kendal's Q to identify the existing trend in the data series.

The Mann-Kendal test mainly used to test whether a univariate time series data has monotonic trend (Upward or Downward), and it assumes in the data that, there should not be any autocorrelation. The hypothesis under this test is as follows.

H_0 = No trend

H_1= There exists a trend (Upward or Downward)

Test statistic is given by

$$S = \sum_{i=1}^{n=1} \sum_{j=i+1}^{n} \text{sign}(x_j - x_i)$$

Where x_j and x_i sequential data values. Variance of S is given as follows

$$\text{Var} = \frac{1}{18}\left[n(n-1)(2n+5) - \sum_t f_t(f_t-1)(2f_t+5)\right]$$

Where t-varies over set of tied ranks and f_t is the frequency that rank t appears and the test statistic will be

$$z = \begin{cases} \frac{(s-1)}{se}, & S > 0 \\ 0, S = 0 \\ (s+1)\, S < 0 \\ /se, \end{cases}$$

Where se=Standard deviation: if there is no monotonic trend.

Sen's slope Q

The magnitude of the trend can be estimated using Sen's Slope Q, which is based on the median values of variables (X_{ij}). The test statistics is given by

$$Q = \begin{cases} \beta_{\frac{(N+1)}{2}} \text{ Where N is odd} \\ \frac{1}{2}\left(\beta_{N/2} + \beta_{\frac{(N+2)}{2}}\right) \text{ Where N is even} \end{cases}$$

A positive Q values indicates upward trend and Negative represents downward trend. After trend analysis, the data has been checked for unit root presence using ADF test and found all dairy units' data series has unit root (ACF & PACF plots). Therefore, data is differenced to make stationery.

Auto regressive Integrated Moving Average (ARIMA): It is an important time series technique given by Box and Jenkins (Box and Jenkin, 1970). This model uses the linear combination of lagged values and errors.

The ARIMA model can be represented as

$$Y_t = \emptyset_0 + \varphi_p Y_{t-1} + \cdots + \varphi_p Y_{t-p} + et - \emptyset_i e_{t-1} - \cdots - \emptyset$$

Where, Y_t are the actual values and e_t is the random error at time t and è, and ö are the model parameters to be estimated and p and q are the integer values. In ARIMA model, it is assumed that the underlying data should be white noise and error component is

identically and independently distributed with constant variance and zero mean (e ~ iid (0, ó²)). The ARIMA models are used by the various researchers of allied field to predict the milk production and other dairy related products (Sagar and Paramasivam, 2016; Deluyker et al. 1990; Lark et al. 1999; Sankar and Vijayalakshmi, 2017; Sánchez et al. 2014; Mgaya, 2019), and to predict various agricultural interrelated phenomenon (Paul et al. 2014). These models are sophisticated and better than any other models when the underlying data is linear.

ANN models have become more popular in the late 90's and then neural networks were used for a wide variety of applications. It is used in many fields of science including agriculture, veterinary, medical science, and other science related fields. Many researchers have applied the ANN technique (Shahriary and Mir, 2016; Dahikar and Sandeep, 2014) for predicting the various agriculture and allied products, animal genetics, and diet formulation, (Shahinfar et al. 2012; Saxena and Parasher, 2019; Ferna´ndez, 2006; Ehret, et al. 2015). The comparative analysis of ANN and other linear models includes multiple linear regression (Gandhi. et al. 2010) and ARIMA models etc. (Murphy et al. 2014; Adebiyi et al. 2014; Li et al. 2018) are also being conducted. In the present study, the models were chosen based on the accuracy criteria viz., root mean square error (RMSE), akaike information criteria (AIC), bayesian information criteria (BIC), coefficient of determination (R-Square), mean absolute percentage error (MAPE), and percentage prediction error (PPE).

Results and Discussion

Trend analysis of milk production from different dairy units in Karnataka was performed using mann kendal's Z and sen's slope Q statistic. The results of the trend analysis along with the descriptive statistics of the data series is shown in table 1. The time series data of cow, TMP and goats were found to be positively skewed while time series data of buffalo was negatively skewed distribution and except goat's data all other found to have flat curve which is indicated by negative coefficient of kurtosis values; therefore, time series data of all dairy unit have non normal distribution. The goats milk production was highly inconsistent (CV- 66.05%) followed by cow (CV- 44.547 %), TMP (CV- 35.137 %) and then buffalo milk production (CV- 21.17%). The data series of all dairy units were non-stationary according to augmented dickey fuller test values (Table 1).

The evidence from the Mann-Kendal's and Sen's Slope estimators, the time series data of all dairy units has a positive and significant trend. The values of Mann-Kendal's Z reveal that the rate of increase in milk production due to cow was higher (7.545) and for buffalo (5.33) was lesser compared to other units. It can be concluded that the rate of increase of total milk production (7.204) in Karnataka is attributed to rate of increase in the milk production from cows. The sen's slope estimator results revealed that the rate of total milk production was 145.870 MT/ year while cow milk production was increasing at a rate of 113.717 MT/year. The rate at which the buffalo (27.633 MT/year) and goat's milk production (2.210 MT/year) were progressing in the state was comparatively less and its contribution to the overall milk production was significantly lesser.

The ARIMA models of different p, d, q parameters fitted to milk production data of dairy units (Table 2) were selected based on lower AIC, SBC criteria and the residuals characteristics of the models. The ARIMA model with parameters p-1, d-1 and q-0 was best fitted to the cow milk production and exhibited least AIC (406.919), SBC (409.851) values, and the residuals from fitted model were found to be independent (x^2 = 0.995 at Lag 12). The parameters of the model found significant (MU-141.002, AR1 - 0.556) at 5% significance level. The autocorrelation and partial autocorrelation function plots for residuals from the model showed that the spikes were well within 5% significance level (Fig.2). The observed values of cow yield closely predicted by the model (1,1,0) which is depicted Fig 1.

For the buffalo's milk production ARIMA with first differencing (d-1) parameter found good fit (Fig. 3 &4) and this model had lowest AIC (398.195), SBC (401.127) values and the residuals were independent ($\mathbf{x^2}$ =0.883 Lag-12). The differencing (MU-25.369, t-value- 0.92) and autoregressive (AR- 0.249, t value- 1.38) parameter of the model was non-significant and therefore, the

Table 1: Descriptive, trend analysis and stationary test of milk production in Karnataka during 1985-86 to 2017-18

Variables	Mean	Standard Deviation	Median	Skewness	Kurtosis	C.V (%)	Mann-Kendal's Z value	Sen's Slope Q Value	ADF Significance
Cows (MT)	2591.86	1154.60	2598	0.464	-0.628	44.547	7.545	113.717	-0.483 (0.976)
Buffaloes (MT)	1468.93	311.03	1427	-0.128	-1.080	21.174	5.330	27.633	-2.062 (0.548)
TMP (MT)	4099.46	1440.45	4124	0.265	-0.788	35.137	7.204	145.870	-.210 (0.532)
Goats (MT)	38.65	25.53	39.00	1.171	2.137	66.049	6.749	2.210	-0.927 (0.933)

* CV-Coefficient of Variation, MT-Metric Tonnes, Values in the parenthesis are *p* value

model failed to produce better accuracy criteria. For predicting the goat (Fig.5) and total milk production (Fig.7) in Karnataka, ARIMA (1,2,0) and ARIMA (1,1,0) found best fit models and these models produced better accuracy criteria, respectively. The model parameters of ARIMA (1, 2, 0) and ARIMA (1, 1, 0) AR-1.00 and AR-0.533 found significant (*t-value* = 5.84 (Goat's), *t-value*= 3.15 (Total Milk Production)) at p-0.01 significance level, respectively. The model information criteria viz., AIC and SBC values showed that the models were best fit to the data set (Table-2 & 3). The graphical representation also showed that these models closely predicted the observed series of Goat and Total Milk production. The ACF and PACF plots showed that the residuals were within the limit (Fig. 4, 6 & 8). As per the model forecast, the total milk production from three dairy units is

Table 2: ARIMA models fitted along with their respective information criterion (AIC, SBC) for Milk production in Karnataka during 1985-86 to 2017-18

