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RESEARCH ARTICLE

Detection of cotton seed oil in cow ghee using triglyceride profiling

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Abstract: The triglyceride profiling of ghee shows significant variations in different regions of India, influenced by factors such as feeding practices, fatty acid composition, lactation stage, and season. Our study focused on detecting and identifying ghee adulterated with cottonseed oil and ghee sourced from the cotton tract area. Triglyceride profiling was employed on pure, adulterated, and cotton tract area ghee samples. Our findings revealed consistent lower levels of triglycerides with carbon numbers 24 to 30 in cow ghee, while higher levels were observed for carbon numbers 38 to 40 and 50 to 54. However, in ghee adulterated with cottonseed oil, triglycerides with carbon numbers 34 to 38 significantly increased ($p<0.05$), while those with carbon numbers 50 to 52 decreased significantly ($p<0.05$) with increasing concentrations of cottonseed oil. To verify the authenticity of the ghee samples, we analyzed normal cow ghee from Livestock Research Centre, National Dairy Research Institute using parameters including S-total, S2, S3, S4, and S5. The observed values for these parameters (98.77 ± 0.23, 103.30 ± 0.19, 99.55 ± 0.22, 97.42 ± 1.31, and 98.34 ± 0.27, respectively) fell within the range specified by International Organization for Standardization. In conclusion, our study highlights the potential of triglyceride profiling as an effective tool for detecting and differentiating ghee adulterated with cottonseed oil and ghee sourced from the cotton tract area. This method is suitable for detecting the presence of cottonseed oil to the tune of 10% in ghee. This technique provides valuable insights into assessing the authenticity and quality of ghee, contributing to the prevention of adulteration practices in the ghee industry.

Keywords: Adulteration, cotton tract area ghee, Gas chromatography, Mass spectrometry, S-values, Quality assurance

Abbreviations:

Abbreviation	Full name
DAHD	Department of Animal Husbandry and Dairying
GC	Gas chromatography
ISO	International Organization for Standardization
IDF	International Dairy Federation
LRC	Livestock Research Centre
NDRI	National Dairy Research Institute
PHVO	Partial hydrogenated vegetable oil
CSO	Cotton seed oil
CTA	Cotton tract area
MMT	Million metric ton
TG	Triglyceride
AOAC	Association of Official Analytical Chemists

Introduction

India, as the leading milk producer globally, witnessed milk production of 209.96 MT in 2020-2021 and 221.06 MT in 2021-2022, exhibiting an annual growth rate of 5.29% (Annual report; DAHD, 2022). Approximately 27.5% of the total milk produced in India is utilized for ghee production (Atbhaiya et al. 2022), making ghee the largest segment among indigenous milk products. Ghee packaging consists of about 60% bulk packaging and 40% consumer packs (Atbhaiya et al. 2022). The production levels of ghee vary across regions, with 57% in the northern region, 9.5% in the eastern region, 23.5% in the western region, and 10% in the southern region (Ramani et al. 2019). Ghee holds a superior position among fats intended for human consumption due to its unique properties, such as better digestibility and potential anti-cancer properties attributed to short-chain fatty acids (Sharma et al. 2020).

Due to its high price and demand, ghee is vulnerable to adulteration by middlemen. Adulterants commonly found in ghee include vegetable oil, cottonseed oil, and animal body fat.

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Differentiating between ghee sourced from the cotton tract area and ghee adulterated with cottonseed oil poses a major challenge. Previous researchers have utilized physico-chemical tests to detect the presence of non-dairy fats and adulterants in butter fat (Kumar et al. 2009; Gandhi et al. 2020; Atbhaiya et al. 2022). Additionally, Joachim (2007) recommended analyzing the lipid composition of milk fat to determine its quality, while Kala (2013) analyzed the triacylglycerols (TG/TAG) composition of milk fat to detect small amounts of extraneous fat. The International Organization for Standardization (ISO) developed a reference method (ISO 17678:2010) to determine the purity of cow milk fat based on the triglyceride (TG) composition consisting of even carbon numbers (24 to 54) using gas liquid chromatography. However, this method is limited to detecting animal body fat and vegetable oils up to 2% levels (Kala et al. 2016; Hazara et al. 2017). Triglyceride profiling has been explored to distinguish cow and buffalo ghee from designer fats used to manipulate the Reichert-Meissl value of ghee (Pathania et al. 2021). Studies have observed structural similarities in the distribution and composition of TG in milk from different bovine breeds (Christie, 1983). Feeding cows diet rich in stearic and linoleic acids, which are modified by rumen microflora, resulted in increased levels of stearic, palmitic, and oleic fatty acids (Christie, 1983). Multiple regression analysis models have been developed to detect the presence of non-dairy fats in pure milk fat. The European Union (EU) has adopted an official reference method based on TG analysis using low-resolution gas chromatography coupled with capillary or packed columns to detect foreign or non-dairy fat in dairy fat. ISO and International Dairy Federation (IDF) have also documented a TG composition-based method, along with standardized formulae (referred to as standardised (S) values), for detecting specific adulterants (ISO, 2010).

Differential scanning calorimetry has been employed to detect ghee adulterated with animal body fat at higher levels (Upadhyay et al. 2016), while Fourier transform infrared (FTIR) spectroscopy has emerged as a sensitive and rapid technique for detecting various types of adulteration in milk and ghee (Gandhi et al. 2022). Recent applications of FTIR include detecting adulteration with common sugar, added urea, soymilk, synthetic milk, goat fat, and pig fat in pure ghee (Jaiswal et al. 2015; Jha et al. 2015; Upadhyay et al. 2016; Upadhyay et al. 2018).

Considering the aforementioned research gaps, this study aimed to determine the triglyceride profile of cow ghee sourced from the cotton tract area and ghee adulterated with cottonseed oil using GC-MS/MS.

Materials and methods

Ghee samples were collected from various cotton tract regions in India, including Maharashtra, Gujarat, and Haryana. Sahiwal breed cows in mid lactation were fed with cottonseed cake at different percentages (15% and 30%) along with regular feed at the Livestock Research Centre (LRC), NDRI, Karnal, and milk samples were collected at specific time intervals (0, 20, 40, 60, and 85 days). All reagents and chemicals used in the analysis were of HPLC or analytical grade and purchased from Sigma Aldrich India. Standard triglyceride mixes (Catalogue No. T7140) were purchased from Sigma Aldrich St. Louis, USA for analysis.

Sample preparation

Ghee was prepared using the creamery butter method described by De (2010), utilizing unripened cow cream. Milk samples from various cotton tract areas were collected for ghee preparation. Pure ghee was then adulterated with cottonseed oil at different concentrations (1, 5 and 10%) on a weight-to-weight basis.

Triglyceride profiling using GC-MS/MS

Samples were analyzed for triglyceride profiling according to the ISO 17678:2010/IDF 202:2010 standard. Initially, 50 grams of melted ghee were weighed, and 0.5 to 1.0 grams of previously dried sodium sulfate at 90°C for 1 hour were added to the ghee. The mixture was then filtered using filter paper. Subsequently, 0.5 milliliters of the melted and filtered ghee were combined with 5 milliliters of n-hexane in a 15-milliliter centrifuge tube. The mixture was vortexed for 1 minute. Afterwards, 1.5 milliliters of the resulting test portion were transferred to GC vials for analysis using gas chromatography-mass spectrometry (GC-MS).

The samples were analyzed using a GC-MS/MS instrument (Shimadzu O207051) equipped with an autosampler and a fused silica *SLB®-35ms* capillary column (30 meters length, 0.25 mm internal diameter, and 0.25 µm particle size, Supelco, Sigma-Aldrich, USA). The derivatized sample was injected into the column using a volume of one microliter, while keeping the injector temperature at a constant 330°C. The GC oven program started with an initial temperature of 80°C (held for 0.5 minutes) and then increased incrementally at a rate of 50°C per minute up to 190°C. Subsequently, the temperature was further increased to 330°C at a rate of 6°C per minute. The final temperature of 330°C was held constant for 10 minutes. The carrier gas (helium) flowed at a constant rate of 3 milliliters per minute in constant flow mode, with a purge flow of 3 mL/min. The interface temperature was set to 350°C, and the split ratio was maintained at 50:1.

Statistical analysis

All the experiments were conducted at least in triplicates (n=3). All the results were expressed as mean of replicates analysis. The results of triglycerides profile were statistically analyzed using SPSS 20.0 software (IBM SPSS version 20.0.NY).

Results and discussion

Triglyceride composition

Table 1 presents the triglyceride composition of cow and buffalo ghee, consisting of 16 triglycerides (C24 – C54). The triglyceride profile of ghee is influenced by factors such as fat content, lactation stage, and season (Palmquist et al. 2005). The triglyceride composition of milk also affects various physicochemical and functional properties of dairy-based products (Smiddy et al. 2012).

In cow ghee, the levels of triglycerides with carbon numbers ranging from 24 to 30 were lower, while those with carbon numbers from 38 to 40 and 50 to 54 were higher (Fig. 1). However, in ghee adulterated with cottonseed oil, there was a significant increase ($p<0.05$) in triglycerides with carbon numbers ranging from 34 to 38, while those with carbon numbers from 50 to 52 decreased significantly ($p<0.05$) with increasing concentrations of

Fig. 1 GC-MS chromatogram showing peaks of triglycerides of normal cow ghee obtained from LRC, NDRI

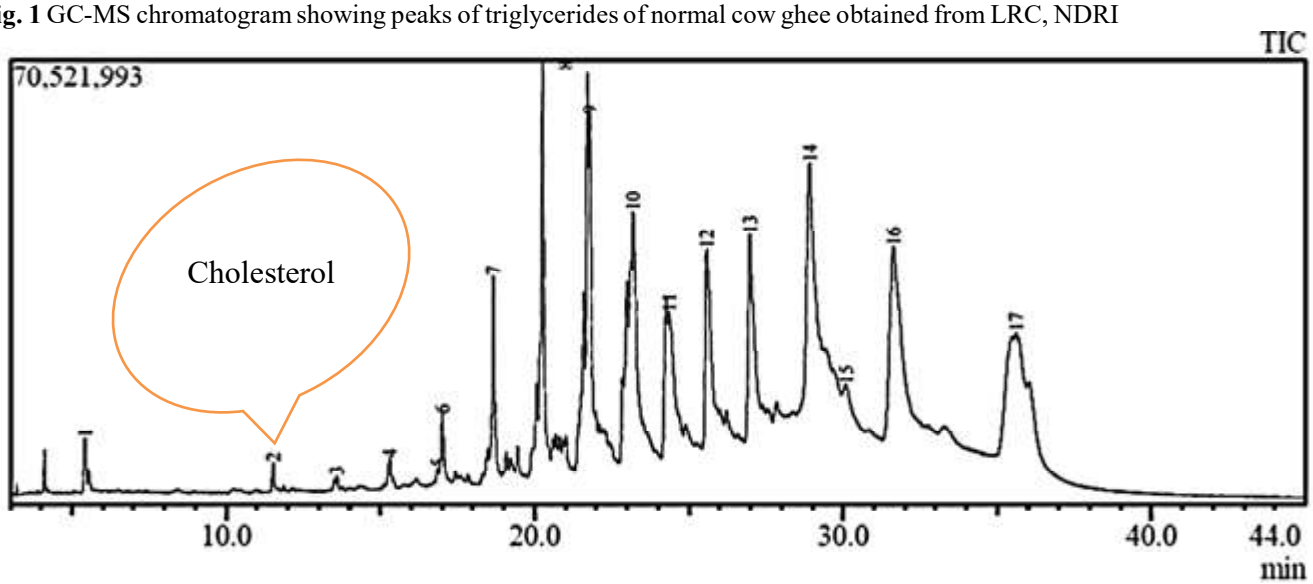

Table 1 Percentage relative triglycerides composition of the milk fat of cow and buffalo

	Pure Cow milk fat	Cow milk procured from Maharashtra	Cow milk procured from Gujarat	Cow milk procured from Haryana	Cottonseed oil 1%	Cottonseed oil 5%	Cottonseed oil 10%
C24	0.10±0.08^{a}	0.28±0.16^{c}	0.22±0.14^{c}	0.25±0.08^{c}	0.06±0.03^{a}	0.19±0.05^{bc}	0.19±0.05^{bc}
C26	0.17±0.06^{a}	0.24±0.06^{b}	0.23±0.10^{b}	0.15±0.07^{a}	0.21±0.01^{b}	0.09±0.00^{c}	0.38±0.02^{d}
C28	0.21±0.09^{a}	0.15±0.02^{c}	0.20±0.08^{a}	0.51±0.31^{d}	0.28±0.02^{e}	0.06±0.02^{f}	0.46±0.04^{g}
C30	0.64±0.48^{a}	0.76±0.09^{c}	0.51±0.10^{a}	0.74±0.06^{a}	0.65±0.48^{a}	0.38±0.03^{d}	1.48±0.03^{be}
C32	1.13±0.13^{a}	1.16±0.07^{a}	1.41±0.23^{ac}	1.45±0.09^{ac}	1.15±0.11^{a}	2.38±0.05^{d}	2.20±0.01^{de}
C34	3.72±0.32^{a}	3.31±0.24$^{a\,c}$	3.38±0.20^{ac}	3.52±0.43^{ac}	3.71±0.31^{a}	4.85±0.01^{ab}	7.45±0.05^{d}
C36	7.61±0.15^{a}	7.60±0.10^{a}	7.22±0.22^{ab}	7.36±0.05^{bc}	7.67±0.26^{ab}	11.86±0.02^{d}	8.93±0.06^{de}
C38	10.41±0.29^{a}	10.23±0.01^{a}	10.17±0.09^{a}	10.36±0.27^{a}	10.38±0.33^{a}	12.57±0.05^{c}	13.25±0.02^{d}
C40	10.44±0.14^{a}	10.56±0.27^{a}	10.19±0.10^{a}	10.15±0.11^{a}	10.40±0.10^{a}	6.93±0.03^{b}	9.46±0.05^{c}
C42	8.34±0.04^{a}	8.30±0.04^{a}	8.42±0.36^{a}	8.35±0.08^{a}	8.34±0.04^{a}	3.29±0.07^{b}	8.57±0.02^{a}
C44	7.65±0.34^{a}	7.32±0.01^{a}	7.54±0.38^{a}	7.41±0.26^{a}	7.49±0.31^{a}	7.64±0.01^{a}	7.08±0.04^{a}
C46	6.96±0.43^{a}	7.36±0.34^{b}	8.25±0.71^{c}	7.77±0.46^{b}	7.12±0.38^{a}	9.53±0.05^{d}	6.08±0.00^{ae}
C48	7.67±0.23^{a}	7.91±0.07^{a}	7.66±0.79^{a}	7.51±0.34^{a}	7.45±0.33^{a}	0.58±0.02^{b}	9.22±0.05^{c}
C50	14.55±0.11^{a}	14.79±0.12^{a}	14.59±0.20^{a}	14.48±0.13^{a}	14.45±0.20^{a}	12.36±0.02^{c}	8.79±0.03^{d}
C52	11.47±0.30^{a}	11.46±0.25^{a}	11.28±0.16^{a}	11.35±0.16^{a}	11.41±0.26^{a}	6.66±0.09^{b}	8.63±0.02^{c}
C54	8.79±0.00^{a}	8.59±0.09^{a}	8.19±0.65^{a}	8.34±0.19^{a}	9.10±0.53^{b}	19.42±0.05^{c}	7.14±0.05^{d}

Values are means ± standard deviation; different superscript letters in a row indicate significant differences (One-way ANNOVA with least significant Difference Tukey and Duncan tests, $P<0.05$). Calculation was based on triplicate measurement per milk sample, standard deviation were calculated over samples at least in triplicates.

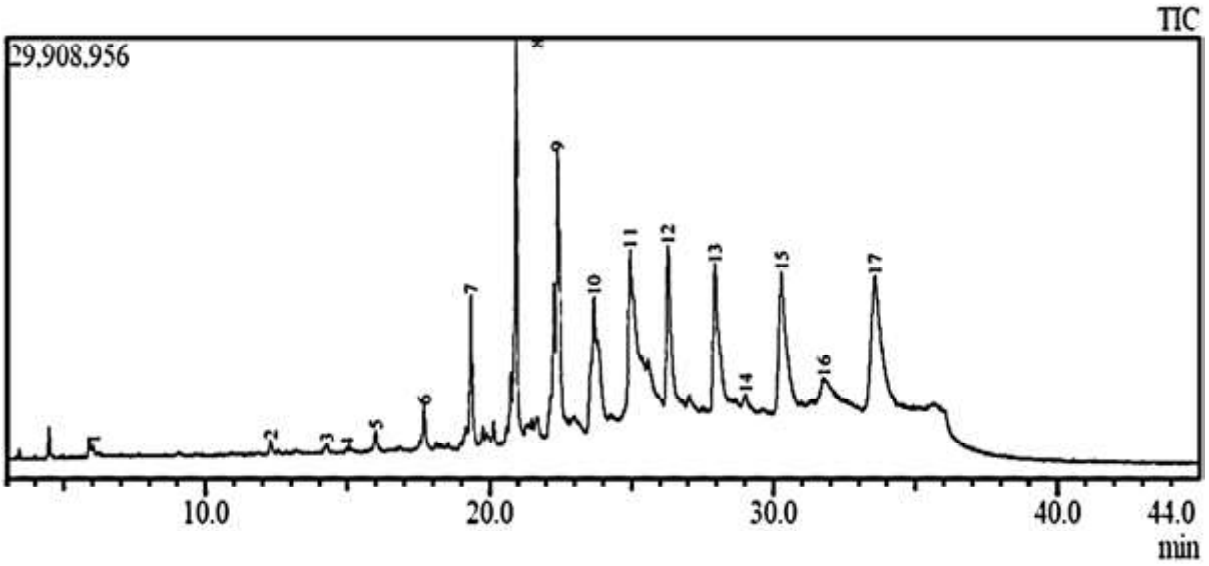

Fig. 2 GC-MS chromatogram showing peaks of triglycerides of ghee adulterated with 5% cotton seed oil.

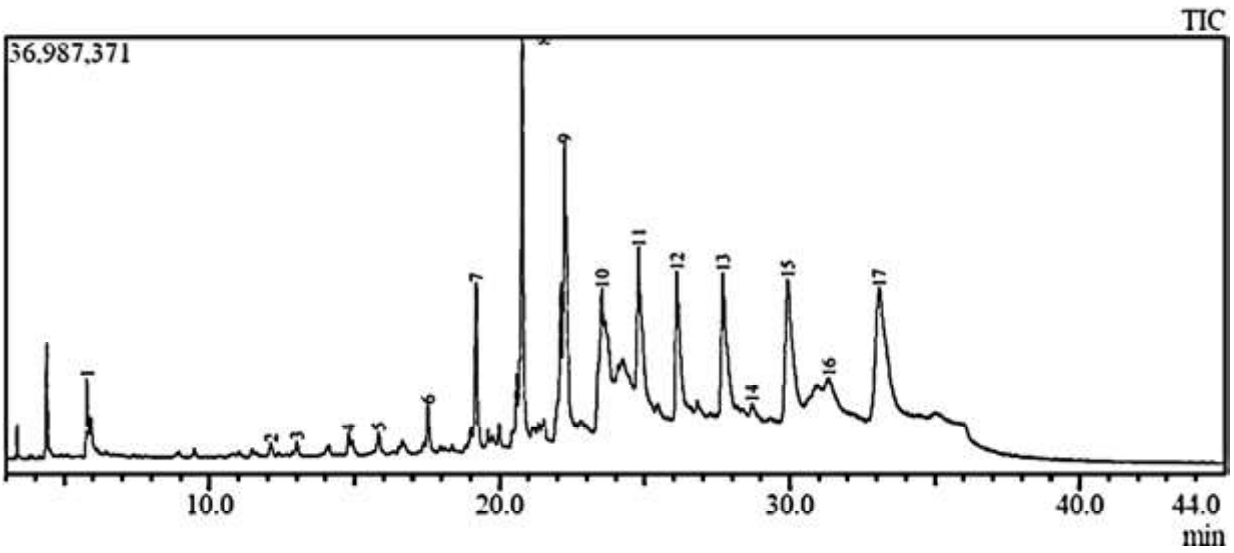

Fig. 3 GC-MS chromatogram showing peaks of triglycerides of ghee adulterated with 10% cotton seed oil

cottonseed oil (Fig. 2 and 3). Van Ruth et al. (2010) and Kala et al. (2013) reported that during the summer period, when cows were fed on grass, the triglyceride with carbon number 52 was highest, while in the winter season, when cows were fed with bran, the maximum levels were found for triglycerides with carbon number 50. Triglycerides C38, C50, and C52 were observed to be at their maximum in cow milk fat (Smiddy et al. 2012).

The triglyceride profiles of pure cow and buffalo milk fat differed, particularly with significantly ($p<0.05$) higher levels of C24 to C30 and C38 in buffalo milk fat compared to cow milk fat. However, triglycerides with carbon atoms ranging from 50 to 54 were significantly ($p<0.05$) lower in pure buffalo ghee compared to pure cow ghee. It is evident from the above discussion that the triglyceride profile of milk fat varies among different species, primarily influenced by genetic factors and feeding habits. Fontecha et al. (1998) reported that the triglyceride content of milk fat not only varies among different species but can also be observed within breeds of the same species.

A similar trend was observed in the ghee adulterated with cottonseed oil, with a significant increase ($p<0.05$) in the triglyceride profile of C34 to C38, while the profile of C50 to C52 significantly decreased ($p<0.05$) compared to other values like C40 to C48 and C54.