Variables	ARIMA Model	AIC	SBC	Ch-Square p-value for residuals
Cows (MT)	ARIMA (1,1,0)	406.919	409.851	0.995 (lag-12)
Buffaloes (MT)	ARIMA(1,1,0)	398.195	401.127	0.883 (Lag-12)
TMP (MT)	ARIMA(1,1,0)	427.100	430.032	0.923 (Lag 12)
Goats (MT)	ARIMA(1,2,0)	217.0915	219.9595	0.871 (Lag-12)

* AIC- Akaike Information Criterion, SBC-Schwartz Information Criterion, MT-Metric Ton

Table 3: Parameter estimates and significance of ARIMA models fitted to milk production in Karnataka during 1985-86 to 2017-18

Sl. No	Variables	Parameters of ARIMA	Model Estimate	Error Estimate	t-value
1	Cow	MU	141.002	52.150	2.70*
		AR1,1	0.556	0.214	2.60*
2	Buffaloes	MU	25.369	27.56	0.92
		AR 1,1	0.249	0.181	1.38
3	TMP	MU	166.416	66.995	2.48*
		AR1,1	0.533	0.169	3.15**
4	Goat	MU	0.78466	7.778	0.10
		AR1,1	1.00	0.171	5.84**

p-value * - 0.05 level, ** - 0.01 level

Table 4: Out sample forecast of ARIMA for Milk Production in Karnataka from 2018-19 to 2022-23

Units	Year	Forecast ('000 Tonnes)	Std Error	95% Confidence Limits	
Cow	2018-19	5637.572	135.497	5372.002	5903.142
	2019-20	5936.195	250.625	5444.979	6427.412
	2020-21	6164.842	355.931	5467.229	6862.454
	2021-22	6354.578	450.421	5471.770	7237.386
	2022-23	6522.678	535.153	5473.798	7571.559
Buffalo	2018-19	1791.634	118.231	1559.905	2023.363
	2019-20	1808.127	189.241	1437.221	2179.034
	2020-21	1831.280	244.704	1351.668	2310.893
	2021-22	1856.096	290.729	1286.277	2425.915
	2022-23	1881.328	330.622	1233.319	2529.336
TMP	2018-19	7522.743	185.727	7158.725	7886.761
	2019-20	7805.799	340.021	7139.371	8472.228
	2020-21	8034.445	479.210	7095.211	8973.680
	2021-22	8234.062	602.921	7052.359	9415.766
	2022-23	8418.192	713.178	7020.388	9815.995
Goats	2018-19	124.920	7.779	109.674	140.166
	2019-20	167.220	11.001	145.659	188.781
	2020-21	169.340	19.053	131.996	206.684
	2021-22	211.640	24.598	163.429	259.851
	2022-23	213.760	33.906	147.306	280.214

expected to achieve 8034.445 Metric tons by 2020-21. The cow milk production 6164.842 MT, Buffalo milk production 1831.280 MT and Goat's milk production expected to reach 169.34 million ton by 2020-21 (Table-4) with the 95 % significance level. Out sample forecasts was depicted in the table 5 from NNAR model. The total milk production for Karnataka during 2018-19 was 7901 MT (National Dairy Development Board, 2019) and the ARIMA (1, 1, 0) closely predicted the value (7805.79 MT for 2018-19) with 4.79 % prediction error, which means the models were better in predicting the milk production in Karnataka (Ranjit Kumar Paul et al. 2014).

ANN model with a single hidden layer was fitted to dairy unit's under study and linear activation function in the hidden layer. The results of the equipped NNAR models were the average of twenty networks fitted simultaneously to the data set. Nonlinear Autoregressive (NNAR) model with a single input, hidden and output model found better for predicting cow, goat, and total milk production while for buffalo, network with two input and two hidden units being used. In case of cow, goat and total milk production the single input i.e., 1 lag period (Y_{t-1}) being used as an input to build the forecasting network while 2 (Y_{t-2}) lag periods were used for building prediction model for buffalo milk yields. The fitted models exhibited better accuracy measures (RMSE), model fitted to total milk production had high RMSE value (203.575) followed by NNAR (1, 1) for cow (131.622), NNAR (2,2) for buffalo (66.356) and NNAR (1,1) for goat's (7.193) milk production (Table 6). The predicted values from NNAR models were plotted against the observed values which is shown in the figures (Fig.9,10,11).

On comparing ARIMA and NNAR models, ARIMA model performed better in predicting Cow's, total milk production and Goat's milk production based on R-Square and MAPE values. While Buffalo milk production was predicted accurately by NNAR model (Fig.12) than the ARIMA (1, 1, 0) model both in terms of R-Square (0.945) and MAPE (3.143) values (Table-7). Total milk production for 2018-19 was validated and found that the NNAR (1,1) predicted the milk production with prediction error 5.61 % which is slightly higher than that of prediction error for ARIMA model.

The ARIMA models performed better in explaining variation in the data series and prediction power especially when the data is stationary(Kumar and Lyngdoh, 2020). When the data is not stationary, differencing may make the data stationary (Jai Sankar and Vijayalakshmi, 2017). The fitted ARIMA models performed better in terms of accuracy criteria viz., AIC, BIC, RMSE, MAPE (Sánchez et al. 2014) and R-Square values except in the case of forecasting of buffalo milk production. The residuals of the fitted ARIMA models were independent. When the data is non linear and complex, ANN models usually perform better than linear time

Table 5: Out sample forecast of NNTAR for Milk Production ('000 tonnes) in Karnataka from 2018-19 to 2022-23

Year	Cow Milk Production	Buffalo Milk Production	Total Milk Production	Goat Milk Production
2018-19	5543.402	1573.521	7457.845	189.825
2019-20	5876.919	1482.109	7784.027	302.898
2020-21	6200.688	1532.715	8109.538	355.302
2021-22	6501.138	1624.260	8426.666	358.888
2022-23	6766.826	1725.561	8727.486	359.013

*Milk production in Metric Tons

Table 6: Fitted Neural Networks and their performance indicator in predicting milk production in Karnataka

Variables	Neural Network Type	Model	Output-Activation function	RMSE
Cow	NNAR(1,1)	1-1-1	Linear	131.622
Buffaloes	NNAR(2,2)	2-2-1	Linear	66.356
TMP	NNAR(1,1)	1-1-1	Linear	203.575
Goat	NNAR(1,1)	1-1-1	Linear	7.1935

*RMSE- Root mean square error

Table 7: Comparison of prediction performance of ARIMA and Artificial Neural Network model for milk production in Karnataka

Variables	R-Square		MAPE	
	ARIMA	ANN	ARIMA	ANN
Cow	0.988	0.986	3.237	3.7462
Buffaloes	0.876	0.945	4.652	3.1425
TMP	0.984	0.978	2.619	3.204
Goat	0.920	0.918	11.395	12.996

* MAPE-Mean Absolute Percentage Error

series models, since ANN models have a faster learning rate and convergence capacity than those linear models (Adebiyi et al. 2014; Li et al. 2018, Lahane, 2008).

Conclusion

It was found that the milk production in the Karnataka state is on significantly increasing trend across dairy units and the ARIMA, and ANN networks were able to capture the variability efficiently in the data sets. The fitted ARIMA models i.e., ARIMA (1, 1, 0) for cow's total milk production and ARIMA (1, 2, 0) for goat's milk production captured the data variation accurately. These model parameters could be used for future prediction of milk production in Karnataka. ANN models also provided consistent predictions but fell short compared to ARIMA model, however, ANN (NNAR [2,2]) model predicted the buffalo milk production with better accuracy than ARIMA models. Therefore, the above models could be potentially utilized by the policy makers for efficient management and while taking policy decisions for the development of dairy industry in the state of Karnataka.

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RESEARCH ARTICLE

Future scenario of Dairy Entrepreneurial Ecosystem (DEE) of Kerala

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Abstract: The present study was undertaken to forecast the future of dairy entrepreneurial ecosystem for a short term, medium term and long term in the state of Kerala (India) during 2020-21; owing to the high ranks of the state in dairy progressiveness, production systems and marketing infrastructure. Delphi survey was used to project the future into conceivable scenarios. Four likely scenarios were estimated for each period based on the important driving factors and their probability of occurrence of change. The constant factor taken to decide the scenario axis for the three periods was 'dairy entrepreneurial growth'; while for the short term, the second contributing factor was 'support services'; for medium term it was 'cooperative sector'; and for the long term, it was 'advanced technology'. The study presented the narration of the scenarios which can be utilized by policy makers and planners to design in advance the appropriate modifications and interventions to develop dairy entrepreneurship; and thereby create a desirable dairy entrepreneurial ecosystem for the state in the future.