The relative weight percentages of triglycerides C48, C50, C52, and C54 were higher in beef tallow, lard, and partially hydrogenated vegetable oil (PHVO), while C34, C36, and C38 were higher in coconut oil compared to milk fat triglyceride profile. The reference method for triglyceride profiling does not have documented detection levels for these adulterants. Therefore, a study was conducted to investigate the triglyceride profile of ghee prepared from standardized milk (which represents mixed milk and is commonly used for ghee production in established dairy industries in India) adulterated with the aforementioned foreign fats. The triglyceride profiles of the ghee showed a decrease in C24, C26, C28, and C30 for all types of adulterants.

Table 2 Standardized (S)- limits of NDRI cow ghee, NDRI buffalo ghee, cotton tract area ghee and ghee adulterated with cotton seed oil ghee samples

S- limits for pure cow milk fats as per ISO standard		NDRI cow ghee	Cotton tract area ghee			Ghee adulterated with different concentration of cotton seed oil		
S- equations	Limits	(n=3)	Maharashtra (n=3)	Gujarat (n=3)	Haryana (n=3)	1% (n=3)	5% (n=3)	10% (n=3)
S2	98.05-105.95	103.30±0.19^{a}	104.45±0.86^{a}	103.45±0.75^{a}	102.91±0.80^{a}	102.10±0.10^{b}	100.13±0.63^{b}	89.87±0.29^{c}
S3	99.42-100.58	99.55±0.22^{a}	99.62±0.35^{a}	99.69±0.24^{a}	99.75±0.21^{a}	99.57±0.25^{a}	99.46±0.08^{a}	99.65±0.20^{a}
S4	95.9-104.10	97.42±1.31^{ab}	98.89±1.43^{bc}	97.75±1.48^{ab}	97.29±1.65^{ab}	98.82±0.33^{bc}	100.23±1.33^{c}	96.17±0.35^{a}
S5	97.96-102.04	98.34±0.27^{a}	98.90±1.52^{ab}	100.46±1.47^{bcd}	99.32±1.24^{abc}	101.06±0.08^{cd}	101.83±0.32^{d}	99.06±0.21^{ab}
S-total	95.68-104.32	98.77±0.23^{ab}	99.15±2.79^{ab}	96.81±1.52^{a}	97.50±1.68^{a}	100.40±0.44^{b}	97.49±0.54^{a}	96.85±0.24^{a}

Soy bean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed and fish oil); S3 (Coconut and palm kernel fat); S4 (Palm oil and beef tallow) and S5 (Lard)

Table 3 Standardized (S)- limits of ghee obtained from animal fed cotton seed cake in different concentration at LRC, NDRI

S- limits for pure cow milk fats as per ISO standard		Animal fed cotton seed cake in different concentration at LRC, NDRI								
S- equations	Limits	Control	15%, 20 days (n=3)	30%, 20 days (n=3)	15%, 40 days (n=3)	30%, 40 days (n=3)	15%, 60 days (n=3)	30%, 60 days (n=3)	15%, 85 days (n=3)	30%, 85 days (n=3)
S2	98.05-105.95	103.30±0.19^{a}	102.78±0.25^{ab}	102.35±0.37^{bc}	101.81±0.36^{d}	102.19±0.45^{cd}	103.58±0.27^{a}	102.40±0.24^{cd}	103.49±0.15^{a}	104.83±0.07^{e}
S3	99.42-100.58	99.55±0.22^{a}	99.79±0.12^{a}	99.49±0.33^{a}	99.67±0.33^{a}	99.74±0.16^{a}	99.65±0.36^{a}	99.48±0.09^{a}	99.49±0.14^{a}	99.49±0.06^{a}
S4	95.9-104.10	97.42±1.31^{ab}	96.74±0.96^{b}	96.97±0.38^{b}	96.97±0.38^{b}	100.15±0.27^{c}	99.51±0.27^{c}	96.38±0.37^{a}	96.79±0.27^{a}	98.75±0.33^{bc}
S5	97.96-102.04	98.34±0.27^{a}	101.89±0.12^{b}	101.08±0.39^{c}	98.32±0.20^{a}	98.75±0.37^{a}	98.43±0.17^{a}	98.41±0.13^{a}	98.45±0.35^{a}	98.69±0.29^{a}
S-total	95.68-104.32	98.77±0.23^{a}	96.94±0.37^{bc}	97.66±0.51^{ab}	97.61±0.41^{ab}	100.60±0.25^{d}	100.50±0.42^{d}	97.53±0.29^{bc}	96.20±0.83^{c}	98.45±0.31^{a}

S2 (Soy bean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed and fish oil); S3 (Coconut and palm kernel fat); S4 (Palm oil and beef tallow) and S5 (Lard)

The standardized milk ghee adulterated with beef tallow and lard at 6.56% and 6.27%, respectively, exhibited an increase in C50, C52, and C54 (Kala, 2013). As discussed earlier, in the present study, pure cow ghee adulterated with cottonseed oil showed an increase in C24 to C30 and a decrease in C50 to C54.

Gutiérrez et al. (2009) detected non-milk fat adulteration in milk samples, using low- resolution GC. Adulteration with PHVO, indicated the increase in C54 height and area, which could be considered as a better marker than the changes in any other TG. Application of GC analysis for TG profiling and estimation of trans fatty acid content, detected PHVO adulteration at 5% level in milk fat (Destaillats et al. 2006). Although the TG profiles and S values predict the possible adulteration, precise fingerprinting of the adulterants used to adulterate the sample cannot not be obtained. PHVO adulteration also could be judged by examining the sterol components (Alonso et al. 1997). TG profiling can also be applied to detect the presence of animal body fats, as they lack in short-chain fatty acid, and have a low amount of 24 – 28 carbon-numbered TG. The increase in peak heights of C50 and C52 compared to C38, as well as the increase in the relative weight percentages of C50, C52, and C54 compared to control ghee samples, could be considered for detecting foreign fats in ghee samples through triglyceride estimations.

Standardized (S)–limits of different ghee samples

The S-limits for different types of ghee samples were determined using the equations outlined in the ISO 17678 (2010) reference method. The S-limits for pure cow ghee were found to be within the range specified by the ISO standards, as shown in Table 2. When comparing the S-values obtained from the equations (Table 2) for various S-limits, it was observed that all corresponding values for the pure ghee samples fell within the specified limits for S-values.

However, if the calculated S-value falls outside the corresponding limits, it indicates that the fat sample is adulterated with foreign fat. For example, when analyzing milk fat adulterated with substances such as sunflower, soybean, rapeseed, olive, wheat gram, linseed, cottonseed, fish oil, and maize germ, the corresponding S-limits were found to be outside the prescribed range (i.e., S2-limit of 98.05 – 101.95) for pure cow ghee.

The S-total and S2 limit for cottonseed oil were found to be 4.13 and 15.35, respectively, which is significantly lower than the values prescribed for pure cow milk fat (S-total: 95.68 – 104.32; S2 limit: 98.05 – 101.95). When comparing the obtained S-values for ghee adulterated with cottonseed oil, the values were within the prescribed limit at 1% and 5% adulteration. However, at a higher level of adulteration (10%), the S-value decreased to 89.70. The other four S-limits (S-total, S3, S4, and S5: 96.71, 99.77, 95.97, and 98.94, respectively) were within the range specified by the ISO for pure cow ghee.

The triglyceride profiles of ghee showed an increase in C24 – C30, C36 – C38, and C48, while there was a decrease in C50 – C54 for ghee adulterated with 10% cottonseed oil. However, in the case of ghee, the S-limits value was outside the range. This discrepancy could be attributed to various factors such as different operating GC conditions and the method of extraction of milk fat.

Table 3 presents the S-values of ghee obtained from cows fed on cottonseed cake at different concentrations at LRC, NDRI, and all values were within the range specified by the ISO standard. Cows fed with 15% cottonseed cake at LRC, NDRI, after 80 days of feeding showed S-values (S-total, S2, S3, S4, and S5: 96.20±0.83, 103.49±0.15, 99.49±0.14, 96.79±0.27, and 98.45±0.35, respectively) within the ISO standard range. Similarly, cows fed with 30% cottonseed cake at LRC, NDRI, after 80 days of feeding displayed S-values (S-total, S2, S3, S4, and S5: 98.45±0.31, 104.83±0.07, 99.49±0.06, 98.75±0.33, and 98.69±0.29, respectively) within the ISO standard range.

Fontecha et al. (1998) also reported that the recommended S-value is not applicable for triglyceride profiling of cheese due to biochemical reactions during ripening and storage. Kala (2013) found that the S-value of market samples of ghee deviated from the recommended value, possibly due to storage conditions after preparation. Another study examined ghee samples containing 5% PHVO, which resulted in S-values outside the range for pure milk fat due to an increase in C50, C52, and C54 triglycerides. The detection level for beef tallow and coconut oil adulteration was found to be 2%. Vegetable fat and lard additions could be detected up to 5% and 6.3%, respectively (Kala, 2013). Furthermore, at a 2% level of adulteration of cow ghee with PHVO, an increase in C50, C52, and C54 was observed, but the S2 value (for vegetable fat adulteration) remained within the prescribed range (Kala, 2013).

The detection of adulteration of milk fat with an unknown adulterant up to a certain level can be achieved by estimating the triglyceride profile and calculating the S-total value. A standardized method can be applied to estimate the amount of foreign fat by specifying a value of 7.46 (for general unknown adulterant) and the S-total value obtained from the triglyceride profile (ISO 2010).

Conclusion

Triglyceride profile analysis of ghee samples is as an effective and reliable method to differentiate between pure ghee and ghee adulterated with cottonseed oil. This technique allows for the identification of specific triglyceride patterns that indicate the presence of adulterants. However, it may not be able to differentiate between ghee sourced from the cotton tract area and ghee adulterated with cottonseed oil especially when the adulteration level is below 5%. In such cases, the triglyceride profile may not exhibit significant variations that can be used for

accurate differentiation. Nevertheless, this method proves to be useful in detecting the presence of cottonseed oil in ghee when the adulteration level reaches 10%. This is evident from the S2 values obtained for such adulterated ghee, which were measured to be 89.87±0.29. These values fall below the prescribed limit of 98.05-105.95, indicating a clear deviation from the triglyceride profile of pure ghee. Therefore, by analyzing the triglyceride profiles of ghee samples, it is possible to identify and differentiate between pure ghee, ghee adulterated with cottonseed oil up to a certain level, and ghee originating from the cotton tract area. In summary, triglyceride profile analysis represents a significant step forward in combating ghee adulteration.

Conflicts of interest

None

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RESEARCH ARTICLE

Optimization and characterization of a value-added fermented ready-to-serve whey-based beverage with high antioxidant potential

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Abstract: This study was conducted to optimize the process parameters for developing a Ready-To-Serve fermented whey beverage using pineapple juice and mint and characterize the drink. The levels of pineapple juice (20-40%), sugar (5-10%), and mint powder (1-3%) on pH, DPPH, TSS, and overall acceptability were investigated using a central composite design. The optimized process conditions achieved were 39.42% (v/v) of pineapple juice, 5.75% (w/v) of sugar, and 1.01% (w/v) of *Mentha*. The beverage had 89.41% mg GAE/ml total phenolic content and 83.88% DPPH radical scavenging activity. FTIR spectrum confirmed the presence of the O-H stretch of phenolic compounds between 3200 cm^{-1}– 3518 cm^{-1} and conformational changes in the secondary structure of the whey protein at 1625-1647 cm^{-1} in the fermented sample. The beverage possessed all essential amino acids, the highest being Leu, Val, and Lys and PUFA (ω6/ω3=1.52) and MUFA levels of 6.7±0.96 and 21.57±0.02 mg/100mL, respectively. In addition, hypercholesterolemic fatty acids (C12:0, C14:0, C16:0) were significantly reduced after fermentation. Therefore, a serving of 100mL would contribute 3.53%, 2.96%, and 2.41% towards the RDA of Fe, K, and Mg, for an adult man, respectively. The whey beverage had acceptable microbiological quality on the 30th day of storage under refrigerated conditions. Fermentation with *Pediococcus pentosaceus* NCDC 273 reduced the coliform count by 5.57% and 4.04% and the Y&M count by 2.2% and 2.9% on the 15th and 30th day of storage, respectively.

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Introduction

Whey is a by-product of cheese or casein production containing about 90% of the milk volume and 55% of milk nutrients (Ryan & Walsh, 2016). However, this nutritive by-product from the dairy industry is the toughest to dispose of due to its high Biological and Chemical Oxygen Demand of around 39-48g/L and 60-70g/L, respectively (Islam et al. 2021; Panghal et al. 2018). Unfortunately, cheese whey is often disposed of as such in water bodies without pre-treatment of the whey, leading to water pollution. The whey output worldwide is approximately 180 million tonnes, with about 1.5 million tonnes of high-value protein and a lactose output of about 8.6 million tons (Rai et al. 2020). However, yearly, the total whey production is increasing by 1-2%, and less than 50% of the total whey produced is effectively utilized worldwide.

Due to its nutritional value, whey can be used to make refreshing and assimilable beverages. Compared to soft drinks available in the market, fruit and whey-based beverages are more likable and healthier due to their refreshing taste (Shukla, 2012). Furthermore, because processing whey to extract lactose and whey protein concentrates is costly, the formulation of refreshing long-life beverages appears to be a logical solution to draining nutritive whey and increasing water pollution from it. Therefore, researchers have investigated the likelihood of utilizing whey as a base ingredient in developing value-added fruit beverages (Ferreira et al. 2019; M'hir et al. 2019).

Pineapple (*Ananas comosus*) is a tropical fruit relished for its sweet acidic taste, distinct flavor, and aroma (Ali et al. 2020). It is ranked third, after banana and citrus, among the fruit production category in the world. Its world production was registered at about 27.82 million tonnes in 2020 (www.statista.com/statistics/298505/global-pineapple-production). Apart from being a good source of polyphenolic compounds such as gallic acid, chlorogenic acid, and ferulic acid, known to exhibit antioxidative, anticarcinogenic, and antimutagenic properties, it also provides benefits against cataracts and cardio-vascular diseases (Baljeet et al. 2013). The enzyme bromelain in pineapple aids digestion

along with antidiabetic, antihypertensive, and anti-cancerous effects (Ali et al. 2020). Pineapple also adds to the supply of dietary fiber (D.F.) to the food matrices that act as 'prebiotics' and improve the host's health by restoring the proliferation and colonization of probiotic bacteria (Dittakan et al. 2018). It is also a good source of specific vitamins such as A, B, and C and minerals such as magnesium, potassium, calcium, and iron (Islam et al. 2021). Mint (*Mentha* spp.) is a prevalent culinary and medicinal herb possessing curative and antioxidative properties that help prevent spoilage of any food. The most common mint plants are peppermint (*Mentha piperata*) and spearmint (*Mentha spicata*). Essential oils from these find several uses in treating headaches, diarrhea, indigestion, colds, and gallstone infections (Smits, 2013).

The present investigation focuses on developing a functional fermented whey beverage blended with pineapple juice and mint powder, fermented by lactic acid bacteria (*Pediococcus pentosaceus* NCDC 273). This strain is known to produce pediocin AcH/PA-1, which belongs to Class II bacteriocins and acts as a primary metabolite possessing antimicrobial activity against *Listeria monocytogenes* and several other pathogens (Lather et al. 2015; Simha et al. 2012). In addition, pediocin AcH/PA-1 produced by this bacterium has also been used to increase the shelf life of several food products, attributed to the production of pediocin, organic acids, and other metabolites (Garsa et al. 2014; Pandey et al. 2019; Verma et al. 2017)

Materials and methods

The study was carried out in the Department of Dairy Science and Food Technology, Institute of Agricultural Science, Banaras Hindu University, Varanasi, Uttar Pradesh, and ICAR-Central Inland Fisheries Research Institute, Barrackpore, Kolkata, West Bengal.

Preparation of ingredients for whey beverage

Chhana whey was prepared from standardized cow milk containing 4% fat and 8.5% SNF, according to Srivastava et al. (2018). Briefly, the milk was procured from the cattle yard, Banaras Hindu University, and after standardization, it was heated to 90°C, cooled to 70°C, and coagulated with 2% citric acid. Whey was collected by filtering the coagulated milk through a clean muslin cloth. A bioprotective culture of *Pediococcus pentosaceus* NCDC 273 was procured from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana. Aseptically, a lyophilized vial was opened and propagated in M.R.S. broth and preserved in 25% glycerol solution at -20°C for further use. Fresh channa whey was pasteurized (in-bottle pasteurization) at 70°C for 30 min.

Experimental Design

Response Surface Methodology was used to optimize the effect of pineapple juice (20-40 %), sugar (5-10 %), and mint powder (1-3%) levels on DPPH activity, TSS, pH, and overall acceptability of the whey beverage. During the investigation, 20 trials (experiments) were generated through Design Expert (7.0.0) software using Central Composite Rotatable Design (C.C.R.D.). Experimental tests suggested were executed, and the responses were fitted to the design. After each experimental trial, the responses were evaluated to see the effect of independent variables on them. A second-order polynomial equation was used to express the responses in terms of the model constant, liner effect of factor, the cross product of factor, the quadratic effect of factor, and residual error, including the experimental errors and lack of fit chosen for the model.

Beverage preparation

After de-crowning and peeling, the pineapple was cut into small pieces. Using a mixer grinder, the small bits were macerated into a pulp and filtered through a clean muslin cloth to extract the juice. The freshly extracted pineapple juice was pasteurized at 60°C for 1-2 mins. Mint powder used in the study was obtained by grinding the dried mint leaves. Next, pineapple juice was added to the whey, followed by sugar and mint powder to prepare the R.T.S. whey beverage. Fermentation was initiated by transferring the inoculum at 1% rate from an overnight fermented skim milk tube with *Pediococcus pentosaceus* NCDC 273. The beverage is then poured into glass bottles and kept for incubation. Based on the literature on this particular bioprotective strain, fermentation time was selected at 16h at 37°C for maximum release of bioprotective metabolites. After fermentation, the bottles were stored under refrigerated conditions.

Physico-chemical analysis

The fermented whey-based beverage infused with pineapple juice and mint powder was analyzed for its moisture, pH, and titratable acidity. The pH of the whey, pineapple juice, and beverage samples was determined directly using a digital pH meter (EUTECH Instruments). The pH and titratable acidity of whey and pineapple juice was recorded to be 5.34±0.003; 0.40±0.07 % lactic acid, and 3.78±0.004; 0.29±0.01 % lactic acid, respectively. The total soluble solids of the whey and pineapple juice, were determined using a digital refractometer (Milwaukee, Romania) and were recorded to be 6.43±0.30 and 8.43±1.11 ° Brix, respectively. The gravimetric method determined the beverage's moisture (on a dry matter basis) (AOAC, 1990). The organic and inorganic matter of the beverage was evaluated gravimetrically by igniting the sample, which had been oven dried until constant weight, in a muffle furnace at 550–600°C. The titratable acidity of the pineapple juice (as % acetic acid) and whey and beverage sample (as % lactic acid) were determined (AOAC, 1990). Five

mL of the sample was titrated against 0.1N NaOH using a few drops of phenolphthalein indicator to an endpoint of faint pink color. The nitrogen and, thereby, the crude protein (C.P.) content of the beverage was measured by the Kjeldahl method (AOAC, 1990) using KEL PLUS Protein Estimation System, Pelican Equipment Ltd., India.

Total Antioxidants

The antioxidant activity of the beverage samples was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Balakrishnan & Agrawal, (2014) with slight modifications. Two mL of the sample was added to 20 mL of 80% methanol solution, followed by proper extraction in a shaking incubator at 37°C for 2hrs at 100 rpm. The extract was centrifuged at 6000 rpm for 20 min at 4°C to collect the clear supernatant. The supernatant was then filtered with Whatman® paper 1. A stock solution of DPPH (0.035 mg DPPH/mL of methanol) was prepared. A volume of 700µl of each sample was added to 700 µl of DPPH stock solution and mixed properly by a vortex machine. Absorbance at 517nm was measured using a spectrophotometer (PerkinElmer) after incubating the samples and control in a dark place at room temperature for 30 min. The antioxidant activity of the beverage samples was determined by DPPH radical scavenging activity in percentage:

$$DPPH\ radical\ Scavenging\ Activity\ (\%) = \frac{Ac - As}{Ac} \times 100$$

where Ac-Absorbance of the control; As-Absorbance of the sample

Total phenolic Compounds

The Total Polyphenolic Content (TPC) of the beverage samples was determined according to Folin-Ciocalteau (F.C.) method described by M'hir et al. (2019) with slight modifications. First, two mL of the beverage sample was added to 20mL of 80% methanol solution. The mixture was left in the shaking incubator for 2hr at 37°C. Next, the extract was centrifuged at 6000 rpm for 20 min at 4°C to collect the clear supernatant. The supernatant was then filtered with a Whatman® filter paper 1. The so obtained filtrate (0.05 mL) was added to 2.75 mL of distilled water. Next, an aliquot of 0.2 mL of F.C. reagent was added to the above mixture, followed by the addition of 0.6mL of 20% sodium carbonate solution. Finally, the above mixture was shaken well using a vortex machine and kept in the dark for incubation at room temperature (30°C) for 30min, after which the absorbance was measured at 750 nm. TPC was calculated by deriving a standard curve where Gallic acid was used for generating the standard curve having concentrations ranging from 100 to 600 mg/mL.

Sensory evaluation

The beverage samples were evaluated for their sensory attributes as described by Sabokbar & Khodaiyan, (2015). The parameters judged were smell, color, flavor, consistency, and overall acceptability by an untrained panel comprising ten panelists from the Department of Dairy Science and Food Technology, Banaras Hindu University, Varanasi, India. An aliquot of 10mL of the prepared beverage was served in transparent bottles. The panelists were asked to record their remarks on the sensory sheet based on a 9point hedonic scale from 1 (extremely disliked) to 9 (extremely liked).

Fatty acid profiling

Fat extraction from the unfermented and fermented beverages was carried out according to the method described by Folch et al. (1957). For that, 50 ml of the drink was homogenized with 300 ml chloroform-methanol mixture in a ratio of 2:1, and 60 ml of water was added to the funnel. For fatty acid methyl esters (FAME), 10 ml fat extract was taken, and FAME was prepared following a standard protocol described by Metcalfe et al. (1966).

The fatty acid compositions of the samples were analyzed by Gas Chromatography-Mass Spectrometry (GC/MS, Thermo Scientific ITQ 900) with a GC (Trace GC Ultra, Thermo Scientific) having a 30 m×0.25 mm × 0.25 µm TR-FAME capillary column and an MS detector (ITQ 900, Thermo Scientific). For the separation of individual fatty acids, the oven temperature program was kept on hold at 50°C initially for 1 min; then, it was raised from 50°C to 150°C with a heating rate of 20°C/min, followed by a hold at 150°C for 15min. Finally, the temperature was increased from 150 to 240°C at a heating rate of 20°C/min with a final hold at 240°C for 3min. As a carrier gas, the inert gas helium was used with a column flow of 1ml/min. The MS conditions maintained were: 70eV ionization voltage, a mass range of 40-400, and the scan time was equal to the GC run time. The particular fatty acids present in the samples were identified and also quantified by comparing their peak areas and retention times to known standards (Fatty acid methyl esters, saturated (ME-19-KT) and fatty acid methyl esters, unsaturated (ME- 14-KT, SUPELCO Analytical) analyzed in the same GC/MS method and also by NIST Library search (version 2.2, 2014).

Amino acid and mineral profiling

The amino acid content of the beverage was evaluated according to the method described by Qu et al. (2002). In 100 ml of drink, 200 mL HCl (6N) was added and kept in an ultrasonic water bath at 90°C for an hour. After cooling, this mixture was transferred to a flask containing 1300 mL acetonitrile added with 1% formic acid. From this admixture, 100 mL was taken and added to 300 mL of saturated ammonium acetate solution to neutralize it. After adding 600 mL ultrapure water to the mixture, a 10-fold diluted sample was finally injected into the HPLC system and analyzed. Formic

acid (1%) and 10 mM ammonium formate in water and acetonitrile were used as mobile phases A and B, respectively. The mineral profile was assessed through ICP-MS (ICP-MS, NexION 1000 Model, PerkinElmer Make, U.S.A.) according to the methodology described by Poitevin (2016).

FTIR spectroscopy

The sample was concentrated using a lyophilizer, and FTIR spectra (Model-Spectrum Two, PerkinElmer) were obtained according to the methodology described by Scholar (2016). Each spectrum was recorded in the 400–4000 cm^{-1} at a resolution of 4 cm^{-1}. The measurements were made for both fermented and unfermented samples. Spectral measurements were repeated 32 times and averaged.

Microbial quality of beverage

Coliform counts using Violet Red Bile Agar medium were determined by incubating the plates at 37ºC for 24-48 hours. For the enumeration of yeast and mold counts, the Potato Dextrose Agar medium was used, and plates were incubated at 25ºC for 3-5 days. These counts were taken on the 0^{th}, $15^{th,}$ and 30^{th} days of storage to assess the shelf life of the beverage at 7ºC.

Statistical analysis

Statistical significance was calculated by t-test using GraphPad Prism. In addition, multifactor analyses of variance (ANOVA) were performed using Design Xpert version 7.0 to determine the statistical significance of differences among samples.

Results and Discussion

The present investigation was conducted to develop and optimize a value-added fermented whey-based beverage blended with pineapple juice and mint powder with different proportions of sugar, pineapple juice, and mint using R.S.M. . Whey fermentation was done with a bio-protective culture of *Pediococcus*

Table 1 Central Composite Rotatable design for the optimization of the fermented whey drink

SL. No		Factors			Responses			
SO	RO	P.Juice	Sugar	MP	DPPH	TSS	pH	OA
10	1	46.82	7.5	2	96.56±0.26	13.86±0.30	4.93±0.003	7
9	2	13.18	7.5	2	81.16±0.55	13.81±0.15	4.39±0.004	6
20	3	30	7.5	2	94.4±1.51	13.64±0.25	4.41±0.003	7
5	4	20	5	3	82.18±0.52	12.28±0.68	4.43±0.007	5
13	5	30	7.5	0.32	93.56±0.89	13.72±0.35	4.36±0.004	8
17	6	30	7.5	2	94.41±1.51	13.67±0.25	4.43±0.003	7
14	7	30	7.5	3.68	86.65±0.32	15.19±0.40	4.52±0.006	4
8	8	40	10	3	86.44±0.60	17.67±0.36	4.66±0.001	5
12	9	30	11.7	2	85.2±0.30	18.75±0.52	4.32±0.003	6
11	10	30	3.3	2	85.36±0.73	9.16±0.37	4.46±0.002	5
15	11	30	7.5	2	94.42±1.51	13.66±0.25	4.40±0.003	7
6	12	40	5	3	95.86±0	12.38±0.2	4.48±0.002	5
1	13	20	5	1	85.45±0.42	12.18±0.20	4.34±0.002	6
18	14	30	7.5	2	94.42±1.51	13.68±0.25	4.42±0.003	7
16	15	30	7.5	2	94.46±1.51	13.66±0.25	4.45±0.003	7
4	16	40	10	1	94.59±0.72	17.64±0.35	4.73±0.002	8
19	17	30	7.5	2	94.45±1.51	13.69±0.25	4.42±0.003	7
3	18	20	10	1	84.21±0.56	17.86±0.15	4.24±0.005	7
2	19	40	5	1	93.78±0.94	12.84±0.2	4.73±0.002	9
7	20	20	10	3	80.56±0.59	17.95±0.15	4.43±0.004	5

Code: SO-Standard Order, RO-Run Order, OA-Overall Acceptability, P.Juice-Pineapple juice, MP-Mint powder, DPPH-2, 2-diphenyl-1-picryl-hydrazyl-hydrate Assay analysis, TSS-Total Soluble Solids, OA: Overall Acceptability

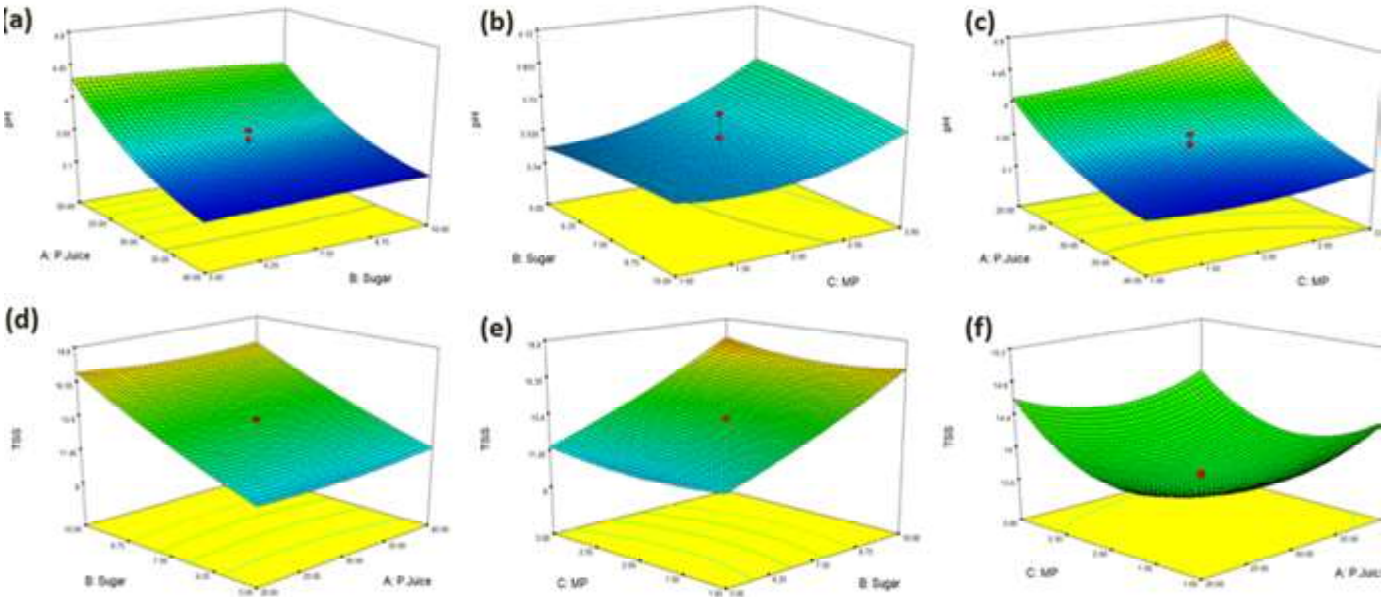

Fig. 1 Response surface plot for (a) pH influenced by the level of sugar and pineapple juice; (b) pH influenced by the level of sugar and mint powder; (c) pH as influenced by the level of mint powder and pineapple juice ; (d) TSS influenced by the level of sugar and pineapple juice; (e) TSS as influenced by the level of mint powder and Sugar; (f) TSS influenced by the level of mint powder and pineapple juice

pentosaceus NCDC 273. This combination of whey pineapple mint fermented with *Pediococcus pentosaceus* NCDC 273 has not yet been used for beverage formulation.

The experiments were designed using Central Composite Rotatable Design. Response Surface Methodology was applied to the experimental data, and parameters taken into account were Pineapple juice (20-40) %, Mint powder (1-3) %, Sugar (5-10) %, and second-order polynomial models were developed (Table 2). In total, 20 formulations were prepared, and the effect of the parameters mentioned above was studied on DPPH radical scavenging activity %, pH, TSS, and overall acceptability (Table 1). The impact of variable quantities of pineapple juice, mint powder, and sugar on responses at linear, quadratic, and interaction levels are given in Table 2. ANOVA was performed for each response to assess the suitability of the selected model (Table 3). All the models displayed statistical significance as indicated by the F value, R^2 values for all models were more than 80%, and lack of fit was found to be insignificant. Therefore, it can be concluded that all models were statistically valid for predicting the response.

Effect of variables on pH

The pH values ranged from 3.18-4.73 for the beverage prepared in this study (Table 1). Acidic pH is an essential parameter for preventing food spoilage by microorganisms. The coefficient of estimation of pH on whey beverages showed that the level of pineapple juice and sugar had a negative effect on pH. On the other hand, the mint powder had a negative impact on the pH but to varying degrees. From Fig. 1(a), it can be observed that with the increase in the level of pineapple juice, a sharp decrease in the pH of the beverage proves the acidic nature of pineapple juice.

In contrast, the sugar level had no such effect on the pH of the beverage. From Fig.1(b), it can be observed that with the increase in the level of mint powder, there is an increase in the pH of the beverage because of the alkaline effect of the mint powder. On the other hand, from Fig. 1(c), it can be observed that with the increase in the level of pineapple juice, there is a sharp decrease in the pH of the beverage.

Effect of variables on TSS

The TSS values ranged from 9.16-17.95 ºBrix (Table 1) after 16hr fermentation at 37°C. TSS mainly measures the sugar content in fruits and beverages. The coefficient of estimation of TSS on the whey beverage showed that the level of pineapple juice, mint powder, and sugar had a positive effect on the TSS but to varying degrees. From Fig. 1(d), it can be observed that the TSS increases sharply with the increase in sugar level. In contrast, with an increasing amount of pineapple juice, there is a slight increase in the TSS of the beverage, considerably lesser than the profound effect of sugar on the TSS of the drink. With increased amounts of mint powder, the TSS of the drink also increased (Fig. 1(e)). However, increasing amounts of pineapple juice initially decreased the TSS, with a slight rise at the end. Similarly, with the increase in mint powder, initially, the TSS decreases somewhat,

followed by a slight increase at the end (Fig. 1(f)). The increase in TSS may be due to the hydrolysis of polysaccharides into monosaccharides and oligosaccharides.

Effect of variables on DPPH

The DPPH values ranged from 80-96% (Table 1) after 16hr of fermentation at 37°C. The coefficient of estimation of DPPH on the whey beverage showed that the level of pineapple juice positively affected DPPH's radical scavenging activity. From Fig. 2(a), it can be observed that with the rise in pineapple juice, the DPPH increases significantly. From Fig. 2(b), it can be observed that DPPH increases considerably with increased levels of both mint powder and pineapple juice. From Fig. 2(c), it is observed that DPPH increases with the concentration of mint powder up to a certain extent, after which it decreases. Antioxidants can delay oxidation in chain reactions, thereby preventing oxidative stress caused due to free radical generation, which can further damage lipids, proteins, and nucleic acids (Jovanovic et al. 2018).

Effect of variables on Overall Acceptability

The coefficient of estimation of the Overall Acceptability of whey beverages showed that the level of sugar and pineapple juice positively influenced the overall acceptability. In contrast, the mint powder had a negative impact on the overall acceptability due to varying concentrations of mint up to a certain level. The terms for the effect of these variables were significant. From Fig. 2(d), it can be observed that with the increase in the level of pineapple juice, there is an increase in the overall acceptability of the beverage. While with the rise in the sugar level, the drink's overall acceptability increases up to a specific limit beyond which it slightly decreases. Still, the increase is considerably lesser than pineapple juice's profound effect on the beverage's overall

Table 2 Analysis of variance and regression analysis

Source	Sum of Squares	DF	Mean Squares	F value	Significant %
pH					
Model	0.027	9	2.992	26.17	**
Residual	1.143	10	1.143		
Lack of fit	1.060	5	2.119	12.65	
Pure error	8.379	5	1.676		
Total	0.028	19			
R^2= 0.9593, Adj R^2=0.9226					
DPPH					
Model	1.62	9	0.18	34.02	**
Residual	0.053	10	5.291		
Lack of fit	0.053	5	0.011	7310.08	**
Pure error	7.236	5	1.447		
Total	1.67	19			
R^2= 0.9684, Adj R^2=0.9399					
TSS					
Model	1.89	9	0.21	27.10	**
Residual	0.078	10	7.755		
Lack of fit	0.078	5	0.016	2763.39	**
Pure error	2.805	5	5.610		
Total	1.97	19			
R^2= 0.9606, Adj R^2=0.9252					
Overall Acceptability					
Model	1.18	9	0.13	29.19	**
Residual	0.045	10	4.480		
Lack of fit	0.045	5	8.961		
Pure error	0.000	5	0.000		
Total	1.22	19			
R^2= 0.9633, Adj R^2=0.9303					

**p<0.0001

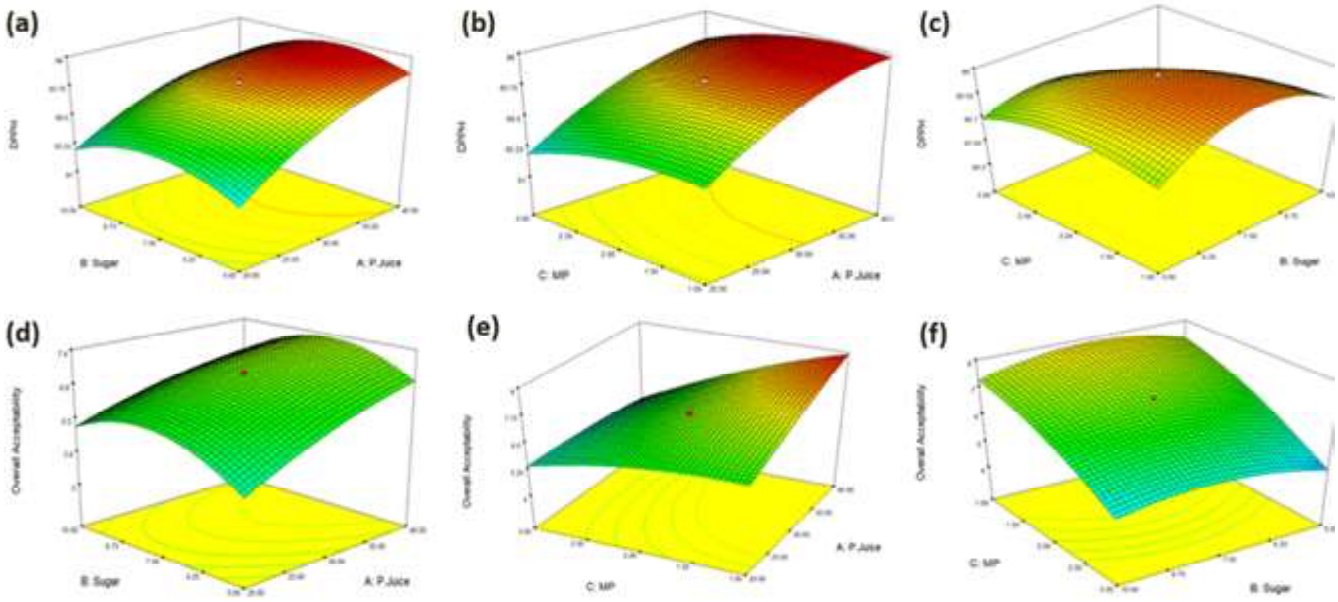

Fig. 2 Response surface plot for (a) DPPH radical scavenging activity influenced by the level of sugar and pineapple juice; (b) DPPH radical scavenging activity influenced by the level of mint powder and pineapple juice; (c) DPPH radical scavenging activity influenced by the level of sugar and mint powder (d) Overall Acceptability influenced by the level of sugar and pineapple juice; (e) overall acceptability influenced by the level of pineapple juice and mint powder; (f) overall acceptability as influenced by the level of mint powder and sugar

Table 3 Analysis of variance as a linear, quadratic, and interaction for each response variable

	Coefficient	Standard Deviation	t.exp	Significant %	Coefficient	Standard Deviation	t.exp	Significant %
Intercept	DPPH				pH			
A-P.Juice	9.72	0.030	9.65	**	2.10	4.361	2.09	**
B-Sugar	0.25	0.020	0.21	**	0.035	2.893	0.029	**
C-MP	-0.046	0.020	-.090	0.0428	-2.799	2.893	-9.246	0.3562
AB	-0.096	0.020	-0.14	0.0006	4.142	2.893	-2.305	0.1828
AC	-0.037	0.026	-.094	0.1826	8.280	3.781	-1.432	0.0533
BC	7.257	0.026	-.050	0.7836	-0.018	3.781	-0.026	0.0009
A^2	-0.070	0.026	-0.13	0.0219	8.280	3.781	-1.432	0.0533
B^2	-0.10	0.019	-0.15	0.0003	0.020	2.817	0.013	**
C^2	-0.17	0.019	-0.21	**	-2.464	2.817	-8.740	0.4022
	-0.078	0.019	-0.12	0.0022	1.736	2.817	-4.540	0.5515
	TSS				Overall Acceptability			
Intercept	3.69	0.036	3.61	**	2.64	0.027	2.58	**
A-P.Juice	4.362	0.024	-.049	0.8584	0.092	0.018	0.052	0.0005
B-Sugar	0.36	0.024	0.31	**	0.014	0.018	-0.027	0.4663
C-MP	0.021	0.024	-.032	0.3954	-0.23	0.018	-0.27	**
AB	-0.021	0.031	-.090	0.5181	-0.021	0.024	-0.074	0.3861
AC	-0.011	0.031	-.080	0.7366	-0.12	0.024	-0.17	0.0006
BC	8.085	0.031	-.061	0.8004	0.021	0.024	-0.031	0.3861
A^2	0.037	0.023	-.015	0.1460	-0.027	0.018	-0.066	0.1579
B^2	0.022	0.023	-.030	0.3653	-0.099	0.018	-0.14	0.0002
C^2	0.065	0.023	.014	0.0183	-0.074	0.018	-0.11	0.0018

**p<0.0001; Coefficients were A, B, C (linear), A^2, B^2, C^2 (quadratic), AB, AC, and BC (interaction) of the model, calculated by software DX7Trial Design expert 7.0.0.

acceptability. On the other hand, with the rise in the level of mint powder, the overall acceptability increases sharply due to the soothing effect of mint on the palate (Fig. 2(e)). From Fig. 2(f), it can be observed that with the rise in the level of sugar, there is a slight increase in the overall acceptability of the beverage up to a specific limit (up to 7.5g), beyond which it decreases slightly, proving that moderate sweetness has a strong influence on the consumer's palate.

Nutritional attributes of the fermented whey beverage

The results of chemical parameters for fermented whey beverage (FPMWB) and Unfermented whey beverage (UFPMWB), such as moisture content, acidity, total soluble solids (TSS), carbohydrate, protein, fat, and ash content of the fermented and unfermented whey beverage have been summarised in Table 4. Several researchers have found similar results for the chemical composition of fruit and whey beverage (AbdulAlim et al. 2018; Cuhna et al. 2022; Islam et al. 2021; M'hir et al. 2019). The beverage's acidity has increased after fermentation due to pineapple juice and acid produced during fermentation. Other studies have observed similar results (Sabokbar & Khodiyan, 2015). The reduced ash content (4.03%) of the fermented whey beverage compared to the unfermented one (4.37%) indicate their possible depletion due to microbial growth. Comparable results have been reported by Islam et al. (2019). The beverage's Total Soluble Solids (TSS) were determined to be approximately 12%, and protein content was around 4% in this study which depends upon the addition of fruit pulp and sugar. Similar results have been obtained by Gimhnai & Liyanage, (2018) and Islam et al. (2021). The phenolic content of the fermented whey beverage and control (unfermented) were 89.41% and 83.34% mg G.A.E./ mL. The results obtained agree with the findings of Balakrishnan & Agrawal, (2014) and Islam et al. (2021). The percentage of DPPH radical scavenging activity of the fermented whey beverage and control (unfermented whey beverage) were found to be 83.88% and 78.17%, respectively, comparable to the results obtained by M'hir et al. (2019). High antioxidant potential, often associated with antiaging and anti-inflammatory effects on human physiology, makes this beverage a healthier option than soft drinks.