Keywords: Advanced technology, Cooperative sector, Dairy, Entrepreneurial ecosystem, Future scenario, Support services,

Introduction

The existing literature in entrepreneurship is mostly concerned with the characteristics and behaviours of individuals or firms; but of late, a strong emphasis is placed on the importance of relationships between entrepreneurs and their local economic and social contexts/environment. Several scholars have highlighted the need to pay more attention to the contexts in which entrepreneurial activities take place (Koch et al. 2017). The recently emerged systemic view of entrepreneurship is known as the 'Entrepreneurial Ecosystem' truncated as EE. Stam (2015) defined Entrepreneurial Ecosystem as 'a set of interdependent actors and factors coordinated in such a way that they enable productive entrepreneurship within a particular territory'. Daniel Isenberg (2011) emphasized that every EE is unique as it develops under idiosyncratic circumstances. They are geographically bounded referring to a nation, state or be limited to smaller geographical areas like towns/cities. Moreover, there are also specific industry based entrepreneurial ecosystems; and those spanning over different industries (OECD, 2014).

Entrepreneurial ecosystems are dynamic and evolve over time; and focus on the cultures, institutions and networks that develop within a region (Stam and Spigel, 2016). Roundy (2017) suggested that we need to have future oriented narrative accounts of entrepreneurial ecosystems to chart the ecosystem's future. Studying EE pertaining to primary production sectors (e.g. dairying) of different states shall benefit in deepening our understanding about how entrepreneurship happens in different contexts. Hence a study was conducted to analyze the future of the dairy entrepreneurial ecosystem of Kerala state owing to its high ranks in socio-economic development index (Ohlan, 2012), dairy progressiveness index (Kale *et al*, 2016), dairy production systems index (Patel *et al*, 2019) and milk marketing infrastructure index (Mohapatra and Sendhil, 2020); to forecast the probable future scenarios and recommend points of action for the actors and factors to the development of dairy entrepreneurship in the state for a short term, medium term and long term period.

Methodology

Future scenario of dairy entrepreneurial ecosystem is the most likely alternative future of the dairy entrepreneurial ecosystem based on the predictions derived through the opinion and consensus of the expert/experienced judges of the sector. In the present study, scenario was not intended to forecast or predict a desired future; but to produce an illustration of the possible futures for learning opportunity and be prepared for any unanticipated changes. Delphi technique was used to collect and analyze data to project the future scenario of DEE. The steps

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followed in the process of comprehending the status of Dairy Entrepreneurial Ecosystem were as detailed by Kale et al. (2017); with slight modifications and sketched in the Fig 1:

The time frame for the study was for short term (up to 2025), medium term (2035) and long term (2050) period and the state of Kerala was the study area. Key factors were identified based on desk research and consultation with the experts (using key informant interviews and focus group discussion). Delphi survey questionnaire was prepared based on the same. A group of 10 experts involved in the field of dairy entrepreneurship in Kerala having experience above 15 years was selected by snowball sampling technique. They belonged to the different stakeholder categories, having prominent roles in developing dairy entrepreneurship in the state. The experts were from Kerala Veterinary and Animal Sciences University (KVASU), State Dairy Development Department, State Animal Husbandry Department, Kerala Livestock Development Board, Kerala Cooperative Milk Marketing Federation and a progressive dairy entrepreneur. In Delphi survey, a questionnaire with 34 statements was prepared to select the key factors and measure their significance along with probable changes that may occur over the selected period.

The importance and uncertainty of the key factors influencing the future of dairy entrepreneurial ecosystem of Kerala was analyzed. The key factors were judged on a 5-point scale by the Delphi panel in Round I. The importance of the factors was measured with a scale ranging from least important (1) to extremely important (5) and the probability of occurrence of change in the factors was measured from highly certain (1) to highly uncertain (5). The first round responses were analyzed for measures of central tendency (Mean, Median and Mode) and dispersion (Standard Deviation) (Grisham, 2009) and those results were sent back to the panel in order to see whether agreement/disagreement increases or decreases (Round II).

On completion of the second round, an index value of the statements was calculated to identify the key factors and probability of occurrence of change in the key factors using the formula given below:

$$\text{Index} = \frac{\textit{Obtained Score}}{\textit{Maximum Obtainable Score}} \times 100$$

Based on the index values, the key factors and their change or trends were prioritized. The key factors were then ordered on the basis of priority and clustered into groups with the help of a scenario team. Two key factors with critical uncertainty in terms of occurrence of change and highest impact in the dairy entrepreneurial ecosystem were identified and a matrix was created with four conceivable scenarios. The other factors were interpreted and logically fitted into the scenarios. Scenarios were generated for the short term, medium term and long term period. The scenarios were carefully examined by a scenario team in line with their experiences and literature review, for proper understanding and explanation which forms the basis for future plan of action. The narration of the scenario was written as per the interpretation by the scenario team and also the commentary provided by the panel members during the course of Delphi survey.

Results and Discussion

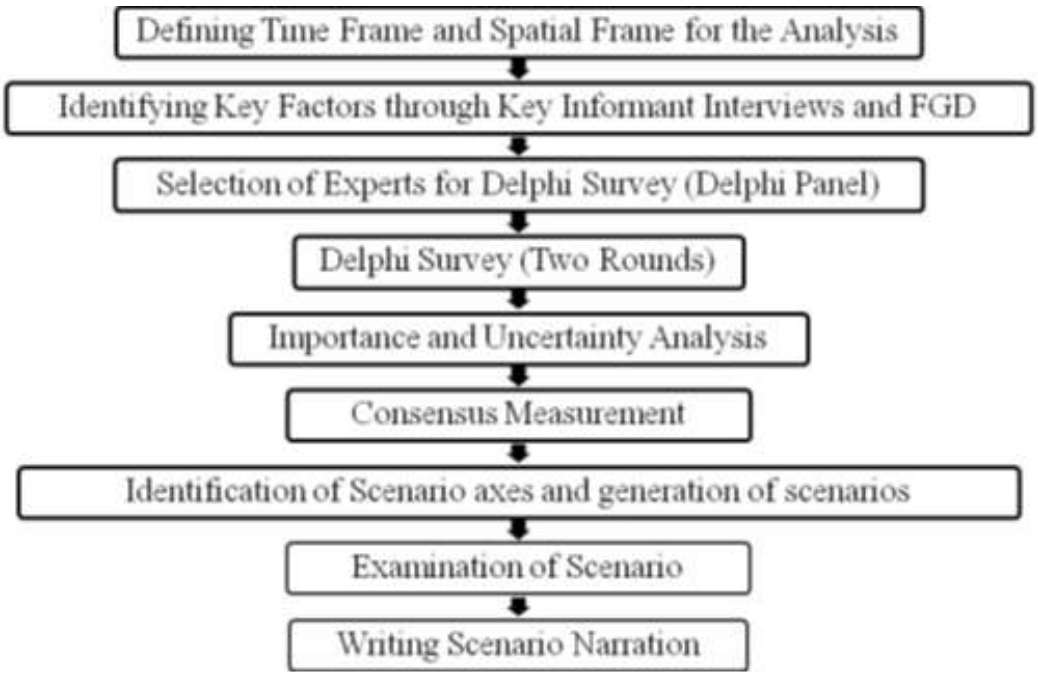

Fig 1: Steps used to project the future scenario of Dairy Entrepreneurial Ecosystem

Table 1: Salient outcomes of scenarios for the Short term period (up to 2025)