FTIR spectra

The FTIR spectra of FPMWB and UFPMWB are presented in Fig. 3(a) and Fig. 3(b), respectively, depicting the predominant

Table 4 Physico-chemical characteristics of the fermented-whey beverage

Parameter	Fermented Beverage (FPMWB)	Control (UFPMWB)
Moisture (% Wet basis)	88.38±1.60^{a}	86.34±0.57^{b}
Dry matter (%)	11.61±1.60^{b}	13.66±0.57^{a}
Ash (%)	4.03±0.85^{b}	4.37±0.69^{a}
Organic matter (%DM basis)	95.96±0.85^{a}	95.63±0.69^{a}
pH	4.13±0.015^{b}	4.34±0.005^{a}
TSS (ºBrix)	12.2±0.1^{a}	12.1±0.1^{a}
Titratable acidity (%)	1.02±0.05^{a}	0.9±0.15^{b}
Crude protein (%)	4.30±0.01^{a}	4.20±0.02^{b}
TPC (mg GAE/mL)	89.41±1.90^{a}	83.34±1.29^{b}
DPPH (%)	83.88±1.77^{a}	78.17±0.99^{b}
Sodium (mg/L)	132.56±0.015^{a}	132.72±0.086^{a}
Potassium (mg/L)	1165.47±0.006^{a}	1036.93±0.044^{b}
Iron (mg/L)	14.66±0.01^{a}	6.72±0.0^{b}
Calcium (mg/L)	27.34±0.02^{a}	25.27±0.02^{b}
Medium chain fatty acid (mg/100mL)	5.8± 0.05^{b}	6.32±0.01^{a}
Long-chain fatty acids (mg/100mL)	49.48±0.27^{a}	41.61±1.08^{b}
Monounsaturated fatty acid (mg/100mL)	24.54±0.2^{a}	21.57±0.02^{b}
Omega 3 fatty acid (mg/100mL)	3.64 ±0.045^{a}	2.6±0.035^{b}
Omega 6 fatty acid (mg/100mL)	7.01± 0.03^{a}	4.01±0.035^{b}
Coliform count (log CFU/mL; % inhibition at 15th and 30th day	1.79±0.04^{a}, 1.97±0.00^{a}	1.69±0.02^{b}, 1.89±0.01^{b} (5.57% ,4.04 %)
Yeast and mold count (log CFU/mL; % inhibition at 15th and 30th day	1.89±0.02^{a}, 1.98±0.00^{a}	1.86±0.02^{b}, 1.92±0.03^{b} (2.2%, 2.9%)

All the values are Mean ± S.D (n = 3). Mean values in a row with different superscript differ significantly from each other (P > 0.05).

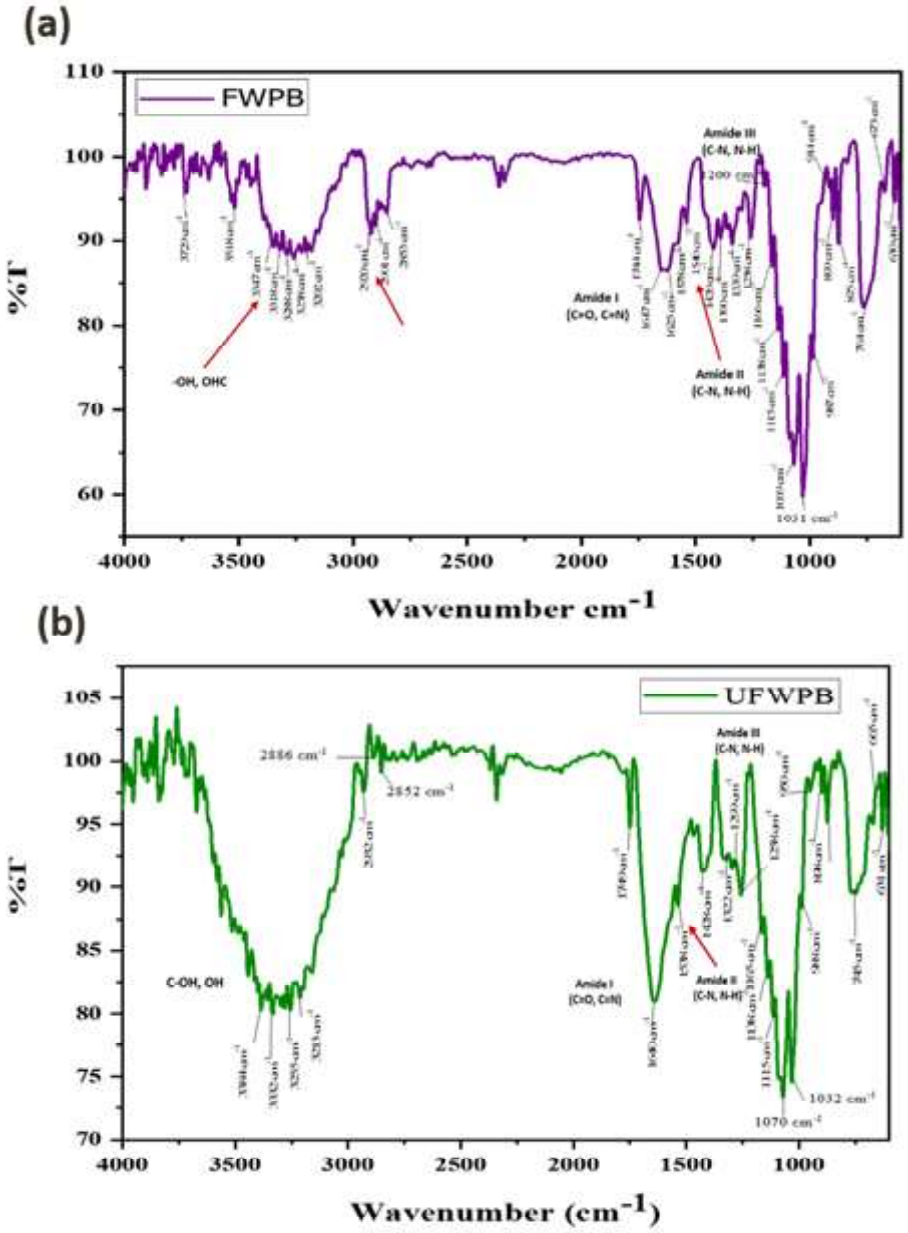

Fig. 3 (a). FT-IR spectrum for Fermented whey beverage; (b). FT-IR Spectrum for unfermented beverage (FWPB-Fermented Whey Pineapple-Mint Beverage, UFWPB- Unfermented Whey Pineapple Mint Beverage)

functional groups present in the beverage and changes in the biomolecules after fermentation. The samples exhibited different absorption bands and heights of the peaks, which may be attributed to the structural changes in proteins' chemical interaction or production of specific metabolites after fermentation.

FPMWB showed increased absorption bands and heights at (1750–1250 cm^{-1} and 2500–3000 cm^{-1}, 3400–3000 cm^{-1}, and 3000–3500 cm^{-1}). Significant differences in the functional groups (Amide-I band) present in the beverage before and after fermentation and comparable results have been reported by Souza et al. (2019) and Wen-Qiong et al. (2021). The Amide-I (C=O coupled with C=N) band at 1640 cm^{-1} in the control sample has been changed to shorter bands appearing at 1625-1647 cm^{-1} in the fermented sample, clearly showing the conformational changes in the secondary structure of the whey protein due to fermentation and interaction with pineapple juice which contains plant polyphenolic compounds and organic acids. The Amide-II band at 1538 cm^{-1}in control and 1540 cm^{-1}in the fermented sample and the Amide-III band at 1258 cm^{-1} in both samples does not show much difference. The bands in the region 3202-3258 $cm^{-1}$of the fermented sample appeared because of stretching vibrations of -O.H. linked to $–NH_2$ (Amide-A), while bands in the area 2901-2932 cm^{-1} are due to asymmetric stretching of $–CH_2$ group (Amide B). The spectral bands occurring between 3200 cm^{-1}– 3518 cm^{-1}clearly demonstrate the presence of the –O.H. group (-O-H stretch) of phenolic compounds. These band ranges are assigned to the stretching vibration of O.H. groups that interact by hydrogen bonding (Heredia-Guerrero et al. 2014). The non-

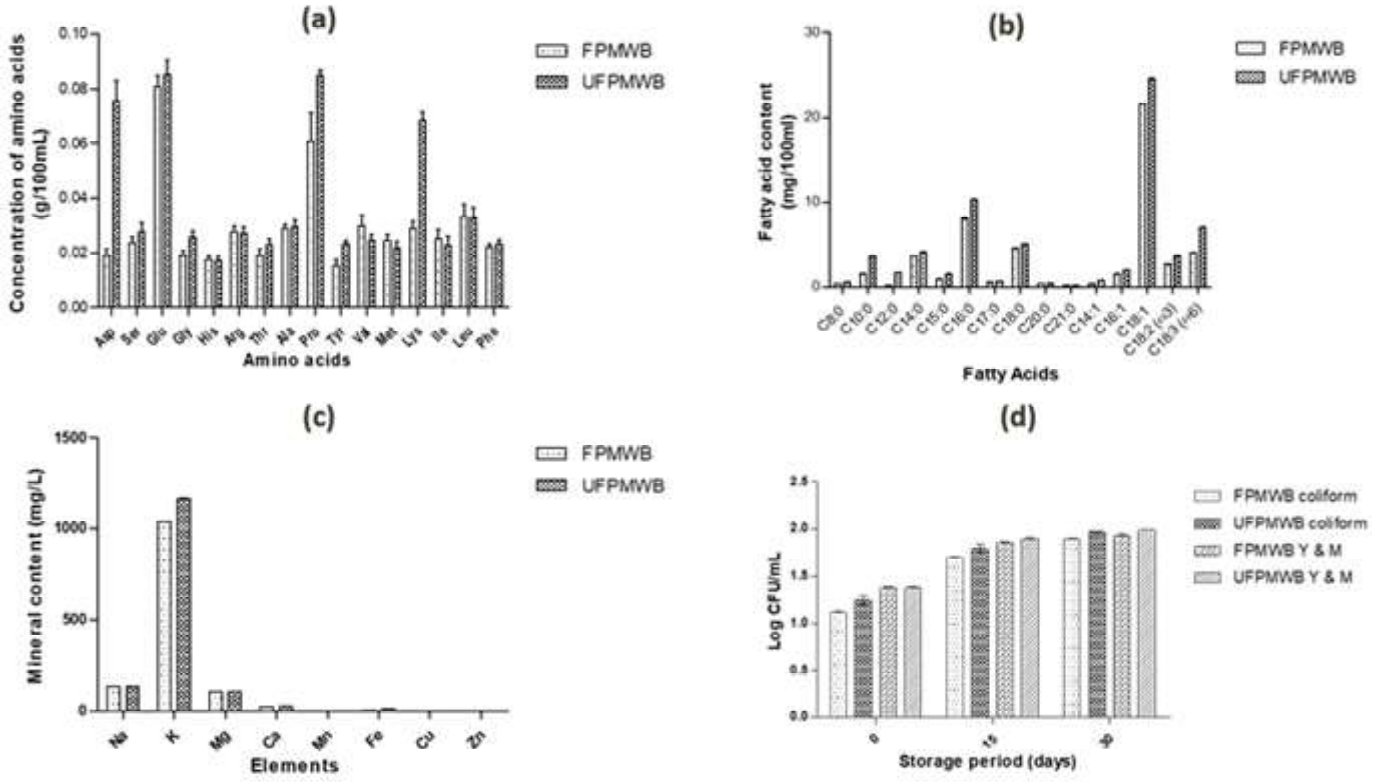

Fig 4 a) Amino acid profile of fermented & unfermented beverage b) Fatty acid profile of fermented & unfermented beverage c) Mineral profile of fermented & unfermented beverage d) Microbial counts of fermented & unfermented beverage (FPMWB- Fermented pineapple-mint whey beverage, UFPMWB- Unfermented pineapple mint whey beverage)

hydrogen bonded or free O.H. groups of alcohols and phenols absorb at 3650-3584 $cm^{-1,}$ which is not visible in these spectra. Apparently, -O.H. groups dominate the fermented sample because fermentation produces more functionalities containing the –O.H. group.

The bands recorded at 1031 $cm^{-1}$with C-O stretch vibration of alcohol functions, 1070 cm^{-1} with C-O, C-C, and C-H stretch vibration, 1165 cm^{-1}, and 1200 $cm^{-1}$with C-O-C ether stretching show the presence of lactose in both the samples. The formation of lactic acid from lactose during fermentation is indicated in the fermented sample by the presence of bands at 1744 cm^{-1} (-C-O stretch), 1420 cm^{-1} (CH_3 bending), and 1390 cm^{-1}(-C.H. bending). The presence of bands at 1744 cm^{-1}in the fermented sample and at 1749 cm^{-1} (-C-O stretch) in the control sample also indicates the presence of other organic acids, including phenolic acids. The bands at 1420 cm^{-1} -1428 cm^{-1} correspond to the stretching vibration of C-C of the aromatic ring, and bands between 900 cm^{-1} -630 $cm^{-1,}$ which result from C-H out of plane deformation, show the presence of aromatic rings such as plant polyphenolic compounds of pineapple juice, and aromatic amino acids of whey protein.

Amino acid, fatty acid, and mineral content

All essential amino acids except tryptophan were found in the fermented and unfermented beverages (Fig.4(a)). Among essential amino acids, the order of concentration was: Leu>Val> Lys>Arg>Met>Ile>Phe>His>Tyr. The concentration of amino acids (especially Asp, Pro, and Lys) is reduced after fermentation as amino acids are consumed by LAB for their growth. The whey beverage had a comparable amino acid content to the results obtained by other researchers (Gulec et al. 2021; Yasmin et al. 2013). Fig. 4(b) shows the fatty acids profile of fermented and unfermented whey beverages. It is clear from the graph that fatty acid content decreased after fermentation. The amount of different types of fatty acid calculated according to Barlowksa et al. (2018) and Florence et al. (2012). Overall, the whey beverage had the highest amount of long-chain fatty acid (41.61± 1.08 & 49.48±0.27 mg/100ml), followed by medium-chain fatty acid (6.32± 0.01 and 5.8± 0.05 mg/100ml) for fermented and unfermented whey beverage. PUFA levels observed in the beverage were 2.6±0.035 & 3.64 ±0.045 mg/100ml (n-3) and 4.01±0.035 and 7.01± 0.03 mg/ 100ml (n-6) for fermented and unfermented whey beverage respectively. The omega-6 to omega-3 fatty acid ratio was 1.52±0.00 and 1.92±0.03 for fermented and unfermented beverages, respectively, showing the positive effects of LAB

fermentation by reducing more omega-6 fatty acid. MUFA levels were 21.57±0.02 and 24.54±0.2 mg/100ml for fermented and unfermented beverages. Similar results have been registered by Silveira et al. (2019). The fatty acids associated with coronary heart disease, termed hypercholesterolemic fatty acid (C12:0, C14:0, C16:0), have been significantly reduced after fermentation, making this beverage healthier.

The whey beverage possessed a fair amount of macro and micro minerals (Fig. 4(c)). Potassium, sodium, magnesium, and calcium concentration ranged from 1.04±0.08, 0.13±0.15, 0.10±0.006 and 0.03±0.02 mg/ml, respectively. The order of concentration of macro-elements was: K> Na> Mg> Ca>Fe. Potassium was highest in macro-minerals and is a healthier choice than sodium due to its hypotensive effects (Machin et al. 2014). The dietary mineral intake for an adult man who consumes 100 ml of the fermented whey beverage would be 3.53%, 2.96%, 2.41%, 0.66%, and 0.25% for Fe, K, Mg, Na, and Ca, respectively, according to the RDA suggested by ICMR, 2020 . Micro-minerals such as Mn, Fe, Cu, and Zn ranged from 1.11±0.006, 6.72±0.01, 0.01±0.001 and 0.60±0.001 mg/L, respectively. Except for Ca, these results corroborated sufficiently with the results of other researchers (Luis et al. 2015; Souza et al. 2019). The mineral content of dairy foods may vary depending on the manufacturing procedure, type, and concentration of ingredients used in the product's manufacturing (Luis et al. 2015).

Shelf-life of beverage

Fermented and unfermented whey beverage had an acceptable amount of coliform and Yeast and molds on the 30th day of storage under refrigerated conditions (Fig 4(d)). The coliform count was recorded as 1.11±0.03, 1.69±0.02, and 1.89±0.01 log CFU/mL, while yeast and mold counts were 1.39±0.02, 1.86±0.02, 1.92±0.03 log CFU/mL at 0th, 15th and 30th day of storage. The percent inhibition in fermented whey beverage due to antimicrobial metabolites of *Pediococcus pentosaceus* NCDC 273 for the coliform count was 5.57% and 4.04%. In comparison, it was recorded as 2.2% and 2.9% for yeast and mold count on the 15th and 30th day of storage, respectively (Table 4). Similar improvements in the microbiological quality of dairy products have been observed previously (Pandey et al. 2019).

Conclusion

The present study illustrates the easy development of value-added healthy fermented RTS beverages from whey, a by-product of the dairy industry. This RTS whey beverage had a refreshing taste and several nutritional attributes, such as high potassium content, total phenolic content and DPPH radical scavenging activity, and a fair amount of PUFA, MUFA, and essential amino acids. Fermentation with a well-known bioprotective culture has improved its nutritional value and shelf life. In India, chhana whey is available in plenty, and this type of RTS beverage presents a logical solution to the problem of environmental pollution due to panner/chhana whey disposal.

Conflict of interest

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Application of carrot powder in preparation of low-fat frozen yoghurt

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Abstract: Low-fat frozen yoghurt was prepared using carrot powder as one of the ingredients. Response surface methodology (RSM) was used to optimize the level of carrot powder, stabilizer-emulsifier mixture and sugar. The responses were sensory attributes such as flavour, body and texture, melting quality, colour and appearance and total score as well as physical attributes like melted quantity and overrun. Based on the output, RSM suggested the rate of addition of carrot powder, stabilizer-emulsifier mixture and sugar to be 3.93%, 0.41% and 14.30% respectively. The experimental frozen yoghurt was prepared as per the suggestions from RSM and compared with control low-fat frozen yoghurt where carrot powder was replaced with vanilla flavour. The experimental frozen yoghurt was statistically similar in terms of compositional parameters except moisture and carbohydrate content while the experimental sample was superior to control sample in terms of sensory attributes. Aerobic plate count of both the samples were statistically similar while coliform as well as yeast and mold were absent in the product.

Keywords: Carrot, Frozen yoghurt, Response surface methodology, Sensory

Introduction

Frozen yoghurt is a frozen fermented milk product manufactured by freezing of either stirred yoghurt prepared by fermentation of ice-cream mix or a mixture of stirred yoghurt and ice-cream mix with addition of colouring and flavouring material. Frozen yoghurt contains the live strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*. Hence, it contains the nutritional benefits of yoghurt and sensory benefits of ice-cream and therefore, it is enjoyed by the people of every age group (Tamime and Robinson, 2007).

Carrot is a root vegetable which is produced, exported and consumed in different parts of the world. Carrot is most valuable member of the apiaceae family due to its nutritional quality, phytochemical content, antioxidant capacity, and health advantages (Leja et al. 2013; Hassan and Barakat, 2018). Carrot roots may be extensively processed into nutrient-rich finished products such as juice, dry powder, concentrate, tinned, candy and pickle in addition to the traditional uses for salad and sweet dishes like carrot *halwaa*, which is the most well-known variant in north India. The acceptability of carrot is significantly influenced by peel to core ratio, total soluble content, carotenoid concentration, and fibre content (Sharma et al. 2012).

Response surface methodology (RSM) has been widely used in recent years for the development of new products as well as improvement in existing products. RSM delineates the effect of the independent variables on responses of importance and is regarded as an effective method to optimize the new product formulations. It is a robust tool for data analysis that focuses on an adequate approximation relationship between input and output variables and determines the best operating circumstances for a system (Myers et al. 2004).

With a changing lifestyle and increasing awareness towards health and nutrition, consumers are moving towards low-fat diet to reduce the risk of obesity, coronary heart disease, atherosclerosis and hypertension (Dharaiya et al. 2021). High fat diet is also linked with psychiatric disorders (Jeong et al. 2019). Fat, being a costliest constituent in milk, increases the cost of final product and make the product unaffordable by low income group people. However, reduction in fat content of frozen yoghurt influences sensory and rheological characteristics of the product. Incorporation of carrot will make up for the deterioration taken place in the quality of frozen yoghurt by reduction of fat along with improvement in the nutritional quality of the final product.

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Therefore, in current investigation, low-fat frozen yoghurt has been developed with incorporation of carrot powder.

Materials and methods

Whole milk and skimmed milk were obtained from nearby commercial dairy plant. Skimmed milk powder of *Sagar* brand, marketed by Gujarat Cooperative Milk Marketing Federation (GCMMF) Ltd., Anand was used for standardization. Commercial grade cane sugar of *Madhur* brand was obtained from local market. Carrots (*Daucus* carota subsp. Sativus) were purchased from local vegetable market. Starter cultures *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* were obtained from Dairy Microbiology department of the institute. Sodium alginate, guar gum, carrageenan and pectin were supplied by HiMedia, Mumbai. Glycerol monostearate (GMS) was supplied by Loba Chemical, Mumbai. Vanilla flavour (International Flavours & Fragrances India Pvt. Ltd., Chennai) was used to prepare control frozen yoghurt.

Preparation of carrot powder

The carrot fruits were washed in portable water, peeled and shredded; the shredded carrots were blanched for 3 minutes in hot water containing sodium metabisulphite to prevent browning and discoloration. The sulphited carrots were immediately cooled by exposing to air and dried in a vacuum tray drier at 50°C for 12 hours. The dried carrot was ground to fine powder and sieved with a 0.150 µ sieve and was packaged in black polythene bag for further uses (Phebean et al. 2017).

Preparation of stabilizer-emulsifier blend

A blend of stabilizers (such as sodium alginate, guar gum, carrageenan and pectin) and emulsifier (such as glycerol monostearate) was prepared and used in frozen yoghurt at a level suggested by RSM. The blend contained sodium alginate, guar gum, carrageenan, pectin and GMS in the ratio of 2:1:1:2:2. The ratio was decided on the basis of preliminary trials.

Preparation of frozen yoghurt

Frozen yoghurt has been prepared using the method suggested by Agarwal and Prasad (2013) with minor modifications. The detailed method is illustrated hereafter:

Whole milk and skimmed milk are blended together and heated to 55°C. All the dry ingredients such as sugar, skimmed milk powder, carrot powder and stabilizer-emulsifier blend were mixed together and added to whole milk and skimmed milk blend. The mixture was then homogenized at 65°C followed by heat treatment at 85°C/30 min. The heated mix was cooled to 42±2°C and inoculated with starter cultures *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* at the rate of 2% (w/w) of the quantity of mix followed by incubation at 42±2°C till the acidity reaches to 0.6% LA. The yoghurt mix was then cooled to 4°C and stirred. The stirred mix was aged at 4°C for 6 h followed by freezing, packaging and hardening at -25°C for 24 h. The frozen yoghurt was stored at -18°C after hardening.

Analysis of carrot incorporated low-fat frozen yoghurt

Whole milk and skimmed milk were analysed for fat, total solids and acidity as per the method described by FSSAI (2015). The prepared frozen yoghurt was analysed for fat, protein, ash, total solids, titratable acidity and overrun as per the method described by FSSAI (2015). Carbohydrates were calculated by difference. Viscosity of yoghurt mix was analysed by the method suggested by Muse and Hartel (2004) using Brookfield viscometer. Melting resistance of frozen yoghurt was analysed using the method given by Muse and Hartel (2004). Overrun of frozen yoghurt was calculated as per the formulae used by Ilansuriyan and Shanmugam (2018). Aerobic plate count, coliform count and yeast and mold count were analysed using the method given by FSSAI (2012).

Sensory evaluation of carrot incorporated low-fat frozen yoghurt

The frozen yoghurt samples were stored at -13±2°C for 24 h before serving to the semi-trained judges (n=12). The judges were from the faculty of the institute who have basic idea about the product. Sensory analysis of the product was performed in isolated sensory booths illuminated with incandescent light maintained at 22±2°C. The well-labelled samples were presented in polystyrene cups in completely randomized order. The frozen yoghurt samples were evaluated using 100-point score card (Marshall et al. 2013).

Statistical analysis

The minimum and maximum levels of carrot powder, stabilizer-emulsifier blend and sugar were selected as 2 and 6%, 0.2 and 0.5% as well as 12 and 16% respectively, on the basis of preliminary trials. A central composite rotatable design (CCRD) of the response surface methodology (RSM) technique was adopted for the optimization of carrot powder, stabilizer-emulsifier blend and sugar. The CCRD of three factors contained 20 combinations, including lower and upper limits, along with their responses for sensory parameters as well as hardness and melting time are displayed in Table 1. The data generated for different responses were analyzed using Design Expert® software (13.0.2 version) (Stat-Ease, Inc., 2021 E. Hennepin Avenue, Minnepolis, USA). A general polynomial equation given below was fitted for each response.

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

where Y represents the predicted response; a_0 the constant coefficient; a_{11}, a_{22} and a_{33} denote quadratic coefficients; a_{12}, a_{23} and a_{13} denote interaction coefficients; x_1, x_2 and x_3 denote rate of addition of carrot powder, stabilizer-emulsifier blend and sugar respectively.

Adequacy of the model was evaluated using coefficient of determination (R^2) and statistical significance was examined by F value. The effect of independent variables and individual responses was described at $P<0.01$ and $P<0.05$. t-test for two samples assuming equal variance was applied using Microsoft Excel for comparison of predicted values with the actual values of the responses. The variation between control and carrot incorporated low-fat frozen yoghurt samples was analysed using independent t-test.

Results and discussion

The optimization of the rate of addition of carrot powder, stabilizer-emulsifier blend and sugar was carried out on the basis of sensory properties of carrot incorporated low-fat frozen yoghurt such as flavour, body & texture, meting characteristics, colour & appearance and total score as well as quantity of frozen yoghurt melted in specific time and firmness in terms of penetration in cone penetrometer. Experimental design matrix showing factors and their responses is displayed in Table 1. The successive regression analysis of the responses produced the quadratic models for each response. The variation in experimental data of fitted quadratic model was given by coefficient of determination (R^2) which ranged from 87 per cent to 93 per cent (Table 2). The model F-value of the fitted quadratic model for all responses was found to be significant. The sufficient accuracy for predicting all response variables of the frozen yoghurt prepared from any combinations of variables within the range was evaluated by non-significant lack of fit. These indicate that the obtained quadratic model fitted the data strongly. The signal to noise ratio called *Adequate precision value (APV)* for a well fitted model should be above four. This measure also fulfilled for the obtained mode with APVs ranging from 6.81 to 12.09. All these results firmly recommended that the model could be used to develop carrot incorporated low-fat frozen yoghurt. Regression equation for predicting sensory score, melted quantity and firmness of carrot incorporated low-fat frozen yoghurt is indicated in Table 3.

Effect of variables on colour and appearance: Colour and appearance is the first sensory parameter that is observed while carrying out sensory evaluation. The colour and appearance score of the frozen yoghurt was ranged between 3.13 and 4.63. The product obtained minimum score for colour and appearance when carrot powder, stabilizer-emulsifier mixture and sugar were added at the rate of 7.36%, 0.35% and 14% respectively while maximum score was obtained when the variables were added at the rate of 4%, 0.35% and 14% respectively. The rate of addition of carrot powder significantly ($P<0.05$) improved colour and appearance of the frozen yoghurt at linear level as the judges liked the light reddish orange colour given by carrot powder but significantly ($P<0.05$) deteriorated colour and appearance at quadratic level due to high intensity of the colour which was disliked by the judges. The appearance score was increased from 6.66 to 7.90 with increase in rate of addition of carrot pulp from 2.0 to 4.0 per cent followed by reduction to 7.80 when carrot pulp was added at the rate of 5.0 per cent which is attributed to formation of gel like coagulum and reduction in wheying off (Agarwal and Prasad, 2013). Ateteallah et al. (2019) and Pandey et al.

Table 1 Experimental design matrix showing factors and their responses for the development of carrot incorporated low-fat frozen yoghurt

Std Run	A: Carrot powder (% w/w)	B: S+E@ (% w/w)	C: Sugar (% w/w)	Response 1: Flavour	Response 2: Body & Texture	Response 3: Melting character-ristics	Response 4: C&A#	Response 5: Total score*	Response 6: Melted quantity, %	Response 7: Overrun, %
1	2.00	0.20	12.00	36.88	24.63	4.38	3.88	84.77	68.35	72.15
2	2.00	0.50	16.00	43.88	25.88	4.50	3.88	93.14	54.39	98.66
3	4.00	0.35	14.00	44.12	28.50	4.25	4.50	96.37	46.94	90.35
4	6.00	0.20	16.00	35.75	25.25	4.00	3.50	83.50	69.35	84.26
5	6.00	0.20	12.00	36.63	24.50	3.50	3.88	83.51	61.88	76.19
6	2.00	0.20	16.00	43.63	26.63	4.00	3.88	93.14	73.45	77.81
7	4.00	0.10	14.00	35.88	24.50	3.88	4.13	83.39	67.89	73.16
8	4.00	0.35	14.00	43.63	28.88	4.63	4.63	96.77	47.05	91.12
9	4.00	0.60	14.00	36.88	27.88	4.25	3.50	87.51	42.16	81.62
10	2.00	0.50	12.00	38.63	26.50	4.50	4.50	89.13	48.65	84.18
11	6.00	0.50	12.00	36.88	24.25	3.63	3.63	83.39	45.37	86.63
12	4.00	0.35	17.36	39.50	26.14	4.25	3.50	88.39	58.98	98.99
13	4.00	0.35	14.00	43.88	28.00	4.25	4.50	95.63	46.99	89.98
14	6.00	0.50	16.00	37.88	25.38	4.25	3.63	86.14	56.18	79.44
15	4.00	0.35	14.00	44.25	28.00	4.50	4.63	96.25	47.11	90.51
16	7.36	0.35	14.00	35.88	22.63	3.00	3.13	79.64	49.12	85.32
17	4.00	0.35	10.64	35.88	23.88	4.13	4.00	82.89	48.63	86.95
18	4.00	0.35	14.00	44.00	28.88	4.25	4.50	96.63	47.01	90.94
19	4.00	0.35	14.00	43.88	28.63	4.50	4.50	96.51	46.96	90.32
20	0.64	0.35	14.00	37.13	24.88	3.13	3.88	84.02	54.18	82.81

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

(2019) also observed initial improvement in colour and appearance followed by deterioration at higher level.

Effect of variables on flavour

Flavour is an amalgamation of taste, odour and mouthfeel. It is a major factor in sensory evaluation and consumer acceptance for majority of the dairy products. The flavour score of the frozen yoghurt between 35.85 and 44.15. The minimum flavour score for the frozen yoghurt was obtained when carrot powder, stabilizer-emulsifier blend and sugar were added at the rate of 6%, 0.2% and 16% respectively while maximum flavour score was obtained when carrot powder, stabilizer-emulsifier blend and sugar were added at the rate of 4%, 0.35% and 14% respectively (Table 1). Carrot powder and sugar significantly ($P<0.05$) improved flavour of the final product at linear level. Similarly, the interaction of both of them also significantly ($P<0.05$) improved the flavour while all the three variables significantly deteriorated flavour at quadratic level because high intensity of carrot flavour and very sweet taste were disliked by the judges. Flavour release was also reported to be slow by the experts when stabilizer-emulsifier mixture was added in higher amount. Agarwal and Prasad (2013) also observed improvement in flavour profile of low-fat frozen yoghurt prepared using carrot pulp. The flavour score increased from 6.76 to 7.90 with increase in rate of carrot pulp addition from 2.0 per cent to 5.0 per cent while increase in rate of addition of stabilizer from 0.5 per cent to 3.0 per cent also increased flavour score from 7.02 to 7.62. Moeenfard and Tehrani (2008) reported non-significant influence of rate of addition of stabilizers on flavour. The flavour score of ice-cream prepared using carrot pulp increased initially with increase in addition of carrot pulp followed by decrease at higher level (Dias et al. 2015; Ateteallah et al. 2019; Pandey et al. 2019).

Effect of variables on body and texture

Body and texture is an important sensory characteristic for frozen yoghurt. The body and texture score of the low-fat frozen yoghurt containing carrot was ranged from 22.63 to 28.88. The frozen yoghurt

Table 2 Regression coefficients and ANOVA fitted quadratic model for the responses of carrot incorporated low-fat frozen yoghurt

Partial coefficients	Flavour	Body & texture	Melting characteristics	Colour & appearance	Total score	Melted quantity, %	Overrun, %
Intercept	43.86	28.46	4.37	4.53	96.23	46.81	92.16
A-Carrot powder	1.31*	-0.59*	-0.15	0.20*	2.24*	-1.51	-0.28
B-S+E[#]	0.44	0.48*	0.21*	-0.04	1.10	-8.21*	2.16*
C-Sugar	1.32*	0.51*	0.16*	-0.06	1.86*	3.43*	0.83*
AB	0.03	-0.16	-0.03	-0.09	-0.25	1.14	0.26
AC	1.47*	0.06	0.19	0.03	-1.19	0.93	0.24
BC	0.03	-0.28	0.06	-0.03	-0.22	0.50	0.15
A^2	-2.12*	-1.54*	-0.46*	-0.32*	-4.46*	2.86*	0.52
B^2	-2.20*	-0.69*	-0.55*	-0.09	-3.06*	4.15*	-1.38*
C^2	-1.69*	-1.09*	-0.39*	0.07	-2.92*	3.59*	0.80*
			Model fit statistics				
Lack of fit	< 0.0001	0.0313	0.0455	0.0022	0.0001	< 0.0001	< 0.0001
Model F value	8.22	11.72	9.43	7.49	12.81	16.00	8.35
R^2	0.88	0.91	0.89	0.87	0.92	0.93	0.88
APV	6.81	9.81	12.09	7.87	10.09	11.96	9.51

#Stabilizer-emulsifier blend; *significant effect at 5% level

Table 3 Regression equation for predicting sensory score, melted quantity and firmness of carrot incorporated low-fat frozen yoghurt

Property	Equation
Flavour	$43.86 + 1.31A + 0.44B + 1.32C + 0.03AB + 1.47AC + 0.03BC - 2.12A^2 - 2.2B^2 - 1.69C^2$
Body & texture	$28.46 - 0.59A + 0.48B + 0.51C - 0.16AB + 0.06AC - 0.28BC - 1.54A^2 - 0.69B^2 - 1.09C^2$
Melting characteristics	$4.37 - 0.15A + 0.21B + 0.16C - 0.03AB + 0.19AC + 0.06BC - 0.46A^2 - 0.55B^2 - 0.39C^2$
Colour & appearance	$4.53 - 0.2A - 0.04B - 0.06C - 0.09AB + 0.03AC - 0.03AC - 0.32A^2 - 0.09B^2 + 0.07C^2$
Total score	$96.23 - 2.24A + 1.10B + 1.86C - 0.25AB - 1.19AC - 0.22BC - 4.46A^2 - 3.06B^2 - 2.92C^2$
Melted quantity	$46.81 - 1.51A - 8.21B + 3.43C + 1.14AB + 0.93AC + 0.50BC + 2.86A^2 + 4.15B^2 + 3.59C^2$
Overrun	$92.16 - 0.28A + 2.16B + 0.83C + 0.26AB + 0.24C + 0.15BC + 0.52A^2 + 1.38B^2 + 0.80C^2$

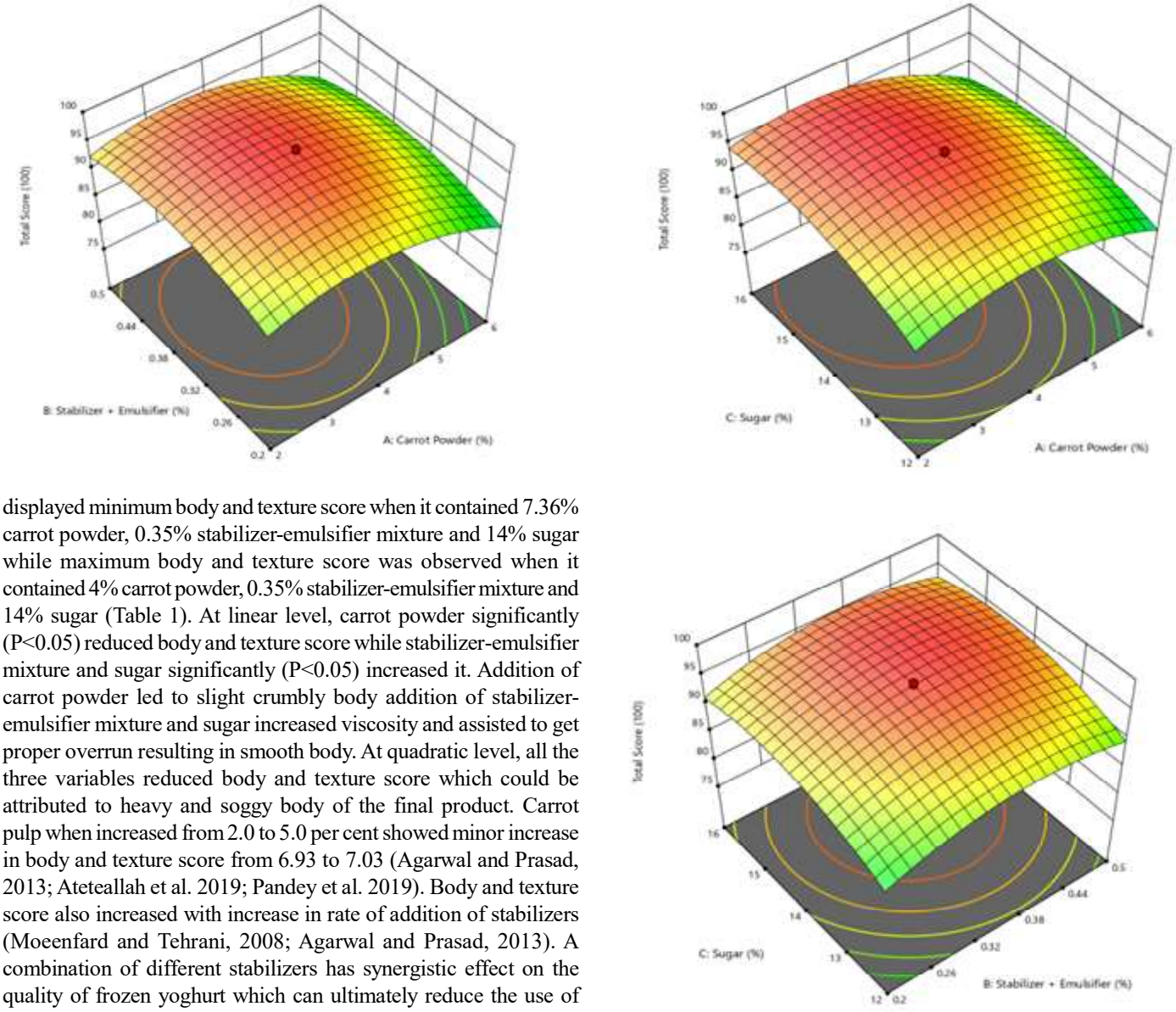

Fig. 1 Effect of different variables on total score of carrot-incorporated low-fat frozen yoghurt

displayed minimum body and texture score when it contained 7.36% carrot powder, 0.35% stabilizer-emulsifier mixture and 14% sugar while maximum body and texture score was observed when it contained 4% carrot powder, 0.35% stabilizer-emulsifier mixture and 14% sugar (Table 1). At linear level, carrot powder significantly ($P<0.05$) reduced body and texture score while stabilizer-emulsifier mixture and sugar significantly ($P<0.05$) increased it. Addition of carrot powder led to slight crumbly body addition of stabilizer-emulsifier mixture and sugar increased viscosity and assisted to get proper overrun resulting in smooth body. At quadratic level, all the three variables reduced body and texture score which could be attributed to heavy and soggy body of the final product. Carrot pulp when increased from 2.0 to 5.0 per cent showed minor increase in body and texture score from 6.93 to 7.03 (Agarwal and Prasad, 2013; Ateteallah et al. 2019; Pandey et al. 2019). Body and texture score also increased with increase in rate of addition of stabilizers (Moeenfard and Tehrani, 2008; Agarwal and Prasad, 2013). A combination of different stabilizers has synergistic effect on the quality of frozen yoghurt which can ultimately reduce the use of stabilizers (Milani and Koocheki, 2011). Hence, a combination of stabilizers and emulsifiers has been used in current investigation.

Effect of variables on melting characteristics: The behaviour of frozen product upon melting has utmost importance. The sensory score for melting quality ranged between 3 and 4.5 The minimum score for melting quality was obtained when the product contained 7.36% carrot powder, 0.35% stabilizer-emulsifier mixture and 14% sugar while maximum body and texture score was observed when it contained 4% carrot powder, 0.35% stabilizer-emulsifier mixture and 14% sugar (Table 1). Stabilizer-emulsifier mixture and sugar significantly ($P<0.05$) improved melting quality of the product at linear level which could be attributed to uniform melting at optimum rate. The addition of all the three variables at higher level resulted in very slow melting as well as presence of visible curd particles on melting, leading to lower score. Ateteallh et al. (2019) observed improvement in melting characteristics with increase in carrot pulp initially followed by reduction at higher level while in current investigation carrot powder failed to impact melting quality which could be due to use of different stabilizer.

Effect of variables on total score

Total score is sum of the scores of all the sensory attributes. The total score ranged from 79.64 to 96.63. The frozen yoghurt containing 7.36% carrot powder, 0.35% stabilizer-emulsifier mixture and 14% sugar showed minimum total score while one containing 4% carrot powder, 0.35% stabilizer-emulsifier mixture and 14% sugar showed maximum total score. The rate of addition of carrot powder and

sugar significantly (P<0.05) increased total score while all the three variables significantly (P<0.05) reduced total score. The impact of different factors on total score is shown in Figure 1. Agarwal and Prasad (2013) also reported similar results in low-fat frozen yoghurt prepared using carrot pulp. The frozen yoghurt containing 0.1 per cent carrageenan was rated better than the ones containing 0.05 and 0.15 per cent while in case of corn starch sensory properties improved with increasing corn starch from 1 per cent to 3 per cent (Skryplonek et al. 2018). Ateteallah et al. (2019) and Pandey et al. (2019) also reported similar results.

Effect of variables on melted quantity

Frozen product should have optimum melting time. Rapid or slow melting causes inconvenience for the consumer. Melting time is inversely related to melted quantity. The melted quantity of the frozen yoghurt ranged between 42.16% and 73.45%. The frozen yoghurt containing 4% carrot powder, 0.6% stabilizer-emulsifier mixture and 14% sugar showed minimum melting while the one containing 2% carrot powder, 0.2% stabilizer-emulsifier mixture and 16% sugar showed maximum melting. At linear level, melting rate was significantly (P<0.05) reduced with increase in stabilizer-emulsifier quantity which could be attributed to increase in amount of bound water with addition of stabilizer and increased overrun while was significantly (P<0.05) increased with increase in sugar quantity leading to higher freezing point depression. At quadratic level, all the three variables resulted in significantly (P<0.05) higher melting rate due to higher viscosity resulting in lower overrun and higher freezing point depression. Moeenfard and Tehrani (2008) also observed increase in melting resistance of frozen yoghurt with increase in stabilizer and emulsifier content. Increase in carrageenan content from 0.05 to 0.15 per cent reduced melting rate by almost 13 per cent while increasing corn starch from 2.0 to 3.0 per cent reduced melting rate by 4 per cent (Skryplonek et al. 2018).