Scenarios			
Futile Uncertainty dimensions: Strong support services; but low dairy entrepreneurial growth	Changing (✓) – desirable Uncertainty dimensions: Strong support services; with high dairy entrepreneurial growth	Constant Uncertainty dimensions: Weak support services and low dairy entrepreneurial growth	Neglected Uncertainty dimensions: Weak support services; however high dairy entrepreneurial growth
Outcomes			
Favourable government support in the background of COVID-19 with new schemes and services.	Encourages entry of nascent entrepreneurs; imposed choice for income generation for youth, displaced workers and expatriates. Migration to dairying due to loss in other sectors.	Lesser subsidies and schemes for dairy development due to financial constraints caused by COVID-19 pandemic. Primary production sectors are prone to be neglected at times of financial crisis.	Overlooking support, new entrepreneurs may take up dairying as a voluntary or forced choice of income generation by investing their own resources without waiting for support from government.
Hike in milk productivity due to breeding support through high quality semen and insemination facilities.	Consumer trend moves to unpacked farm fresh milk; hence expansion of local milk market with better price.	Though cattle induction from neighbouring states has contributed to increase in milk production, it has created an allusion of spread of animal diseases which apprehends the number of takers for government schemes during the recent times.	There shall be media hype and celebration of dairy entrepreneurial success with more appealing stories appearing in media. This naturally fascinates youth towards the sector and shall pave ways for new entrants to dairying.
Bank loan facilities are available at lower interest; but mortgaging shall be a must for bigger dairy units.	Liquidity of the sector shall attract investment and number of animals in farms shall increase for higher profitability. (Minimum profitability with 8-10 animals by 2025)	Demand for liquid milk shall remain steady or shall increase marginally until 2025. It is the lowest among the South Indian states and this shall negatively impact the growth of dairy sector.	The demand for fresh milk, out of quality and health concern is increasing among the urban families especially for feeding infants and children. This indirectly creates a market for new dairy entrepreneurs who take up direct marketing.
Government subsidies shall continue at the present pace; but the number of beneficiaries shall be limited due to fund constraints.	Society shall give due respect to dairy entrepreneurs and there shall be recognition and awards for dairy entrepreneurship.	Number of smallholder dairy farmers shall remain stagnant or lessen during the short-term period. Cost of milk production shall increase and profits for small holders may be less, which is negative for dairy entrepreneurial growth.	Special ghee, spiced paneer, dairy health supplements etc. are gaining popularity creating opportunities for product diversification. This market demand shall develop the sector in spite of deprived support services.
There shall be more agencies and more schemes in the animal and owner insurance sector. But entrepreneurs may evade insurance owing to high premium.	Effective support services extended by Government departments, public sector undertakings, banks and cooperatives shall boost the growth of dairy entrepreneurship.	The breeding policy of the state refrain the use of pure exotic semen which impedes the creation of high productive animals. The entrepreneur's interests may thus be curtailed which negatively affect dairy entrepreneurial development.	Diversification can lead to dairy entrepreneurs themselves starting consultancy services, sales of inputs especially feed and fodder; and starting training activities.
Skilled and unskilled labour availability increases due to unemployment, movement of displaced workers from other fields and migration of labour from other states		Competition with cooperatives in selling price is a disadvantage for the entrepreneur; for he is constrained to sell his product at a lower price.	

Table 2: Salient outcomes of scenarios for the medium term period (2035)

Scenarios			
Protective	Competitive (✓) - desirable	Status quo	Transitional
Uncertainty dimensions:Strong cooperative sector; but low dairy entrepreneurial growth	Uncertainty dimensions: Strong cooperative sector and high dairy entrepreneurial growth	Uncertainty dimensions: Weak cooperative sector and low dairy entrepreneurial growth	Uncertainty dimensions: Weak cooperative sector; nevertheless high dairy entrepreneurial growth
Outcomes			
Government support is usually given through dairy cooperatives in the form of subsidies, incentives and pension; available to its members. This shall benefit smallholders; and not farm owners, whose dependency is less on cooperatives and also avoid meagre pensions.	The number of dairy entrepreneurs shall increase due to consistent entry of youth, use of technology and product diversification (also including dung and urine products) in dairying during the medium term. Also there shall be growth of secondary trading entrepreneurs.	A decreasing trend in milk production continued through the short term to medium term period shall weaken the cooperative sector, resulting in increased inflow of cheaper milk from neighbouring states, through cross-border milk traders. This will increase competition and is detrimental to dairy entrepreneurship.	Despite the weakening of cooperative sector, the number of dairy entrepreneurs shall marginally increase and dairy farms may transform to larger ones with capital-intensive operations, advanced breeding and feeding technology, increased scale of production, variety products and direct marketing alternatives.
The small holder dairy farmers who are at present in their fifties shall remain in the field for a maximum of another 15 years. In addition, they have disinterest/risk in local sales (apathetic to household sales due to delay in getting milk price); hence their milk shall always flow to cooperatives.	There shall be availability of funds from private agencies for dairying in the medium term, especially funding in the form of venture capital for processing start-ups or private investment as part of contract farming by retail chains, supermarkets, processing industries etc.	The number of milch animals may decrease in the medium term because of drop out of small holders (the pillar of cooperatives) due to high cost of production, lack of land availability and strict environment protection laws and compulsory licensing etc. It is also forecasted that commercial dairy farms shall compensate the decrease in animals and hence static milk production.	Availability of training and incubation facilities for dairy entrepreneurship shall increase over the period in line with those already established by public institutes in livestock sector. Online forums shall emerge as the main platforms for training to cater expected change in technologies.
There shall be increase in the number of dairy cooperatives and processing plants continuing the current trend due to political interests and government support. Also there shall be revamping and merger of cooperatives for economies of scale and export. The government in power has a tendency to capture the administration of regional milk unions; by increasing the number of cooperatives having political affiliation to the ruling party.	There are chances of low cost feed substitutes/formulations using locally available resources entering market in the medium-term period. New customized equipment/devices at low cost shall enter market as part of mechanisation. Labour cost shall decrease and condition shall shift from labour scarcity to surplus.	There shall be merger/amalgamation of cooperatives or conversion to producer companies in order to gain better scale of production and avoid liquidation during the medium term. Also a good number of dairy cooperatives and dairy plants shall become defunct due to non-viability and tough competition.	ICT sources; especially use of mobile and social media shall increase for selling of milk and milk products directly to consumers (through apps and portals); already initiated by private agencies. Milk shall be available in different types, quantities and packaging options.
The incremental increase in procurement and selling price of milk shall continue from the short term to the medium term period; prompting small holders to bind to cooperatives. It shall indirectly affect the sales of dairy entrepreneurs, pressured to sell their product at the price of cooperatives.	Animal identification and insurance shall change from optional to mandatory to rear cattle; and insurance schemes shall be tailored as per the requirement of the entrepreneur; benefitting him. Also paid consultancy and single window services shall be popular.	The increasing trend in cost of animals, input and utility services shall continue. It shall reduce the profit margin, with falling interest in dairy entrepreneurial activity. Also Government support with incentives shall come down.	Private veterinary hospitals shall increase by 2035. Number of professional private consultants in dairy field shall escalate during the medium term, utilized by large farm owners. Ayurvedic and homoeopathic treatment of animals shall get popularized.

Table 3: Salient outcomes of scenarios for the long term period (2050)