Effect of variables on overrun

Overrun, directly related to amount of air incorporated in ice cream, is an important characteristic as it influences product quality and profit of the producer as well as is also involved in meeting legal standards. Too high overrun results in fluffy ice ceam while too little overrun produces soggy and heavy body (Patel et al. 2015). Overrun (%) of the frozen yoghurt ranged between 72.15 per cent and 98.99 per cent. At linear level, the rate of addition of stabilizer-emulsifier mixture and sugar significantly (P<0.05) increased overrun due to increased water binding and thus viscosity while at quadratic level stabilizer-emulsifier mixture had significantly (P<0.05) negative impact on overrun due to drastic increase in viscosity leading to poor air incorporation (Syed and Shah, 2016). Sugar had significantly (P<0.05) positive impact even at quadratic level. Overrun increased from 41.16 per cent to 45.46 per cent with increase in rate of addition of stabilizer-emulsifier mixture from 0.144 per cent to 0.254 per cent (Moeenfard and Tehrani, 2008). The rate of addition of stabilizer-emulsifier mixture was quite lower by Moennfard and Tehrani (2008) than current investigation where rate of addition varied from 0.10 to 0.60 per cent. Increase in carrageenan content from 0.05 to 0.15 per cent reduced overrun by almost 5.0 per cent while increasing corn starch

Table 4 Goals set for constraints to optimize the carrot incorporated low-fat frozen yoghurt

Constraint	Goal	Lower limit	Upper limit
Carrot powder, %	Maximize	2	6
S+E*, %	In range	0.2	0.5
Sugar, %	In range	12	16
Flavour	Maximize	35.75	44.25
Body & texture	Maximize	22.63	28.88
Melting characteristics	Maximize	3.00	4.63
Colour & appearance	Maximize	3.13	4.63
Total score	Maximize	79.64	96.77
Melted quantity, %	Target – 50	42.16	73.45
Overrun, %	Target - 90	72.15	98.99

*Stabilizer-emulsifier blend

Table 5 Comparison of predicted values and observed values for carrot incorporated low-fat frozen yoghurt

Attribute	Predicted value	Observed value	t-value
Flavour	43.29	43.27	NS
Body & texture	27.47	27.43	NS
Melting characteristics	4.43	4.42	NS
Colour & appearance	4.45	4.46	NS
Total score	94.66	94.58	NS
Melted quantity, %	50.13	50.14	NS
Overrun, %	90.05	90.12	NS

Table 6 Comparison of carrot incorporated low-fat frozen yoghurt with control frozen yoghurt

Parameter	Control frozen yoghurt	Carrot incorporated low-fat frozen yoghurt	t-value
Chemical composition			
Moisture, %	68.18±0.11	64.51±0.09	0.11
Fat, %	2.47±0.07	2.44±0.08	NS
Protein, %	5.18±0.10	5.15±0.12	NS
Ash, %	1.09±0.05	1.13±0.07	NS
Carbohydrates, %	23.10±0.18	26.67±0.20	0.20
Physical characteirstics			
Melted quantity, %	50.62±0.42	50.14±0.37	NS
Overrun, %	91.45±1.15	90.12±1.13	NS
Sensory characteristics			
Flavour	40.85±0.51	43.27±0.62	0.35
Body & texture	25.54±0.69	27.43±0.55	0.26
Melting characteristics	4.23±0.16	4.42±0.22	0.13
Colour & appearance	4.49±0.14	4.46±0.20	NS
Total Score*	90.11±0.64	94.58±0.71	1.07
Microbial analysis			
APC (log_{10}cfu/g)	9.04 ± 1.24	8.97 ± 1.37	NS
Coliform	Absent in 1 g		
Y&M	Absent in 1 g		

from 2.0 to 3.0 per cent reduced overrun rate by 6.0 per cent (Skryplonek et al. 2018).

Optimization of variables for preparation of low-fat frozen yoghurt

The optimization of different variables such as carrot powder, stabilizer-emulsifier mixture and sugar was carried out using numerical optimization technique. The criteria used for optimization are summarized in Table 4. Among the variables, carrot powder was maximized while stabilizer-emulsifier mixture and sugar were kept in range. Among the responses, the sensory parameters were maximized while melted quantity and overrun were set to target of 50% and 90% respectively for the optimization process. RSM suggested the rate of addition of carrot powder, stabilizer-emulsifier mixture and sugar to be 3.93 per cent, 0.41 per cent and 14.30 per cent respectively with desirability of 0.90. Carrot-based low-fat frozen yoghurt was prepared by adding carrot powder, stabilizer-emulsifier mixture and sugar as suggested by RSM. The predicted values for flavour, body and texture, melting quality, colour and appearance, total score, melted quantity and overrun for the frozen yoghurt were 43.29, 27.47, 4.43, 4.45, 94.66, 50.13% and 90.05% respectively. It is evident from the Table 5 that the observed values were not significantly ($P>0.05$) different from predicted values with respect to all attributes. Hence, it was confirmed that the selected level of addition of carrot powder, stabilizer-emulsifier mixture and sugar is most suitable for the preparation of carrot-based low-fat frozen yoghurt with optimum sensory and physico-chemical attributes.

Analysis of carrot incorporated low-fat frozen yoghurt

Carrot-based low-fat frozen yoghurt was analysed and compared with control frozen yogurt for its compositional parameters, physical characteristics as well as sensory attributes and analysed statistically using t-test. Moisture content of experimental frozen yoghurt was significantly ($P<0.05$) lower while carbohydrates content was significantly ($P<0.05$) due to addition of carrot powder. Sensory attributes flavour, body and texture, melting quality and total score of experimental frozen yoghurt were significantly ($P<0.05$) higher than those of control frozen yoghurt. Aerobic plate count of the experimental and control frozen yoghurt were statistically at par. Both the yoghurt samples were free from coliform as well as yeast and mold (Table 6).

Conclusion

Carrot-based low-fat frozen yoghurt was prepared using response surface methodology and the rate of addition of carrot powder, stabilizer-emulsifier mixture and sugar were optimized to obtain sensorially acceptable product and similar physical characteristics to those of control. At linear level, carrot powder improved flavour as well as colour and appearance of the frozen yoghurt while stabilizer-emulsifier mixture improved body and texture, melting quality as well as overrun. It also reduced melted quantity of frozen yoghurt. Sugar also improved flavour, body and texture, melting quality as well as overrun. At quadratic level, all the variables had negative impact on sensory attributes and rate of melting was also increased. Stabilizer-emulsifier mixture reduced overrun while sugar had opposite effect. On the basis of the outcomes, RSM suggested to prepare frozen yoghurt using 3.93 per cent carrot powder, 0.41 per cent stabilizer-emulsifier mixture

and 14.30 per cent sugar. The final product was highly acceptable. Hence, acceptable quality carrot-based low-fat frozen yoghurt can be developed by using response surface methodology.

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RESEARCH ARTICLE

Listeria monocytogenes isolated from raw milk: phenotypic and molecular characterization, pathogenicity testing, and multidrug resistance profiling

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Abstract: Foodborne infections are a worldwide public health emergency that actually impact a variety of disorders. In the current research, a total of 300 samples comprising raw milk (100), milk products (100), and chicken (100) were screened for detection of pathogenic *Listeria* species by using the USDA method. On conventional biochemical characterization, three isolates were identified as *Listeria monocytogenes* (from raw milk samples) indicating an overall prevalence of *Listeria monocytogenes* to the tune of 1%. All other samples comprising milk products and chicken samples (100) showed negativity for the presence of any of the *Listeria* species. Further, all three isolates were subjected to polymerase chain reaction (PCR) targeting genus-specific (*prsA* and *iap*) and species-specific gene (*isp*) in which all three were turn out positive for both the genes, endorsing their identification as *Listeria monocytogenes*. All three confirmed *Listeria monocytogenes* isolates were phenotypically assessed for *in-vitro* pathogenicity tests like hemolysis on 7% sheep blood agar, CAMP test, and PI-PLC assay. The results revealed their highly pathogenic nature. Subsequently, all these isolates were also assessed for their virulent nature by PCR, targeting the array of markers including virulence-associated genes viz. *hlyA, actA, plcA*. The results endorsed pathogenic nature of all isolates showing amplification of all targeted virulence genes. The antibiotic resistance profiling revealed occurrence of multidrug-resistant pathogenic *L. monocytogenes* in foods of animal origin with maximum multiple antibiotic resistance (MAR) index of 0.6 and a minimum MAR index of 0.48 in MDR, which is a matter of concern from public health point of view.

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Introduction

Listeria monocytogenes is a psychrophilic, Gram-positive, facultative aerobic bacteria and one of the world's most common foodborne diseases (Farber and Peterkin, 1991). The number of species in the genus *Listeria* has increased recently with the discovery of several new ones. There are now 26 species in the genus *Listeria* including *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. marthii, L. costaricensis, L. rocourtiae, L. fleischmannii, L. newyorkensis, L. weihenstephanensis, L. floridensis, L. aquatica, L. thailandensis, L. cornellensis, L. riparia, L. booriae, L. goaensis and L. grandensis, L. valentina, L. farberi, L. portnoyi, L. cossartiae, L. rustica, and L. immobilis*. The most prevalent microbe in both humans and animals among these species is *L. monocytogenes*, while *L. ivanovii* is the one that sickens animals. (Barbuddhe et al. 2022). It leads to severe invasive illness in humans; the main signs are septicemia, abortion, stillbirth, perinatal infections, meningitis, gastroenteritis and meningoencephalitis, particularly in aged and immunocompromised individuals (Posfay-Barbe and Wald, 2004). The incidence of listeriosis caused by this bacterium has skyrocketed in recent years. *Listeria monocytogenes* has been isolated from various foodstuffs, including milk (Barbuddhe et al. 2002), and meat (Lunden et al. 2003 and Bhandare et al. 2007). Typically, this bacterium is found in dairy products like cheese and ice cream manufactured from raw milk (Brooks et al. 2012). In comparison to traditional approaches, molecular techniques like polymerase chain reaction (PCR) provide faster and more reliable results (Borucki et al. 2003). The potential of *Listeria* species to quickly develop resistance to any antimicrobial drug creates a significant and growing hazard to both human and animal health (Luque-Sastre et al. 2018). The current study was designed with the objective to assess the prevalence of vital foodborne pathogen *Listeria monocytogenes* in foods of animal origin viz. milk and meat along with phenotypic and molecular characterization, pathogenicity testing, and multidrug resistance profiling of recovered listerial isolates.

Materials and methods

Sample collection

A total of 300 samples were collected and screened for microbiological evaluation in the current study, including raw milk (100) and milk products (100) from small scale milk vendors and farmers, and chicken (100) from meat shops in the Udgir tehsil of Maharashtra state. Milk product and chicken samples were collected in sterile zip-lock bags while, raw milk samples were collected in sterile milk sampling bottles (35 ml, International Scientific Supplies Ltd., UK).

Bacterial strains

The standard strains of *L. monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 12600), and *Rhodococcus equi* (ATCC 6939) were used in the present study which were obtained from Himedia, Mumbai.

Isolation and Phenotypic characterization of *Listeria* species

Samples were collected aseptically and processed immediately after collection, for the isolation of *Listeria* species as per the protocol suggested by the USDA method as described by Curtis and Lee (1995) with suitable modifications.The protocolincludes two-step enrichment with the University of Vermont (UVM-I and II) and subsequent streaking onto polymyxin-Acriflavin-Lithium chloride Ceftazidime Aesculin-Mannitol (PALCAM) medium as a selective agar. Phenotypically, isolates were characterized by employing a battery of biochemical and sugar fermentation tests. Biochemical testing comprised catalase, oxidase Methyl Red-Voges Proskauer (MR-VP), and nitrate reduction tests, while sugar fermentation tests were carried out with Alpha-Methyl-D-Mannoside, Rhamnose, and Glucose (Dextrose) as per the method described in Cruikshank et al. (1975) and Bergey's Manual of Systematic Bacteriology (1984).

In-vitro pathogenicity testing

In order to assess the pathogenic potential of recovered isolates of *L. monocytogenes* phenotypically, the isolates were subjected to *in-vitro* pathogenicity tests like hemolysis on 7% sheep blood agar (SBA) (Courtieu 1991), Christie, Atkins, Munch-Petersen (CAMP) Test (Christie et al. 1944) and Phosphatidylinositol-specific Phospholipase-C (PI-PLC) assay (Notermans et al. 1991).

PCR confirmation of isolates

The recovered listerial isolates characterized by biochemical tests and sugar fermentation tests were further confirmed by PCR, targeting genus-specific genes (*prsA* and *iap*), and *L. monocytogenes* species-specific (*isp*) gene, adopting the protocol suggested by Rawool, et al. (2016) and Bubert et al. (1992). The PCR conditions and primers used are summarized in Table 1 and 2. The DNA was extracted by using the snap chill method as method described by Rawool et al. (2007). The PCR assay in a final volume of 25 µL was done using the following: 1 × PCR buffer, dNTPs, 50 mM $MgCl_2$, 10 pM of each Primer, HotStar® Taq polymerase (QIAGEN, Hilden, Germany) and standard strain of *L. monocytogenes* MTCC 1143 (Serotype 4b) was used as a positive control. The amplified PCR product was subjected to electrophoresis in a 1.5% agarose gel (in TBE buffer), stained with ethidium bromide (10mg/mL) solution, and finally visualized with a UV transilluminator coupled with a digital gel imaging system (UVP Gel Seq. Software) (Bio-Rad GelDoc Go System, USA).

Moreover, the pathogenic character of confirmed *L. monocytogenes* isolates was also performed by targeting the array of virulence markers viz. *hlyA, actA, plcA* (Rawool et al. 2007) as per the primers and amplification conditions mentioned in Table 1 and 2 and with similar volume and concentration of components as mentioned earlier.

Antimicrobial susceptibility testing

Antimicrobial resistance profiling of recovered isolates was performed by disc diffusion method as per the procedure given by Agarwal(1974). In a nutshell, a single isolated colony of *Listeria* isolates from a Brain Heart Infusion agar plate was grown in Muller Hinton broth for 12-16 hours at 37°C, followed by 1 ml freshly grown culture spread over the Muller Hinton agar plate. Antimicrobial discs were placed at appropriate positions on agar plates and incubated at 37°C. Zones of inhibition were measured after 18 hours and again after 48 hours of incubation using an antibiotic zone scale. The antibiotic susceptibility of recovered *Listeria* isolates was determined based on the data provided in CLSI, (2012). The isolates were tested against a panel of 25 unique antibiotics belonging to different classes and which are routinely used in human and animal treatment as given in Table 3.

Determination of the MAR index

The method given by Osundiya et al. (2013), which divides the number of antibiotics employed in the study by the number of antibiotics an isolate is resistant to (a), was used to calculate the MAR index (b). The following is the calculation formula: Index MAR = a/b.

Results and discussion

Prevalence of *Listeria monocytogenes* in food samples

In this study, on microbiological analysis of 300 food samples comprising raw milk, milk products, and chicken, three presumptive listerial isolates were recovered and identified as *Listeria monocytogenes* on the basis of results of a battery of biochemical tests and molecular detection by PCR, giving an overall occurrence of *Listeria monocytogenes* to the tune of 1%.

The other samples comprising milk products and chicken showed negativity for the presence of any of the *Listeria* species. Amongst these, all three isolates were recovered from raw milk samples revealed a prevalence of 3%.

The prevalence of *Listeria* spp. in milk detected in this study is consistent with the findings of Lovett et al. (1987), who reported a 4.2% overall prevalence of *Listeria* spp., and Gaya et al. (1996), who reported a 2.56% prevalence of *L. monocytogenes* recovered from raw milk samples. Globally, the results of Beak et al. (2000) with 4.4% prevalence from raw milk in Korea, Aygun et al. (2006) with 2.12% prevalence from raw milk in Antakya, Turkey, and Indian data reported by Karthikeyan et al. (2015) and Bhilegaonkar et al. (1997) with 3.5% and 4.9% prevalence *Listeria* spp. in milk, respectively, can be corroborated. These variations of prevalence observed may be attributed to the fact that *Listeria* spp. typically affects raw milk by contamination caused by unhygienic conditions in the environment, gastrointestinal tract, and teat skin of animals.

Furthermore, many researchers, including Aurora et al. (2006), Kalorey et al. (2008), Khan et al. (2013), Shantha and Gopal (2014), Sharma et al. (2017), and Shakuntala et al. (2019), found a 1.69%, 0.1% (2/2060), 0.8%, 0.76%, 1.09%, and 1.7% prevalence of *Listeria* spp. in milk, which were on the low side as that of results of the current study. However, certain studies have reported quite a higher recovery of *Listeria* spp. in milk which includes Mary et al. (2017) with 52.7% (219/415) and Gebretsadik et al. (2011) with 22% (22/100) prevalence. The other factors that also contribute to listerial contamination include lack of hygiene, environmental contamination, and poor milking practices. In this study, raw milk samples were obtained from small scale milk vendors and farmers. It is believed that milk is diluted with water before being sold to customers as a malpractice in order to increase the amount, which could explain why *Listeria* spp. was found in the raw milk sample.

Biochemical characterization and *in-vitro* pathogenicity testing of *Listeria* isolates

On biochemical characterization, all three recovered isolates showed the typical greyish green, glistening, iridescent, and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis on PALCAM agar (Curtis and Lee, 1995), characteristic Gram-positive coccobacilli morphology, tumbling motility in hanging drop technique (Islam et al. 2016) and positivity towards catalase, MR-VP, nitrate

Table 1 Primers and amplicon size for the PCR assays

Target gene	Sequence (5'–3')	Amplicon (bp)	Reference
prs	F=AGCTGAAGAGATTCCGAAAGA R=TTCACCAAGAAGAGCTGCAA	844	Rawool et al. (2016)
iap	F=ACAAGCTGCACCTGTTGCAG R=TGACAGCGTGTGTAGTAGCA	131	Bubert et al. (1992)
isp	F=TGCAGCGAATGCTCTTAGTG R=AGCCAAGCACGGCTACTTTA	713	Rawool et al. (2016)
plcA	F=CTGCTTGAGCGTTCATGTCTCATCCCCC R=CATGGGTTTCACTCTCCTTCTAC	1484	Notermans et al. (1991)
hlyA	F=GCAGTTGCAAGCGCTTGGAGTGAA R=GCAACGTATCCTCCAGAGTGATCG	456	Pazaik-Domanska et al. (1999)
actA	F= CGCCGCGGAAATTAAAAAAAGA R= ACGAAGGAACCGGGCTGCTAG	839	Suarez and Vazquez-Boland (2001)

Table 2 Amplification conditions for the PCR assays

Target gene	Amplification conditions
prs	95°C (5 min), 95°C (30 sec), 53°C (1 min), 72°C (2 min), 72°C (10 min), 40 cycles
iap	95°C (5 min), 95°C (15 sec), 57°C (1 min 20 sec), 72°C (2 min), 72°C (7 min), 40 cycles
isp	95°C (5 min), 95°C (30 sec), 53°C (1 min), 72°C (2 min), 72°C (10 min), 40 cycles
plcA *hlyA* *actA*	95°C (5 min), 94°C (15 sec), 60°C (30 sec), 72°C (1 min 30 sec), 72°C (10 min) , 35 cycles

reduction test and negativity to oxidase test. After confirmation of genus *Listeria*, isolates fermented only the Alpha-Methyl-D-Mannoside, Rhamnose, and Glucose (Dextrose) sugars on sugar fermentation tests, identifying the species of all three isolates as *Listeria monocytogenes* (OIE terrestrial manual 2021 and Nayak et al. 2015). Further, the isolates were judged for their pathogenic potential by employing *in-vitro* pathogenicity tests like hemolysis on SBA, CAMP Test, and PI-PLC assay. In this investigation, all *L. monocytogenes* isolates showed typical beta (β) haemolysis on 7% sheep blood agar and positivity for pathogenicity in CAMP Test and PI-PLC assay revealing their virulent character.

Confirmation of isolates by genus and species-specific PCR

The isolates that exhibit characteristic biochemical and sugar fermentative characteristics were exposed to PCR targeting the *prsA* and *iap* (genus-specific) and *isp* (species-specific) genes, with a product size of 844 bp,131bp, and 713 bp respectively as per results obtained by Rawool, et al. (2016) and Bubert et al. (1992). All 3 listerial isolates showed positivity towards all the three targeted genes, -hence confirming the organisms as *Listeria monocytogenes* (Figure 1 and 2).

PCR targeting virulence-associated genes of *Listeria monocytogenes*

The confirmed *Listeria monocytogenes* isolates were further subjected to PCR that targeted virulence-associated genes to check the virulence character of isolates viz., Actin filament protein (*act*A), haemolysin called listeriolysin O (*hly*A) and invasion associated internalin gene (*Inl*C). The results of the experiment showed that all three isolates (*Listeria monocytogenes*) are positive for all targeted three genes, which were haemolytic on 7% sheep blood agar, CAMP positive, and showed PI-PLC activity, suggesting their pathogenic nature (Figure 3 and 4). The same kind of results was obtained by Rawool et al. (2007) who carried out a PCR employing virulence-associated genes of *L. monocytogenes*, i.e., *plc*A, *hly*A, *act*A, and iap. Also, Arslan and Baytur (2019) targeted *hlyA*, *actA*, *inlA, inlB, inlC, inlJ*, *prfA*, *plcA*, and *iap* virulence-associated genes and reported 100% positivity.

Antibiotic sensitivity test

The inappropriate use of antibiotics for therapeutic purposes in animal and human medicine has led to the development of antibiotic resistance, a major public health issue. Antibiogram study of isolates revealed multiple drug resistance patterns. The panel of 25 different antibiotics along with their different therapeutic concentrations were used, out of which all isolates showed complete resistance (100%) against 12 antibiotics of different groups viz., ampicillin, amoxicillin-clavulanic acid, amoxicillin-sulbactam, cefaclor, cefalexin, cefepime, cefotaxime, furazolidone, oxacillin, streptomycin, sulphadiazine, trimethoprim. The listerial isolates showed 100% sensitivity towards

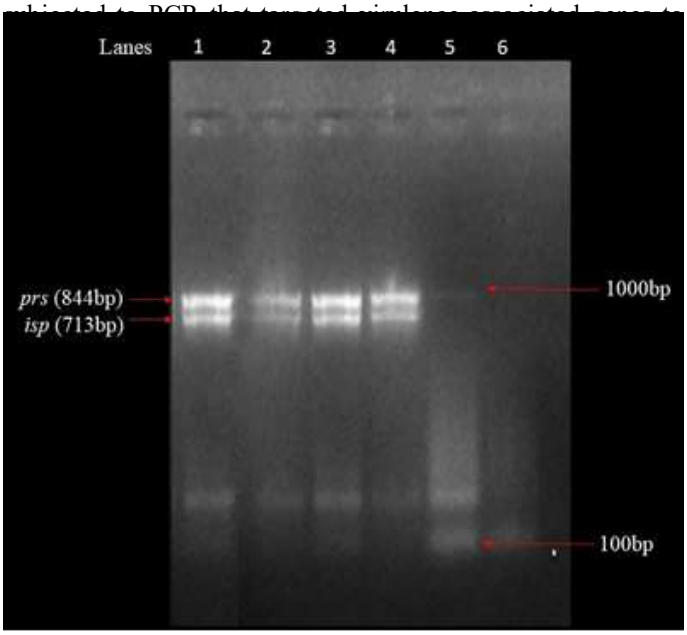

Fig. 1 PCR profile of genus-specific (*prs*) and species- specific (*isp*) genes of *L. monocytogenes*
Lane 1 Positive control template
Lane 2-4 *L. monocytogenes* positive isolates from milk (Both *prs* and *isp* positive)
Lane 5 100 bp DNA ladder (Promega, USA)
Lane 6 NTC (Negative control template)

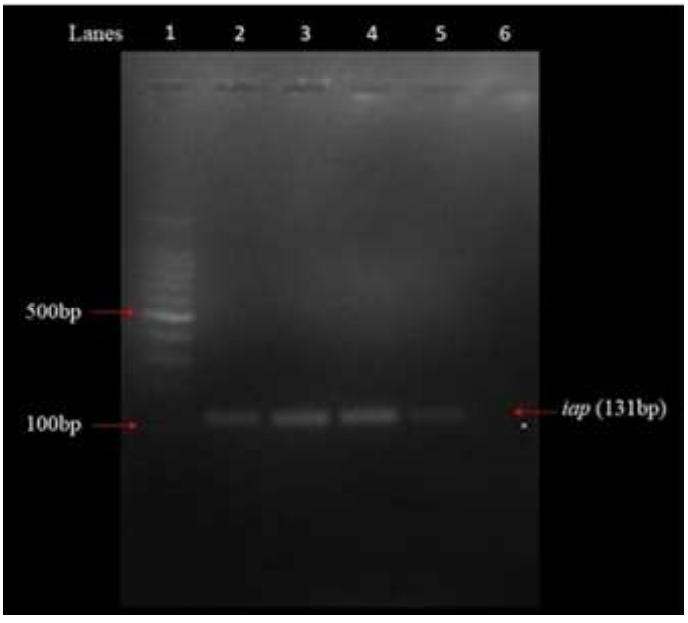

Fig. 2 PCR profile of genus-specific *iap* gene of *L. monocytogenes*
Lane 1 100 bp DNA ladder (Promega, USA)
Lane 2-4 *L. monocytogenes* positive isolates from milk (*iap* positive)
Lane 5 Positive control template
Lane 6 NTC (Negative control template)

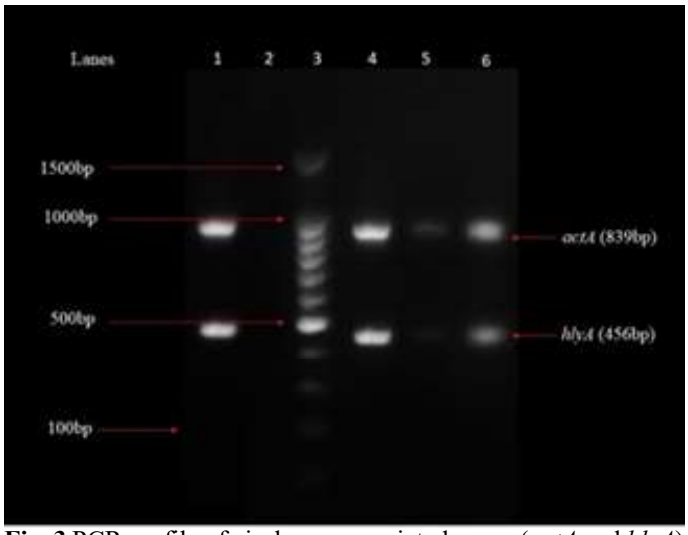

Fig. 3 PCR profile of virulence-associated genes (*actA* and *hlyA*) of *L. monocytogenes*
Lane 1 Positive control template
Lane 2 NTC (Negative control template)
Lane 3 100 bp DNA ladder (Promega, USA)
Lane 4-6 *L. monocytogenes* positive isolates from milk (Both *actA* and *hlyA* positive)

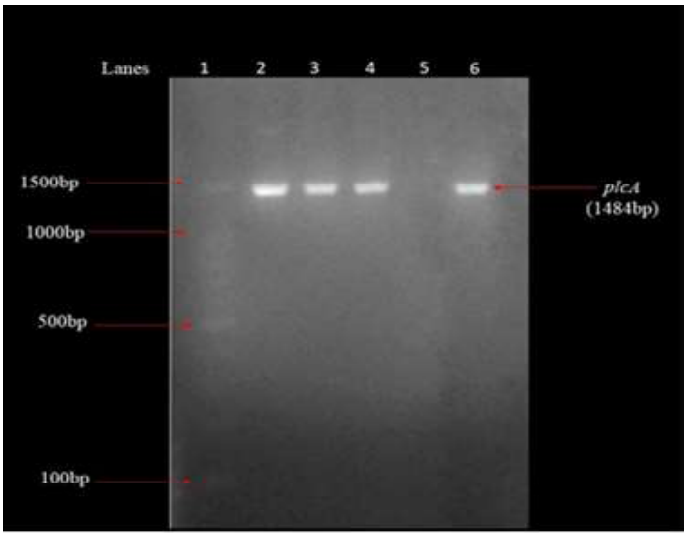

Fig. 4 PCR profile of virulence-associated gene *plcA* of *L. monocytogenes*
Lane 1 100 bp DNA ladder (Promega, USA)
Lane 2-4 *L. monocytogenes* positive isolates from milk (plc*A* positive)
Lane 5 NTC (Negative control template)
Lane 6 Positive control template

Table 3 Antibiogram pattern of *L. monocytogenes* isolates

Sr. No	Antibiotics	Concentration	No. of isolates (n=3)			Percentage (%)		
			S	I	R	S	I	R
1.	Ampicillin (AMP)	10 mcg	0	0	3	0	0	100
2.	Amoxycillin-clavulanic acid (AMC)	25 mcg	0	0	3	0	0	100
3.	Amoxicillin-sulbactam (AMS)	30/15 mcg	0	0	3	0	0	100
4.	Azithromycin (AZM)	10 mcg	3	0	0	100	0	0
5.	Cefaclor (CF)	30 mcg	0	0	3	0	0	100
6.	Cefalexin (CN)	30 mcg	0	0	3	0	0	100
7.	Cefepime (CPM)	30 mcg	0	0	3	0	0	100
8.	Cefotaxime (CTX)	10 mcg	0	0	3	0	0	100
9.	Ciprofloxacin (CIP)	10 mcg	3	0	0	100	0	0
10.	Doxycycline (DO)	10 mcg	3	0	0	100	0	0
11.	Enrofloxacin (EX)	10 mcg	3	0	0	100	0	0
12.	Furazolidone (FR)	50 mcg	0	0	3	0	0	100
13.	Gentamicin (GEN)	50 mcg	3	0	0	100	0	0
14.	Lincomycin (L)	10 mcg	1	1	1	33.33	33.33	33.33
15.	Nalidixic Acid (NA)	30 mcg	3	0	0	100	0	0
16.	Nitrofurantoin (NIT)	200 mcg	2	0	1	66.66	0	33.33
17.	Oxytetracycline (O)	30 mcg	3	0	0	100	0	0
18.	Oxacillin (OX)	5 mcg	0	0	3	0	0	100
19.	Ofloxacin (OF)	2 mcg	3	0	0	100	0	0
20.	Penicillin-G (P)	2 units	3	0	0	100	0	0
21.	Streptomycin (S)	25 mcg	0	0	3	0	0	100
22.	Sulphadiazine (SZ)	100 mcg	0	0	3	0	0	100
23.	Tetracycline (TE)	10 mcg	3	0	0	100	0	0
24.	Trimethoprim (TR)	30 mcg	0	0	3	0	0	100
25.	Vancomycin (VA)	10 mcg	1	2	0	33.33	66.66	0

(S- Sensitive, I-Intermediate, R-Resistant)

Table 4 MAR index isolates

Sr. No.	Origin of isolates	Resistance to antibiotics	Resistance to the number of antibiotics	MAR index
1.	Raw milk	AMS, AMP, AMC, CN, CF, CTX, CPM, S, TR, OX, SZ, FR, VA, L, NIT	15	0.6
2.	Raw milk	AMS, AMP, AMC, CN, CF, CTX, CPM, S, TR, OX, SZ, FR	12	0.48
3.	Raw milk	AMS, AMP, AMC, CN, CF, CTX, CPM, S, TR, OX, SZ, FR, NIT	13	0.52

azithromycin, ciprofloxacin, doxycycline, enrofloxacin, gentamicin, nalidixic acid, oxytetracycline, ofloxacin, penicillin-G, tetracycline, antibiotics as shown in Table 3.

In this research, 100% resistance observed against 12 different antibiotics belonging to different groups can be validated with the results of Prazak et al. (2002) who reported 100% resistance to oxacillin and 85% toward penicillins from food samples, which concerns directly public health as both the antibiotics used as a treatment of listeriosis in combination with the gentamicin (Gomez et al. 2014). Troxler et al. (2000) also stated that all pathogenic *Listeria* species i.e., *L. monocytogenes* and *L. innocua* were naturally resistant to the modern cephalosporins, due to the absence of proper penicillin-binding proteins (PBPs) in the cytoplasmic membrane of *Listeria* which validates our findings. while natural sensitivity towards fluoroquinolones and aminoglycosides also validates our results. The findings of Abdollahzadeh et al. (2016), who also revealed susceptibility showed against tetracyclines by listerial isolates recovered in their research, can be used to support the results observed in present study that listerial isolates were sensitive to tetracyclines.Higher resistance to ampicillin in current research can be related to the work of Soni et al. (2013), who reported 100% resistance towards ampicillin.

MAR index

The multi-drug resistant (MDR) isolates in the present study showed resistance to a minimum of 5 and a maximum of 15 antibiotics. Thus, 3 resistance patterns were observed ranging from 5 to 15 antibiotics with maximum multiple antibiotic resistance (MAR) index of 0.6 and a minimum MAR index of 0.48 in MDR isolates as depicted in Table 4. These findings are comparable with Shourav et al. (2020), who got MAR indices ranging from 0.40 to 0.64 to a panel of 25 antibiotics from recovered *Listeria* isolates. Multidrug resistant *Listeria* were also discovered by Kuan et al. (2017), Swetha et al. (2021), and Elsayed et al. (2022), with MAR indices ranging from 0.11 to 0.56, 0.56 to 0.78, and 0.22 to 0.78, respectively.

Conclusion

To summarise, the current study found 3% prevalence of pathogenic *L. monocytogenes* in milk. Moreover, the listerial isolates revealing pathogenic nature in phenotypic *in-vitro* pathogenic assays were also observed showing the presence of all targeted virulence associated genes viz. *plcA*, *hlyA*, *actA* in PCR, demonstrating excellent correlation between phenotypic and genotypic assays. Further, multidrug-resistant character shown by all pathogenic food borne *L. monocytogenes* isolates are a matter of concern from a public health point of view.

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RESEARCH ARTICLE

Antioxidant property of palm oil blended ghee and its fractions

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Abstract: Ghee is a high-priced product that costs three to six times than the edible vegetable oil. Dry fractionation is one of the cost-effective methods of modifying the physical properties of milk fat. Natural antioxidant present in palm oil is believed to enhance the oxidative stability of ghee. Therefore, in the current study, ghee was intentionally blended with palm oil at levels of 0, 5, 10, and 20%. Then the ghee was fractionated to separate liquid and solid portions by dry fractionation technique. The antioxidant activity accessed by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method shows that as the palm oil (especially tocotrienols and tocopherols) level increased the antioxidant activity in the prepared samples. This could be due to carotenoids and vitamins E which act synergistically as powerful natural antioxidants which also contribute better towards stability against auto oxidation (peroxide value). The liquid fraction (L_{20} with palm oil 20%) has a high level of antioxidant activity. Fractionated ghee, especially the liquid fraction showed higher antioxidant activity as compared to ghee could be due to its higher vitamin E activity. The peroxide value and free fatty acid content of ghee were determined during storage at 30°C and 60°C for 90 days. Liquid fractions showed more peroxide value and FFA as the storage day increased than solid fractions, which contained more peroxide value and FFA than control samples.

Keyword: - Antioxidant activity; Free fatty acid; Ghee; Palm oil; Peroxide value

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Introduction

Ghee is also known as clarified butterfat. It has a pleasant and appetizing aroma and is prepared from cream or butter made from cow or buffalo milk, or a combination of these two. Ghee is most widely used milk product in the Indian subcontinent and is regarded as the best cooking and frying medium. During storage, ghee undergoes oxidative degradation, resulting in changes to major quality parameters such as intensity of colour decrease as the palm oil level increases (0, 5, 10 and 20 per cent), flavour, aroma and nutritive value, affecting its suitability for consumption. The development of rancidity reduces the shelf life of the product, which ultimately affects consumer acceptability. Various studies have found that oxidized lipids may be harmful to one's health (Nerin et al. 2008). Since, India is the primary producer and exporter of Ghee, it is a one of the costliest dairy product in India (Ghee Market in India: Industry Trends 2022). The acceptability of ghee is largely determined by the extent of oxidative deterioration. Several chemical methods have been developed to measure the oxidative changes in oils and fats (Gray, 1978). The methods reported for monitoring the oxidative deterioration of various oils and fats are based on chemical changes that occur at different stages of oxidation, namely the primary and secondary stages. Peroxides, particularly hydroperoxides, are the first compounds formed during oxidation and are known as primary oxidation products. Peroxide value (PV) is most commonly used as an indicator of the early stages of oxidation in fats and oils. Oxidative stability is an important factor in oil quality, and is particularly significant for oils used for frying because of the high temperature and long duration of the frying process. The chemistry of oxidation at high temperatures is very complex since both thermal and oxidative reactions are involved (Marquez-Ruiz and Dobarganes, 2007). Vegetable oils such as palm oil, olive oil, cottonseed oil, peanut oil, and sunflower oil are classed as Oleic – Linoleic acid oils seeing that they contain a relatively high proportion of unsaturated fatty acids (monounsaturated fatty acid like oleic acid and the polyunsaturated fatty acid like linoleic acid) (Dunn, 2005; Gertz et al. 2000). Palm oil is edible plant oil extracted from palm tree fruits. Palm oil (*Elaeis guineensis*) is extracted from the pulp of the palm fruit (Poku, 2002). Palm oil has been used in food preparation for over 5,000 years. Palm oil is the most widely

produced edible vegetable oil in the world, and its nutritional and health benefits have been well documented (Chandrasekharan et al. 2000). The most abundant natural source of tocotrienol is palm oil. Because of its good resistance to oxidative deterioration and better ability to withstand the high temperatures used in frying than other oils, the industrialization of convenience foods and snack food manufacturing opened up additional uses for palm oil (Kheri, 1987; Berger, 1992). Crude palm oil (CPO, also known as red palm oil, RPO) contains both healthy beneficial compounds like vitamin, carotenoids and phytosterols as well as impurities like phospholipids, free fatty acids (FFA), gums and lipid oxidation products, which can be removed through refining processes which causes off-flavour/ odour to oil (Sambanthamurthi et al. 2002). Centrifugation and drying are commonly used to purify the CPO. After purification oil is cooled and kept in a suitable container. CPO is the highest natural source of carotenoids (500–700 ppm), tocopherols (600–1200 ppm), and tocotrienols (600–1200 ppm), all of which contribute to its nutritional value and oxidative stability. Their antioxidant effects, which are primarily directed against reactive oxygen species (ROS), play a role in the prevention of ageing, CVD and cancer. Tocotrienols are natural cholesterol production inhibitors (Edem, 2002). The carotenoids and the vitamins E act synergistically as powerful natural antioxidants. Palm oil is largely made up of unsaturated fatty acids, which can raise blood cholesterol levels as compare to other oil. (Edem, 2002). In the present investigation, the ghee was blended with palm oil (at concentration 0, 5, 10, and 20%.) and fractionated by dry fractionation technique. Antioxidation activity along with FFA and peroxide value from palm oil blended ghee and its fractions.

Materials and methods

Butter was purchased from retail outlet of local market, Bengaluru. Palm oil was purchased from the local Bengaluru market and used for the adulteration purpose.

Preparation of samples

The creamery butter method was used to produce ghee (Parmar and Khamrui, 2017). It is then clarified in a stainless steel vessel over direct flame into ghee with continous strring at a temperature of 115-117C. Ghee was then filtered through muslin cloths, cooled and filled in airtight glass bottles for futher analysis.

Fatty acid profile of cow milk fat

Fatty acid	Cow milk fat (%)
Butyric acid (C4:0)	1.78
Caproic acid (C6:0)	1.44
Caprylic acid (C8:0)	0.99
Capric acid (C10:0)	2.55
Lauric acid (C12:0)	3.15
Myristic acid (C14:0)	10.30
Palmitic acid (C16:0)	24.03
Palmitoleic acid (C16:1)	1.18
Stearic acid (C18:0)	9.36
Oleic acid (C18:1)	20.04
Linoleic acid (C18:2)	1.64
Linolenic acid (C18:3)	0.66
Arachidonic acid (C20:4)	0.11

(Pena-Serna and Restrepo-Betancur, 2020)

Fatty acid profile of palm oil

Fatty acid	Palm oil (%)
Lauric	0.19
Myristic	1.10
Palmitic	46.38
Stearic	4.6
Oleic	38.08
Linoleic	9.33
Linolenic	-
Arachidonic	0.38

(Li et al. 2011)

Dry fractionation technique

Fractionation was done by following Kankare (1974) method with slight modification in temperature and time period of centrifugation. Melting method was used to fractionate ghee into solid and liquid fractions. The crystal nuclei memory was removed by heating ghee to 60°C. It was then progressively cooled to 30°C in an incubator for 12 h to crystallize. After centrifugation (at 2500 rpm for 15 min) using temperature-controlled centrifuge at 30°C, the liquid was separated from the crystals by decantation. At 30°C, solid fraction obtained (S_{30}) was considered as a high melting fraction. The liquid fraction collected at 30°C was then incubated at 20°C for 12 h. Again, the fraction was centrifuged at 20°C under same conditions. The produced crystals were separated. The solid portion obtained at 20°C (S_{20}) was considered a medium melting fraction, whereas the amount that remained liquid at 20°C was referred to as the low melting fraction (L_{20}).

Antioxidant Activity

The percentage of antioxidant activity (AA %) of each fraction (S_{20} and L_{20}) of ghee, control ghee as well as palm oil was assessed by DPPH free radical assay as described by Wu et al. (2006).

FFA and Peroxide

The FFA content was determined as per SP: 18 (Part XI)-(1981). The peroxide value was determined by method as described in AOAC (2000). The prepared ghee samples were stored in 30 ºC for 3 months and accelerated storage at 60 ºC for 3 months.

Statistical analysis

Significant difference between the values was verified by one way analysis of variance (ANOVA) and comparison between means was made by critical difference value by using R software [R. version 4.1.2 (2021-11-01), copyright © 2021, R foundation].

Results and discussion

The antioxidant activity of palm oil was found to be 92.5 %, respectively. The antioxidant activity in ghee added with palm oil (0, 5, 10 and 20 %) measured by DPPH method is shown in Table 1. Ghee with palm oil (20 %) had higher antioxidant activity as compared to other ghee sample. The statistical analysis reveals that significant ($P \leq 0.05$) difference amongst ghee sample with respect to antioxidant activity. The antioxidant activities in liquid and solid fractions of ghee added with palm oil (0, 5, 10 and 20 %) are shown in the Table 1. L_{20} with palm oil (20 %) had higher antioxidant activity as compared to other ghee samples. It was evident from the Table 1 that significant ($P \leq 0.05$) difference amongst liquid fraction of ghee sample respect to the antioxidant activity. Fraction S_{20} with palm oil (20 %) had higher antioxidant activity as compared to other ghee samples. Statistically it was proved that significant ($P \leq 0.05$) difference amongst solid fraction of ghee sample respect to antioxidant activity.