Scenarios			1
Lethargic Uncertainty dimensions:Strong technology; but low dairy entrepreneurial growth	Dynamic(✓) – desirable Uncertainty dimensions: Strong technology with high dairy entrepreneurial growth	Conventional Uncertainty dimensions: Weak technology leading to low dairy entrepreneurial growth	Conservative Uncertainty dimensions:Weak technology; however high dairy entrepreneurial growth
Outcomes			
The technological advancement in production (better animal productivity) and processing sector (continuous methods) shall be high during the long term period. But the use of the technologies by entrepreneurs may be limited owing to cost factors, indifference to adopt and lack of information and training.	Adopting the changes in technology, dairy farms shall prosper in the long term due to increased profitability. Rather than high-tech farms, growth will be for skill-tech farms (using technology skilfully than plentifully). Dairy production and processing shall transform as an entirely business enterprise for youth.	Number of dairy entrepreneurs shall marginally decrease due to cost of milk production, reduced profits and competition from external milk brands. Dairying will vanish from peri-urban areas unless innovative methods for waste disposal evolve. Number of cattle shall also shrink and the per-animal productivity may no longer be able to hold on the level of milk production.	Number of small holders in dairy shall diminish to the level of 50% from the short term period. But there shall be growth of dairy entrepreneurs having land and financial resources particularly in rural areas following an integrated approach with agriculture and dairying.
Competition from milk brands outside the state shall increase steadily. Efficient local brands shall survive competing in the market. Contract farming, online purchase/sales, mobile apps, door delivery etc. shall be highly prevalent. Food Safety and Standards Authority of India (FSSAI) shall make chilling/processing milk mandatory for sales similar to registration/license at present, citing health reasons.	Organic milk, A2 milk and their products may gain importance among an elite group of population. There shall be change in consumer preferences and it depends on quality, price, advertisement, health and accessibility; which can be used by dairy entrepreneurs. There shall be Public-Private-Community-Partnership (PPCP) in milk marketing; and farm branding dominates product branding in the long term.	It is very unlikely for cooperatives and entrepreneurs to beat the effect of cross border price difference due to high cost of production. The local milk will find it hard to compete with incoming milk, due to price difference. The assumption is that there may be influx of additional 60 LLPD to Kerala by 2050 from outside states.	Number of cattle owned by an individual entrepreneur may increase during the long-term period. Dairy farms may transform to larger ones with direct marketing. Availability of cheap labour due to unemployment shall inhibit chances of mechanization.
Profitability may decrease in the long-term period and number of animals in a dairy farm should increase for better profitability. Licensing will be mandatory and Animal Husbandry/Dairy Development Departments shall have a say in issuing license to dairy farms.	Number of dairy smallholders shall reduce to a higher extent by 2050. Many young smallholders may grow to an entrepreneurial level by upgrading their farms through intensive methods and innovative marketing for survival.	Leaving apart the cooperative sector, sale price of milk in unorganized sector shall be different in rural and urban market. Out of competition, it is forecasted that the selling price may reduce in the long term, depressingly affecting dairy entrepreneurs of the state.	The increased productivity of animals in the long term may result in additional infertility and disease problems instigating the entrepreneur to adopt lesser productive and adaptive breeds without affecting the herd size.
Mechanization shall grow as a must in dairy industry and new equipment/devices shall enter market. There shall be customized equipment manufacturers and sellers in market. However, in the long term, the cost of mechanization shall increase; prompting the entrepreneur to select cheaper labour against automated systems.	There shall be sharp drop in the number of dairy cooperatives and many dairy cooperatives may transform to producer companies. In addition, private dairy plants shall take up alternate models of marketing to compete with national/international brands.	There are chances of overhauling or merger of various departments for improved efficiency during the long term. Government intervention shall be restricted to extension, insurance, social security and regulatory laws. Smallholders may be brought under minimum wages norms with financial support.	There are chances of proliferating desi and indigenous milch breeds with better production and climate adaptability as part of state and central government schemes. Climate crisis and natural calamities may also contribute in selection of animals.

The results obtained after the Delphi analysis is expressed for the three periods as given below:

Future scenario of the dairy entrepreneurial ecosystem by 2025 (short term)

The four scenarios produced were named as 'futile', 'changing', 'constant' and 'neglected' based on the strength or weakness of 'support services' leading to high or low 'dairy entrepreneurial growth'; which were the uncertainty dimensions. It was seen that stronger support services shall form the base of a productive entrepreneurial ecosystem and this shall induct a higher number of new entrants into the dairy sector. The salient outcomes during the short term are given in Table 1. 'Changing' dairy entrepreneurial ecosystem is the desirable scenario during the short term.

Future scenario of the dairy entrepreneurial ecosystem by 2035 (medium term)

The scenario matrix was framed depicting the key uncertainty dimensions of 'Cooperative sector' and 'Dairy Entrepreneurial Growth'. Stronger expansion of organized cooperative sector and more intense participation of entrepreneurs in dairying form the base for a further competitive entrepreneurial ecosystem. The four scenarios were named as 'protective', 'competitive', 'status quo' and 'transitional'. The salient outcomes of the scenarios forecasted by 2035 are given in table 2 and 'Competitive' dairy entrepreneurial ecosystem is the desirable one.

Future scenario of the dairy entrepreneurial ecosystem by 2050 (long term)

The scenario matrix was illustrated depicting the key uncertainty dimensions of 'Advanced Technology' and 'Dairy Entrepreneurial Growth'. The four scenarios by 2050 were named as 'lethargic', 'dynamic', 'conventional' and 'conservative' based on the strength or weakness of advanced technology leading to high or low dairy entrepreneurial growth and forecasted in table 3; and 'Dynamic' system being the desirable scenario.

Conclusion

The projection of the dairy entrepreneurial ecosystem of the state of Kerala provides both desirable and undesirable scenarios in the future for the three terms studied. During the short term, support services shall be the main driving factor for high dairy entrepreneurial growth which can be designated as 'changing dairy entrepreneurial ecosystem'. During the medium term cooperative sector shall be the leading major factor; and its strength shall decide an appropriate dairy entrepreneurial growth labelled as 'competitive dairy entrepreneurial ecosystem'. Strength of advanced technology in milk production, processing and marketing shall be the driving factor for desired dairy entrepreneurial growth during the long term, termed as 'dynamic dairy entrepreneurial ecosystem'. Other scenarios too were predicted pertaining to weaker driving factors which lead to diminished dairy entrepreneurial growth. Furthermore, scenarios were described when there was possibility of dairy entrepreneurial growth in spite of weak driving factors; and those when even strong driving factors shall not create desired dairy entrepreneurial growth. Moreover, it is to be noted that the expected outcomes shall be overlapping the periods; as one cannot ignore the possibility of an outcome getting extended from one term to another. The results shall enable the actors of the dairy entrepreneurial ecosystem and also the policy makers to appreciate the plausible future outcomes and plan in advance, to reconsider their actions towards a desirable dairy entrepreneurial development.

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RESEARCH ARTICLE

Trends in herbal pharmaceutical patent protection for Dairy industry: Perspective from Grassroots innovations

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Abstract: The national patent regime had amended Patent Act on innovation as per requirement for protecting pharmaceutical product patents among member states of World Trade Organisation. In this respect, the study was conducted to understand patentability nature and prosecution timelines for patent protected indigenous dairy technologies. Investigators had examined 28 herbal pharmaceutical patent grant(s) from grassroots innovators/outstanding knowledge holders. Patent applications were filed between 2007-2014, prosecution were held and accorded patent grant during 2019-2022. The study noted that Section 3 (d), (e) and (p) were principal factors involved while defending veterinary pharmaceutical patent applications. About 46 percent did not have Section 3 (d) objections inferring these medicinal practices followed by dairy farmers were unique. It was observed that majority of patent grants were in product category illustrating transformation of national patent regime from process patent to product-patent grant system. Overall time taken for availing grant was about ten years with an average prosecution period of 2.6 years. Therapeutic efficacy, synergistic action and suitable claim amendments are pertinent features in responding to prosecution stages. These pharmaceutical product patents from social innovation are of interest to dairy industry. These experimental wisdom provide cost effective sustainable technologies for dairy health system and repurpose existing knowledge of local communities. It is paramount to nurture this bottom-up approach for improvising sustainable dairy health service delivery system.

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Keywords: Pharmaceutical Patents; Product Patents; Grassroots Innovations; Indigenous medicine; Herb; Social Innovation

Introduction

The contribution of outstanding knowledge practices from societal experimentation to dairy health care and production system were recognized (Eiki et al. 2021). There is appreciation of usefulness indigenous knowledge systems, however limited literature were available on their innovation capabilities, protection through patent filing. These new knowledge are key for focussed goals in socio economic development and drivers for industrial growth. Innovation is endogenous to economic progress (Kaplinsky and Mbula, 2022; Singh et al. 2015). Emerging economies like India had society comprising largely informal sector contributing to innovation-economic paradigm. With large pool of informal economy, India had pioneered in recognizing innovative ability of individuals who were not part of formal system (Ustyuzhantseva, 2015).These social innovations were considered as an outcome to overcome limitations in routine activities. Efforts to strengthen this bottom-up approach had created attention among rural region (Ferreiro et al. 2021).These innovations are developed to combat existing problem and for creating new opportunity (Taalbi, 2017). The need to involve multiple process in iterative innovation system is essential to address societal requirements and for seizing opportunities (Poblete et al. 2022). India is among the top three innovation economies of Central and Southern Asia region and ranked 40 in Global Innovation Index. The country positioned first among lower middle-income group of nations (GII, 2022).

Sustainable Development Goals 2030 of United Nations require dairy sector to embrace greener technologies (Granato et al. 2022). These are crucial development for dairy sector, which needs to access new medications with less cost involvement. Herbal medicines play vital role in dairy sector and it is necessary to examine requirements in protecting indigenous knowledge system through pharmaceutical grants. Strengthening these social innovations will be key to support dairy health systems (Ravikumar et al. 2017). The need for supporting local entrepreneurial activity at early stages in development of

technology and practices of community is felt (Cuntz and Peuckert, 2023). A strong Intellectual Property Rights (IPR) protection regime and their joint effect are positively associated with technology adoption (Jayasekara and Fredriksson, 2021).The patent filing rate had grown to 1.6 percent compared to 2019 (Bhardwaj and Arora, 2023) and increasing patent filing activity can attract industries enabling largescale commercial diffusion.

Inorder to meet this purpose, Intellectual Property Rights policies had evolved since entry of India into Trade related aspects of Intellectual Property Rights (TRIPS) through amendment of Patent Act. This engagement was heldbased on sensitization of protecting community practices that otherwise had taken longer prosecution stages to defend *eg*., Turmeric patent (Mashelkar, 2001). Accordingly, national IPR system started permitting pharmaceutical patent grants for *process claim* thereby ensured accessibility of cost effective medicines. Since 1972-2004, pharmaceutical industry in India relied on process-patent system (Haley and Haely, 2012). There is need to introduce product patent grants for scaling up accessibility of medicines. Amendments like Section 3 (d) of Indian Patent Act was introduced by 2005 that led to examination of pharmaceutical patent applications both for process and product claim(s).This Section 3 (d) reiterated 'significant enhanced efficacy of a known substance 'towards clearance of pharmaceutical patent grant.

Patent applications need to disclose appropriate information that establishes novelty, suitable inventive steps, and usefulness in terms of industrial application (Blind et al. 2022). Moreover, disclosure needs to be "sufficiently clear and complete to enable it to be replicated by a person with an ordinary level of skill in the relevant technical field" as per patent office. Industries had adequate attention for protection of herbal products through patent claims. The volume of Indian Pharmaceutical Industry is third largest in the world and thirteenth in value producer (Singh, 2022). This reiterates need for critical engagement in development of herbal drugs, meet with global changes. Indian national patent system had considered these priorities and with several policy changes, IPR policy was adopted in May 2016 [GoI, 2021]. It is to be noted that only 40 percent of innovative technologies developed were transformed into application in developing world, which is 70-85 percent in developed world (Wu and Li 2023). The innovation performances need to be reinforced by innovation policies and appropriate practices for desirable outcome.

Hence, it is necessary to understand emerging trends on herbal pharmaceutical patent prosecution governed by national IPR jurisdiction. The study examined herbal pharmaceutical patents in light change in IPR policies for upscaling domestic innovation system. This insight can help to understand, develop suitable technological approach for dairy industry and to explore necessary practices in harnessing social innovation derived from grassroots/outstanding herbal practices.

Methodology

The study had examined 28 patent grants accorded by Indian Patent Office [IPO] in the field of dairy sector. These patent applications were enlisted based on the grant availed during 2019-2022 by National Innovation Foundation-India [NIF]. The priority date of examined applications were between 2007-2014 and filed from 9 states of India [Table 1, 2]. The investigation had examined the nature of Section 3 objections raised by IPO during prosecution stages of these applications. Investigators were part of correspondence between applicants and patent office in terms of responding to First Examination Report's, Controller reports and hearing/prosecution process. The study also assessed time duration during prosecution period and from priority date of filing for these herbal pharmaceutical grants. Nature of claim amendment(s) and issuance of patent grant based on process or product or both were recorded.

Results and Discussion

These herbal veterinary pharmaceutical patent grants were received for grassroots innovator(s) being incubated by National Innovation Foundation-India. A total of 28 veterinary patent grants were received for green grassroots innovators located from 9 states of the country. These patent rights are crucial for recognizing innovation capabilities of knowledge holder(s)from informal society. Such development can assist in scaling up of social innovation through commercial sphere. These activities distinguished knowledge holders based on technological practice(s) shared by them and confirmed prevalence of novel/ unique medicinal systems, being practiced at farming communities. Intellectual property rights protection is an essential step in commercial product cycle facilitating licensing agreements.

Nature of Section 3 objections

Indian IPR regime had to comply with TRIPS and introduced Section 3 (d). The section examines patentability in terms of mere 'discovery new form of known substance', 'discovery of new use of known substance' and 'mere use of known process'. The study had identified 15 of these grants which had at least Section 3 (d) objections in First Examination Report [FER]. The patentability nature of such applications were put forth by demonstrating significantly enhanced efficacy. Remaining 13 patent grants (46 percent) were not known earlier and reflecting unique dairy technologies. It is pertinent to protect these indigenous herbal wisdom maintained at different geographical communities. Knowledge holders had sustained not only the knowledge of unique herbal medicine but also capability of utilization of practice to address complex dairy health constraints. These innovations are endogenous in nature. Necessary support system can enhance capability to assimilate new information.

Table 1: Prosecution information

Ailment	State	Application Number	Priority date	Date of Grant	Date of FER Response Filed	Nature of Section 3 objections
Ephemeral Fever	Himachal Pradesh	3188/DEL/2012	12-10-2012	31-10-2022	23-08-2019	(c), (d), (i),(j), (p)
Milk Yield	Odisha	1453/KOL/2012	24-12-2012	13-09-2022	24-06-2020	(e) , (i)
Expulsion Of Placenta	Gujarat	1049/MUM/2011	31-03-2011	09-09-2022	11-10-2016	(e)
Mastitis	Tamil Nadu	998/CHE/2014	27-02-2014	30-06-2022	27-06-2019	(d), (e), (p)
Herbal formulation for the treatment or prevention of mastitis	Gujarat	3754/MUM/2014	26-11-2014	30-05-2022	26-12-2019	(p), (d), (e)
Bloat	Gujarat	1115/MUM/2011	31-03-2011	12-04-2022	21-12-2018	(p), (e)
Endoparasitic Infections	Odisha	486/KOL/2011	04-04-2011	30-03-2022	08-06-2019	(c), (e), (j),(p)
Estrus In Animals	Gujarat	1109/MUM/2011	31-03-2011	24-02-2022	19-12-2018	(p), (e)
Anestrus In Animals	Odisha	489/KOL/2011	04-04-2011	21-01-2022	5-12-2019	(d), (i), (p)
Bloat	Bihar	449/KOL/2011	31-03-2011	07-10-2021	19-12-2018	(p), (e)
Estrus In Animal	Gujarat	1114/MUM/2011	31-03-2011	07-10-2021	20-12-2018	(p), (e)
Ectoparasitic Infestation	Tamil Nadu	1039/CHE/2011	31-03-2011	06-10-2021	28-05-2019	(c),(d)(e), (j), (p)
Gastrointestinal disorders	Odisha	467/KOL/2011	01-04-2011	05-10-2021	30-04-2019	(d),(e), 3(p)
Fever Treatment In Animals	Tamil Nadu	1111/CHE/2011	01-04-2011	17-09-2021	25-02-2019	(d),(e),(i),(p)
Bloat	Gujarat	202/MUM/2007	05-02-2007	26-08-2021	23-10-2013	(e)
Bloat	Tamil Nadu	1011/CHE/2011	30-03-2011	07-07-2021	28-03-2019	(d), (e), (i), (p)
Mastitis	Tamil Nadu	1113/CHE/2011	01-04-2011	06-07-2021	15-02-2019	(d), (e), (i), (p)
Milk Yield In Cattle	Gujarat	1028/MUM/2011	31-03-2013	05-07-2021	09-04-2019	(d), (e), (p)

Table 2: Nature of patent grants and timelines

Application Number	Composition or Single Herb	Claim Amendment(s)	Product/ process or both	Time duration during prosecution stage (Days)	Time duration to obtain grant since priority date [Days]
3188/DEL/2012	Composition	Principal claim amended	Product	1165	3671
1453/KOL/2012	Composition	Principal claim amended	Product & Process	812	3550
1049/MUM/2011	Composition	Principal claim amended	Product & Process	2159	4180
998/CHE/2014	Composition	Principal claim amended	Product & Process	1099	3045
3754/MUM/2014	Composition	Principal claim amended	Product & Process	886	2742
1115/MUM/2011	Composition	Principal claim amended	Product	1208	4013
486/KOL/2011	Composition	Principal claim amended	Product & Process	1026	4016
1109/MUM/2011	Composition	Principal claim amended	Product & Process	1163	3983
489/KOL/2011	Composition	No change in Principal claim	Product & Process	778	3945
449/KOL/2011	Composition	Principal claim amended	Product & Process	1023	3843
1114/MUM/2011	Composition	Principal claim amended	Product & Process	1022	3843
1039/CHE/2011	Composition	Principal claim amended	Product & Process	862	3842
467/KOL/2011	Composition	Principal claim deleted	Process	889	3840
1111/CHE/2011	Composition	Principal claim amended	Product & Process	935	3822
202/MUM/2007	Composition	No change in Principal claim	Process	2864	5316
1011/CHE/2011	Composition	Principal claim amended	Product & Process	832	3752
1113/CHE/2011	Composition	Principal claim amended	Product & Process	872	3749
1028/MUM/2011	Composition	No change in the claims	Product & Process	818	3749
1027/MUM/2011	Composition	Principal claim deleted	Process	775	3661
999/CHE/2014	Composition	Principal claim amended	Product & Process	442	2582
2243/CHE/2008	Composition	Principal claim amended	Product & Process	696	4569
997/CHE/2014	Single	Principal claim amended	Product & Process	562	2521
463/KOL/2011	Composition	Principal claim amended	Product & Process	685	3559
1053/MUM/2011	Composition	Principal claim amended	Product & Process	1061	3492
562/KOL/2012	Composition	Principal claim amended	Product & Process	373	3076
648/KOL/2012	Composition	Principal claim amended	Product & Process	675	2663
1051/MUM/2011	Composition	Principal claim amended	Product & Process	419	3080
1033/CHE/2011	Composition	Principal claim amended	Product & Process	371	3039

Twenty patent applications (89 percent) had objections on the ground of Section 3 (e) referring herbal compositions were mere admixture. Except one applications, rest of 27 granted application are in the form of herbal composition. Therapeutic value along with features were illustrated and necessary claim amendment(s) were carried out in overcoming objections for according pharmaceutical grants. FER examination of these applications indicated about 82 percent (23 grants) had Section (p) objections referring as Traditional knowledge. Traditional knowledge cannot be protected with patent system as it is known for generation. Further, Indian Patent system did not allow patent grant on the basis of mere aggregations or duplication of known properties. This was due to plant biodiversity and associated knowledge being maintained at local communities. These knowledge is known and available as prior art and measures to prevent unfair exploitation

In general, these applications have to ensure sufficiency of disclosure interms of working examples, differentiating features of composition through prior art and experimentation to prove these practices are effective. The distinguishing nature of herbal compositions were elaborated during prosecution stage and grants were accorded.

Claim amendment(s) on process and product patent grant

It was noted that for 23 grants received had principal claim amendment(s). The prosecution proceedings had resulted about 82 percent of grants (n=23 grants) as product and process pharmaceutical grants. It was also noted that two applications (n=2) were accorded with product grants. It is essential to protect original product during early stage of Research and Development process. This only provide incentive to industry as it enables exclusivity in the market. Overall only 10 percent (3 applications) of these dairy knowledge applications had resulted in process grant. In access to drugs, India had restricted product patent grant before embarking into WTO (Pre TRIPS) in order to avoid monopoly of drugs. Agreement on Trade related aspects of Intellectual Property Rights (TRIPS) of World Trade Organization had mandated members in protecting patents on pharmaceutical products (Dai and Watal, 2021). Subsequently, the IP system had evolved to meet the international obligations.The study had found that with changes in patent laws, decisions were oriented product patent grant(s). This augments well for domestic innovation system.

Social innovations for dairy health and production

Technologies related to dairy *viz*., ephemeral fever, expulsion of placenta, anestrus, mastitis, bloat, endoparasite, ectoparasite, enteritis and nutritional supplements for milk yield were protected based on societal wisdom at IPO. These technological practices had evolved from experimental nature of dairy farming communities to sustain dairy health production and welfare.

The studies found that majority of patent grants were in product category and directly related to serve the purpose of claim. This provides critical insight to understand social realities in terms of contribution of informal knowledge system in sustainable rural dairy health system. Systemic adaptation to contextual aspects can help in nurturing technological and innovation capabilities (Mugwagma et al. 2022). Knowledge holders had minimized distress of dairy communities and reduced input cost in treatment of mastitis to the tune of Rs. 1800 per animal (Devgania et al. 2015). The compliance with patent legislation provide impetus to these technologies in value chain. IPR are vital during development stage of a medication from conception stage to product. These patent protected technologies can aid in development of new products and minimize factors affecting innovation performance. Innovation waves can happen with support of social transformation, complementary conditions, longer duration, availability of radical innovation and mass production (GII, 2022).The interface of indigenous knowledge with service institutions where farmers largely rely was stressed (Thakur et al. 2022). Sustained efforts are needed to realise potential of technologies into scalable innovation in market place.

Prosecution duration for herbal pharmaceutical applications

Investigators had evaluated prosecution record(s) of each of these 28 pharmaceutical patent grants. As per WTO requirement, developing countries evolved towards open economies and tries to adapt their patent systems (Vu, 2012). Applications have to meet requirements of Access and Benefit Sharing [ABS] agreement as per Biodiversity Act 2002 to receive IPO clearance. The regulation based on Convention on Biological Diversity [CBD] in seeking mutually agreed agreement for equitable benefit sharing upon utilization of material were adhered from knowledge holders. These obligations were based on Nagoya protocol, a supplementary agreement under CBD on ABS implemented from 12th October 2014 (Davis et al. 2015). The study noted that average time duration during prosecution stage was945 days [approximately 2.62 years or31 months]. It had taken on an average 3612 days [approximately 10 years/120 months] to accord patent grant from the date of priority. This empirical contribution is essential for scope of filing patent and appropriability of wisdom.

Conclusion

Herbal practices are the immediate access points for health care system especially in dairy sector. The study had provided insights on innovation capabilities of green grassroots innovators based on 28 patent grants. A total of 13 patents were granted in North; Western regions; 13 patent grants were in Southern regions and 2 patent grants in North Eastern regions of the country. These were from larger geographical regions of the country illustrating complementing role of grassroots innovators in dairy production system.Upon examination of pharmaceutical patent grants it was noted that Section 3 (d), 3 (e) and 3 (p) as major objections during

prosecution stages. Claim analysis indicated that most of these grants had novel poly herbal composition. It also highlighted the changed direction of patent regime moving towards product patent system thereby augmenting domestic innovation(s). The study-reinforced bottom up approach in incubating social innovation and protection of unique technologies may help in accessing new knowledge by dairy enterprises.

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SHORT COMMUNICATION

Effect of vacuum packaging on hardness of *Kradi* cheese stored at different temperatures

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Abstract: This study investigated changes in hardness property of *kradi* cheese stored under vacuum and normal conditions at 25°C, 5°C and -20°C at different intervals. At 25°C storage temperature hardness was affected by atmosphere whereas storage periods did not affect hardness of samples. At 5°C storage temperature both atmospheres as well as storage periods affected the hardness of samples. At -20°C storage temperature, it was observed that both atmosphere as well as storage periods affected the hardness of stored samples. Hardness in normal packed samples decreased more as compared to vacuum packed samples. The vacuum packaging retarded textural changes related to hardness in comparison to normal packaging.

Keywords: *Kradi* cheese; Vacuum storage; Hardness.

The consumption of organic foods is increasing (Akarca, 2020). Artisanal cheese are good sources of nutrients (Herman-Laraet al.. 2019). Demand for artisanal cheeses is growing at national and international level (Cagri-Mehmetoglu, 2018). Kradi cheese is an organic artisanal cheese of Jammu Kashmir manufactured by Gujjar tribes. The sale of *kradi* cheese is increasing annually but no studies has been reported with respect to hardness changes during storage. Studies on *kradi* cheese are very limited and there is no data on the hardness changes which take place during the storage.

The textural degradation of cheese is not desired during storage. Such textural changes induced during storage can alter the quality of product (Foxet al., 2004). Physico chemical, microbiological, microstructural properties, descriptive sensory analysis and chemical changes during storage of *kradi* cheese have been reported (Punoo,et al.. 2018a; Punoo,et al.. 2018b, Punoo,et al.. 2018c, Punoo, H.A, 2020).

As compared to ordinary packaging, vacuum packaging can reduce textural deterioration. The alteration in packaging condition by vacuum packaging can either accelerate or inhibit textural changes during storage of *kradi* cheese. Thus vacuum packaging can be a way of preserving textural quality of *kradi* cheese. The present study was aimed at assessment of hardness quality of textural changes of *kradi* cheese throughout its storage at different periods at refrigeration temperature under vacuum and non vacuum conditions.

It would be immensely interesting, therefore, to know as to how packaging conditions influence the textural properties of *Kradi*. There is no appropriate information available on any of the aspects of this product. The need to undertake the research to study the manufacturing process of *Kradi* on scientific basis with an aim to improve the overall quality of the product in terms of textural properties is immensely felt.

Kradi cheese was made as per the method described (Punooet al.. 2018a). The fresh product was packed in multilayer laminates under vacuum and normal conditions and stored at refrigeration temperature (5±1°C). The products stored were evaluated at one day interval at 25°C, on weekly interval at 5°C and on monthly interval at -20°C to monitor changes in textural properties.

The textural profile of *kradi* cheese was performed using TAXT-2i (Stable Micro System, UK) fitted with a 25 kg load cell. The cubes of *kradi* cheese samples 1x1x1 cm^2 were subjected to mono-axial compression up to 80% of its original height on the textural analyzer. The TPA was carried out at 25°C after tempering the sample for 1 h at this temperature. The textural parameters of hardness was determined according to the method of Bourne (1978).

The data obtained during the present investigation was compared by one-way analysis of variance (ANOVA) with the application of SYSTAT software, version 6.0.1 copyright © 1996, SPSS INC

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and also by Microsoft® Excel StatPro™ (Palaside Corporation, Newfield, NY). Significant difference ($p<0.05$) among treatments were detected using Duncan's multiple range tests.

Texture profile analysis (TPA) reveals the texture of cheeses. The compression of molar teeth during mastication is simulated by Texture profile analysis (TPA). Texture profile analysis (TPA) predicts the texture characteristic of cheese before consumption (Delgadoet al.. 2011). Textural parameters hardness, adhesiveness, cohesiveness, springiness, elasticity, and gumminess are generated by TPA. The force applied by the molar teeth to compress the food is described by hardness. Cheese consumers and manufacturers give importance to textural property of cheese. Consumers perceive texture as one of the indicators of cheese quality. The changes occurred in hardness of *Kradi* cheese samples packed in two types of atmospheres (vacuum and normal) and stored in different temperatures at 25 °C, 5 °C and - 20°C for different periods (Table 1). The hardness of samples exhibited a decreasing trend throughout the entire storage period in all the samples packed in two types of atmospheres (vacuum and normal) and stored at three different temperatures for different periods. It can be observed that initial value of hardness 151.54 N in normal packed samples decreased to 121.52 N on 15th day of storage while in vacuum packed samples it decreased to 132.1N on 20th day of storage at 25°C. This indicates that decrease in hardness was slow under vacuum conditions as compared to normal conditions. The estimation of hardness at 25°C was stopped after 15th day of storage in normal packaging as the samples were not sensorialy accepted while in samples packed under vacuum hardness measurement was continued till 20th day of storage as the samples were sensorialy acceptable. ANOVA (Table 2) revealed that packages had highly significant (pd"0.01) effect whereas storage periods had not significant effect on the hardness of samples stored at 25°C. Patil, G.R and Rao, K.J. (2006) reported decrease in hardness in ready to eat canned paneer cury during storage at 30°C for 90 days. Mohamed, A.G and Shalaby, S.M. (2016) reported decrease in hardness in analogue process cheese stored at 25°C for three months. Punoo,et al.. (2017) reported decrease in hardness in soy paneer prepared from admixtures of skim cow milk and soymilk stored at 25°C for

Table1: Effect of vacuum packaging on hardness characteristic of Kradi cheese stored at 25°C and 5°C

Period of storage (Days)	Temperature of storage (25°C)		Period of storage (weeks)	Temperature of storage (5°C)		Period of storage (months)	Temperature of storage (5°C)	
	Hardness			Hardness			Hardness	
	VP	NP		VP	NP		VP	NP
1	151.54	151.54	1	151.54	151.54	1	151.54	151.54
2	150.31	149.23	2	148.21	145.54	2	146.35	144.52
3	149.21	147.52	3	145.31	139.54	3	142.15	137.40
4	148.11	145.21	4	142.21	132.54	4	138.32	132.52
5	147.25	143.45	5	139.52	128.54	5	134.10	128.20
6	146.11	141.45	6	135.31	123.47	6	127.25	123.21
7	145.21	139.47	7	132.21	119.75			
8	144.36	137.70	8	129.65	115.41			
9	143.63	135.24	9	127.52	111.14			
10	142.58	133.52	10	124.21	107.42			
11	141.65	131.21	11	121.11	105.21			
12	140.25	128.90	12	118.52	103.2			
13	139.62	126.11	13	115.42	98.96			
14	138.52	123.75	14	113.21	97.52			
15	137.52	121.52	15	111.11	-			
16	136.22	-	16	109.61	-			
17	135.41	-						
18	134.11	-						
19	133.51	-						
20	132.11	-						

Table 2: Analysis of variance for hardness characteristic of *Kradi* stored at 25°C, 5°C and - 20°C

Attribute	df (between packaging systems)	Mean sum of squares Packaging system	Time interval	F- Value
Hardness (at 25°C)	1	3461.47	442.05	9.30**
Hardness (at 5°C)	1	1326.12	549.48	98.94**
Hardness (at -20°C)	1	43.33	240.89	17.71**

* * Significant at 1% level of probability

fifteen days. At 5°C of storage temperature, the initial hardness of 151.54 N in air packed samples decreased to 97.52 N after 14th week while in vacuum packed samples it decreased to 109.61N after 16th week of storage. The hardness measurement at 5°C was stopped after 14th week of storage in normal packaging as the samples were not sensorialy accepted while samples packed under vacuum hardness measurement was continued till 16th day of storage as the samples were sensorialy acceptable. ANOVA (Table 2) revealed that effect of packages and storage periods was highly significant (pd"0.01) from the consideration of the hardness of samples stored at 5°C. Mohamed, A.G and shalaby, S.M. (2016) reported decrease in hardness in analogue process cheese stored at 5°C for three months. Dimitreli,Get al.. (2017)) reported decrease in hardness in white soft cheese made from buffalo and cow milk mixtures at 4°C during three month storage. Alamet al.. (2016) reported decrease in hardness of mozzarella cheese at all atmospheres at 7°C throughout entire storage period. Punoo,et al., (2017) reported decrease in hardness in soy paneer prepared from admixtures of skim cow milk and soymilk stored at 5°C for ninety days. Decrease in hardness was reported in vacuum packed ewe's cheese during 90 days storage at 4°C by Caro, A, D.,et al. (2016). Ghisoni, F,et al.. (2022) reported decrease in hardness in taleggio cheese (an Italian cheese) in micro-perforated packaging during 28 days of storage. Mileriene, J,et al.. (2021) reported decrease in hardness in Eastern European curd cheese coated in antimicrobial protein-based (5%, wt/wt) edible coating, during 31 days of storage. Szkolnicka,et al. (2021) found decrease in hardness in quark cheese made from buttermilk during 3-week refrigerated (4°C) storage. At -20°C of storage temperature, the initial hardness of 151.54 N in normal packed samples decreased to 123.21 N after 6 months whereas in vacuum packed samples it decreased to 127.25 N after 6 months of storage. This indicates that decrease in hardness was slow at deep freeze as compared to refrigeration temperature. The results in general are in accordance with the findings of Ghosh (1987), Olson and Johnson (1990) and Lawrenceet al.. (1987), who inferred that values for hardness decreased significantly with time. ANOVA (Table 2) revealed that packages and storage periods had highly significant (pd"0.01) effect on the hardness of samples stored at -20°C. Punoo,et al., (2017) reported decrease in hardness in soy paneer prepared from admixtures of skim cow milk and soymilk stored at -20°C for five months. Decrease in hardness in kradi cheese can be due to increased proteolysis during storage (Punoo, 2020).

Conclusion

The effects of vacuum packaging on *kradi* cheese revealed that decrease in hardness values were retarded as compared to ordinary packaging. Textural quality of product was better maintained at refrigeration temperature of 5°C. Therefore, vacuum packaging could be an alternative to conventional normal storage for fresh *kradi* cheese production by obtaining better hardness score. Therefore, vacuum packaging of *kradi* cheese can maintain the hardness of this traditional regional product and can guarantee the consumers a quality product. Vacuum packaging will therefore offer manufacturers to store the product for longer time with maintained hardness quality.

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