S_{20} ($P \leq 0.05$)

The prepared ghee samples were stored in 30 ºC for 3 months and accelerated storage at 60 ºC for 3 months. The samples were analysed for storage stability viz. hydrolytic (per cent FFA) and oxidative rancidity (Peroxide value). FFA and peroxide value were determined periodically at an interval of 30 days as per ISI (1981) for a period of 90 days.The peroxide value of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0 mM O_2/kg of fat, respectively in all the ghee sample at 0^{th} day and it increased to 2.67, 2.45, 2.15 and 1.89 mM O_2/kg of fat, respectively for ghee blended with 0, 5, 10 and 20 percent of palm oil on 90^{th} day of storage at 30°C (Table 2). The peroxide value in liquid fraction of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0 m.MO_2/kg of fat, respectively in all the sample at 0^{th} day and it was increased to 4.87, 4.57, 3.87 and 3.63 mM O_2/kg of fat, respectively on 90^{th} day of storage at 30°C (Table 2). The peroxide value in solid fraction of ghee added with palm oil (0, 5, 10 and 20 %) were found to be 0 mM O_2/kg of fat, respectively in all the

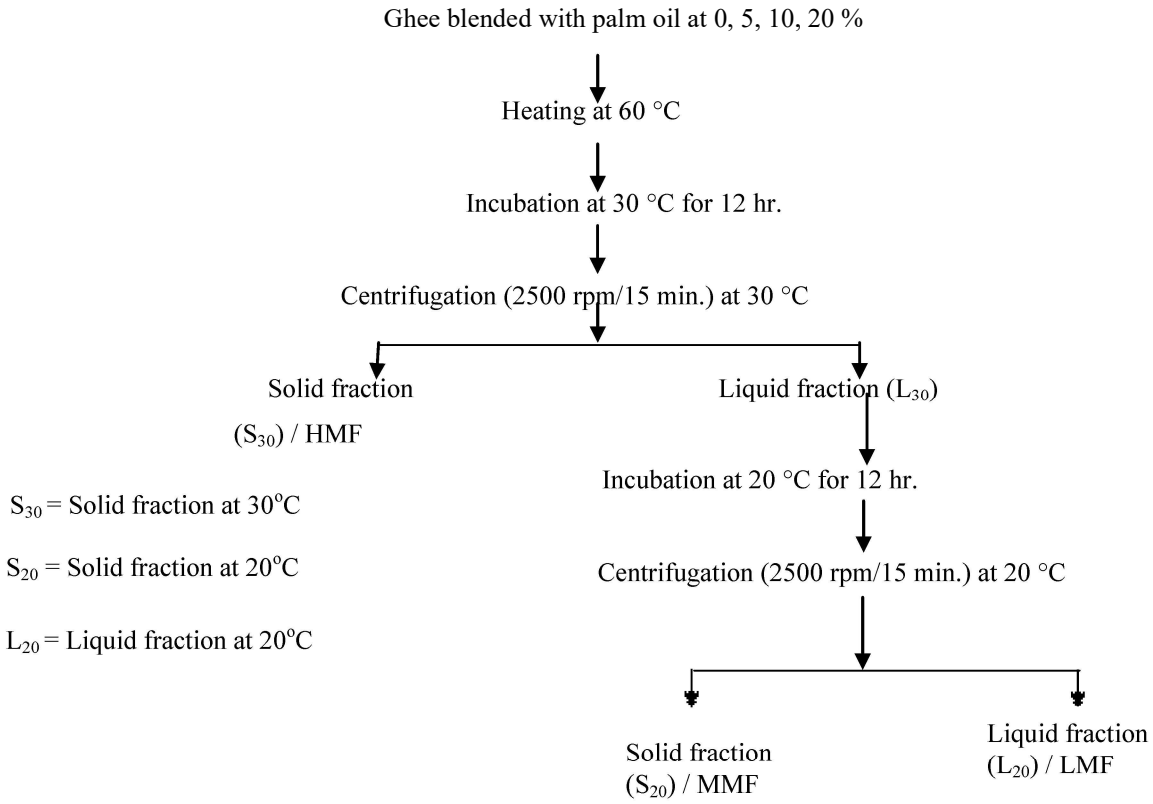

Fig: 1: Dry fractionation technique

sample at 0th day and it increased to 3.77, 3.20, 2.96 and 2.34 mM O_2/kg of fat, respectively on 90th day of storage at 30°C (Table 2).

The peroxide value in ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0 mM O_2/kg of fat, respectively in the entire sample at 0th day and it increased to 8.01, 7.35, 6.45 and 5.67 mM O_2/kg of fat, respectively on 90th day of storage at 60°C (Table 2). The peroxide value in liquid fraction of ghee added with palm oil (0, 5, 10 and 20 %) were found to be 0 mM O_2/kg of fat, respectively in all the samples at 0th day and it increased to 14.61, 13.71, 11.61 and 10.89 mM O_2/kg of fat, respectively on 90th day of storage at 60°C (Table 2). The peroxide value in solid fraction (S_{20}) of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0 mM O_2/ kg of fat, respectively in all the

Table 1 Antioxidant activity of ghee, liquid fraction and solid fraction sample added with palm oil

Sample	Antioxidant activity (%)	Sample	Antioxidant activity (%)	Sample	Antioxidant activity (%)
Palm oil	92.5[a]	L20 (Control)	55.1[d]	S20 (Control)	49.9[d]
Control ghee	62.9[e]				
Ghee with PO (5%)	68.3[d]	L20 with PO (5%)	71.3[c]	S20 with PO (5%)	56.1[c]
Ghee with PO (10%)	77.3[c]	L20 with PO (10%)	84.8[b]	S20 with PO (10%)	58.2[b]
Ghee with PO (20%)	83.1[b]	L20 with PO (20%)	86.4[a]	S20 with PO (20%)	65.3[a]
CD (*P≤0.05*)	0.55	CD (*P≤0.05*)	0.56	CD (*P≤0.05*)	0.50

CD-Critical difference
All the values are average of three trials
Different superscripts within the column are compared with other values

Table 2: Effect of storage on peroxide value of ghee, liquid fraction of ghee and solid fraction of ghee added with palm oil stored at 30 ºC and 60 ºC

Sample	Peroxide value (m.M O2/Kg of fat)							
	Storage period (in Days) 30 ºC				Storage period (in Days) 60 ºC			
Days	0th	30th	60th	90th	0th	30th	60th	90th
Control ghee	0	0.52[a]	1.48[a]	2.67[a]	0	1.56[a]	4.44[a]	8.01[a]
Ghee with PO(5%)	0	0.50[b]	0.85[b]	2.45[b]	0	1.50[b]	2.55[b]	7.35[b]
Ghee with PO(10%)	0	0.47[c]	0.54[c]	2.15[c]		1.41[c]	1.62[c]	6.45[c]
Ghee with PO(20%)	0	0.42[d]	0.50[d]	1.89[d]	0	1.26[d]	1.50[d]	5.67[d]
CD (P≤0.05)	NS	0.01	0.01	0.01	NS	0.01	0.55	0.01
L20 (Control)	0	0.78[a]	3.53[a]	4.87[a]	0	2.34[a]	10.6[a]	14.61[a]
L20 with PO(5%)	0	0.67[b]	3.00[b]	4.57[b]	0	2.01[b]	9.01[b]	13.71[b]
L20 with PO(10%)	0	0.58[c]	2.49[c]	3.87[c]	0	1.74[c]	7.47[c]	11.61[c]
L20 with PO(20%)	0	0.52[d]	1.89[d]	3.63[d]	0	1.56[d]	5.67[d]	10.89[d]
CD (*P≤0.05*)	NS	0.01	0.01	0.01	NS	0.01	0.52	0.74
S20 (Control)	0	0.65[a]	2.56[a]	3.77[a]	0	1.95[a]	7.68[a]	11.31[a]
S20 with PO (5%)	0	0.55[b]	2.00[b]	3.20[b]	0	1.65[b]	6.01[b]	9.60[b]
S20 with PO (10%)	0	0.49[c]	1.86[c]	2.96[c]	0	1.47[c]	5.58[c]	8.88[c]
S20 with PO (20%)	0	0.45[d]	1.59[d]	2.34[d]	0	1.35[d]	4.77[d]	7.02[d]
CD (P≤0.05)	NS	0.01	0.01	0.01	NS	0.01	0.6	0.57

CD-Critical difference
All the values are average of three trials
Different superscripts within the column are compared with other value

sample at 0th day and it was increased to 11.31, 9.60, 8.88 and 7.02 mM O_2/kg of fat, respectively on 90th day of storage at 60°C (Table 2).

The FFA content in ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0.32, 0.68, 1.00 and 1.70 percent oleic acid (O.A), respectively at 0th day and it was increased to 0.69, 0.92, 1.53 and 2.15 per cent O.A, respectively on 90th day of storage at 30°C (Table 3). The FFA content in liquid fraction of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0.34, 0.70, 1.20 and 1.90 per cent O.A, respectively at 0th day and it were increased to 0.89, 0.96, 1.75 and 2.34 per cent O.A, respectively on 90th day of storage at 30°C (Table 3).The FFA content in solid fraction of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0.33, 0.69, 1.10 and 1.80 per cent O.A, respectively at 0th day and it was increased to 0.77, 0.95, 1.59 and 2.19 per cent O.A, respectively on 90th day of storage at 30°C (Table 3).

The FFA content of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0.32, 0.68, 1.00 and1.70 per cent O.A, respectively at 0th day and it was increased to 0.69, 0.92, 1.53 and 2.15 per cent O.A, respectively on 90th day of storage at 60°C (Table 3).The FFA in liquid fraction of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0.34, 0.70, 1.20 and 1.90 per cent O.A, respectively at 0th day and it was increased to 2.67, 2.88, 5.25 and 7.02 per cent O.A, respectively on 90th day of storage at 60°C (Table 3).The FFA content in solid fractions of ghee added with palm oil (0, 5, 10 and 20 per cent) were found to be 0.33, 0.69, 1.10 and 1.80 per cent O.A, respectively at 0th day and it was increased to 2.31, 2.85, 4.77 and 6.57 per cent O.A, respectively for 0, 5, 10 and 20 per cent samples on 90th day of storage at 60°C (Table 3).

The assay involved 50 mg/ 100 ml (ethanol) DPPH solution, leading to the detection of even up to 5% level of palm oil adulteration in ghee. Ramani et al. (2019) described the effect of a chromogenic analytical method for detecting palm oil adulteration in ghee. Palm oil was added to ghee in quantities of 5, 10, 15, and 20%. The detection limits were approximately 5%. (activity 68.3) The method proposed proved to be simple and appropriate for the detection of palm oil in ghee even at 5% level. In the current study L_{20} fraction with PO (20%) have showed highest antioxidant activities (86.4 %) as compared to control ghee (activity 55.1%). This could be due to the presence carotenoids and vitamins E which acts synergistically as powerful natural antioxidants and also provides better stability toward auto oxidation (Mba et al. 2015). The results obtained was comparable with those of Alyaqoubi et al. (2014), reported that anti-oxidant activity of ghee was 60.81 % by DPPH method. Similar results were also found by Ramani et al. (2018) reported that DPPH reaction (qualitative) of pure ghee and ghee adulterated with palm oil (5, 10, 15 and 20 %). During storage, as the palm oil level increased the antioxidant activity also increased. Therefore, it could be concluded that the carotenoids and the vitamins E acts as an strong antioxidants and hence enhances the shelf life of the ghee.

Table 3: Effect of storage on FFA value in ghee, liquid fraction of ghee and solid fraction of ghee added with palm oil stored at 30 ºC and 60ºC

Sample	FFA (% OA)							
	Storage period (in Days) 30ºC				Storage period (in Days) 60ºC			
Days	0th	30th	60th	90th	0th	30th	60th	90th
Control ghee	0.32^{d}	0.45^{d}	0.58^{d}	0.69^{d}	0.32^{d}	1.35^{d}	1.74^{d}	2.07^{d}
Ghee with PO(5%)	0.68^{c}	0.74^{c}	0.83^{c}	0.92^{c}	0.68^{c}	2.22^{c}	2.49^{c}	2.76^{c}
Ghee with PO(10%)	1.00^{b}	1.20^{b}	1.38^{b}	1.53^{b}	1.00^{b}	3.60^{b}	4.14^{b}	4.59^{b}
Ghee with PO(20%)	1.70^{a}	1.90^{a}	2.05^{a}	2.15^{a}	1.70^{a}	5.70^{a}	6.15^{a}	6.45^{a}
CD (*P≤0.05*)	0.09	0.01	0.01	0.01	0.09	0.52	0.47	0.55
L20 (Control)	0.34^{d}	0.70^{d}	0.78^{d}	0.89^{b}	0.34^{d}	2.10^{d}	2.34^{c}	2.67^{c}
L20 with PO(5%)	0.70^{c}	0.82^{c}	0.89^{c}	0.96^{b}	0.70^{c}	2.46^{c}	2.67^{c}	2.88^{c}
L20 with PO(10%)	1.20^{b}	1.50^{b}	1.66^{b}	1.75^{a}	1.20^{b}	4.50^{b}	4.98^{b}	5.25^{b}
L20 with PO(20%)	1.90^{a}	2.04^{a}	2.27^{a}	2.34^{a}	1.90^{a}	6.12^{a}	6.81^{a}	7.02^{a}
CD (*P≤0.05*)	0.01	0.01	0.01	0.74	0.01	0.01	0.76	0.58
S20 (Control)	0.33^{d}	0.55^{d}	0.70^{d}	0.77^{a}	0.33^{d}	1.65^{d}	2.10^{d}	2.31^{c}
S20 with PO (5%)	0.69^{c}	0.78^{c}	0.86^{c}	0.95^{a}	0.69^{c}	2.34^{c}	2.58^{c}	2.85^{c}
S20 with PO (10%)	1.10^{b}	1.34^{b}	1.44^{b}	1.59^{a}	1.10^{b}	4.02^{b}	4.32^{b}	4.77^{b}
S20 with PO (20%)	1.80^{a}	1.95^{a}	2.10^{a}	2.19^{a}	1.80^{a}	5.85^{a}	6.30^{a}	6.57^{a}
CD (P≤0.05)	0.33^{d}	0.01	0.01	0.65	0.09	0.50	0.38	0.68

CD-Critical difference
All the values are average of three trials
Different superscripts within the column are compared with other value

Peroxide value in ghee was decreased with increasing levels of palm oil (5, 10, 20 % respectively). It confirms with work carried out by Niranjan, (2017) that the control ghee sample had a peroxide value of 1.4 mM O_2/Kg of fat on 15th day and then it raised drastically to a peroxide value of 6 mM O_2/Kg of fat on 25th day of storage. Similar results were also found by Archana, (2019) report that ghee stored at 29 °C for 0 day to 60 days had a peroxide value of 0 to 1.50 mM O_2/Kg. This is probably due to the unsaturated fatty acids in fat are oxidised, and the principal oxidation products are hydroperoxides. Though peroxides are not responsible for the development of off-flavours in and of themselves, their measurement provides a good indication of the degree of auto-oxidation. Several elements that determine the quality of ghee have been reported to alter the rate of auto-oxidation. Ghee prepared using the desi method developed peroxides more quickly than creamery ghee (Lalitha and Dastur, 1983). When ghee was clarified at a higher temperature (120 °C), it produced peroxide at a much slower rate than when it were clarified at a lower temperature (Narayanan et al. 1996). Other elements that affect the keeping quality of ghee include storage temperature, antioxidants, metal contamination, dissolved oxygen, and light exposure. It confirms with work carried out by Niranjan, (2017) The desi ghee samples exhibited a peroxide value ranging from 1.4 to 1.7 mM.O_2 /kg fat on 15th day of storage at 60ºC,while the value at the end of the 25th day was ranged from 6.25 to 7.6 mM O_2 /kg fat at 60 ºC. Similar results were also found by Archana, (2019) and report that ghee stored at 60 °C for 0 to 45 days had a peroxide value of 0 to 10.45 mM O_2/Kg. The result obtained are comparable with those of Narayanrao, (2007), who analyzed the ghee, S_{20} (Solid fraction at 20 ºC) and L_{20} (Liquid fraction at 20 ºC) sample contain 0 mM O_2/Kg at 0 day. As the storage day increased the peroxide value increase more in liquid fraction than solid fraction which containing more peroxide value than control sample. At 7 day of storage the ghee, S_{20} (Solid fraction at 20 ºC) and L_{20} (Liquid fraction at 20 ºC) sample contain 0.153, 1.4145 and 2.344 mM O_2/Kg fat.

The FFA value in ghee was increased with increasing levels of palm oil (5, 10 and 20 %) as similarly reported by Archana, (2019), who report that ghee stored at 29 °C for 0 to 60 day had FFA value of 0.33 to 0.60 (% OA). Niranjan, (2017), also reported that FFA content were ranged between 0.27 to 0.72 % OA in the market ghee samples against the value of 0.60 % OA recorded for control ghee sample at 0th day of storage. The rate of increase in FFA was very slow and gradual at the end of the 25th day of storage. The FFA content of control ghee was 0.80 % OA while the market samples were ranged between 0.57 to 0.96 % OA. The FFA content were ranged between 0.40 to 0.93 % OA in the desi ghee sample against the value of 0.60 % OA recorded for control ghee sample in the 0th day of storage. The rate of increase in FFA were very slow and gradual, at the end of the 25th day of storage the FFA content of control ghee were 0.80 % OA while the desi ghee samples ranged between 0.72 to 1.28 % OA for desi ghee Niranjan, (2017). Similar result were also found by Archana, (2019) and analysed that ghee stored at 29°C for 60 day had FFA content of 0.33 to 0.60 (% OA). Archana, (2019) also reported that ghee stored at 60 °C for 45 days had FFA content ranges from 0.42 to 0.61 (% OA). The shorter-chain homologues are principally responsible for the rancid flavour represented by butyric acid; FFA is undesirable in milk fat products (Munro et al. 1992). No work has been done on fractionated ghee in terms of the content of FFA.

Conclusion

It may be concluded from the present study, that as the palm oil level increased the antioxidant activity in the blended samples increased, this could be due to presence of higher levels of carotenoids and vitamins E in palm oil which act synergistically as powerful natural antioxidants and have also contributed towards stability against auto oxidation (peroxide value). The liquid fraction (L_{20} with palm oil 20%) has a higher antioxidant activity. Finally, the adoption of fractionation technique (especially liquid fraction) for the detection of adulteration with palm oil could be an effective method even at 5% level of adulteration. Still there is a scope for the detection at lesser concentration especially with DPPH method.

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RESEARCH ARTICLE

Storage related changes in short-set cream cheese manufactured using thermophilic starter culture and direct acidification technique

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Abstract: Cream cheese is a fresh acid curd variety with semi-soft body, manufactured by the gradual, quiescent acid gelation of milk. The typical flavour profile of cream cheese is cultured diacetyl with little bit lactic acid flavour as well as aroma. It is envisaged that demand of cream cheese will increase in upcoming years due to its application as spread and it having higher nutritional value compared to similar products available in the market. The purpose of the study was to observe changes in sensory, physicochemical and microbial quality during storage in short-set cream cheese samples manufactured by using thermophilic starter culture and direct acidification technique and was compared with control cream cheese manufactured using mesophilic starter culture. All the three samples i.e., control cream cheese made using mesophilic culture (MC), cream cheese made using thermophilic culture (TC) and cream cheese made using laboratory grade lactic acid (SLA) were stored in re-closable polypropylene tubs at 7 ± 2°C. During storage study, flavour score, FFA, tyrosine value were significantly affected by storage period (P), treatment (T) and the interaction of T × P. Moisture, pH, titratable acidity, sensory parameters (except flavour) were significantly affected only by storage period (P) and treatment (T). All the three samples had storage stability up to 15 days at 7 ± 2°C.

Keywords: cream cheese, short-set, thermophilic culture, mesophilic culture, direct acidification

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Introduction

Generally, fermented dairy products have been contemplated as essential foods because they provide good nutrition and immunity boosting effects to consumers. Cheese belongs to first and most popular manufactured food products. Cream cheese is a soft, mild, rich, unripened cheese and is a creamy white, slightly acidic product with a diacetyl flavor (Hirpara *et al*, 2016b). As per FSSAI (2020), cream cheese (Rahmfrischkase) is defined as a soft, unripened cheese made by coagulating pasteurized cow and/or buffalo milk, or mixtures thereof, and pasteurized cream with cultures of harmless lactic acid-producing bacteria with or without the addition of suitable coagulating enzymes. It should have not more than 55 % moisture and not less than 70 % fat on dry matter basis. It is usually manufactured by the coagulation of cream or mixture of milk and cream by acidification with starter culture (Krishna and Ghosh, 2019). Cream cheese had a more acidic flavour and contained less saturated aldehyde and ketone compounds, such as, hexanal and 2-nonanone (Pettersen et al. 2005). It is used widely as a spread to replace butter which contain ~80 percent fat. The high fat level not only increases its cost but also makes it unsuitable for those who are fat conscious. In cream cheese, the presence of non-fat solids makes it nutritionally more balanced (Hirpara *et al*, 2016a). In upcoming years, it is envisaged that the demand for cream cheese would continuously increase due to its application as value -added ingredient in various products, like spread in bread, a major ingredient in Cheese cake, etc. due to its superior nutritive value (especially protein). Though Cream cheese belongs to unripened type of cheese, around 24 to 48 h are required after manufacturing to get Cream cheese with proper fat crystallization and product structuring (FAO/WHO, 2007). Short-set cream was manufactured using thermophilic lactic culture as well as by direct-acidification technique that reduced setting time to a greater extent. Cold pack cream cheese had a shelf-life up to 3 weeks while in case of hot pack Cream cheese, the product remained acceptable till about 3 months (Kosikowski and Mistry, 1999; Lucey, 2003). One of the main issues with cream cheese that reduces its shelf life is microbial deterioration. When kept over an extended period of time, Cream cheese is more prone to growth of bacteria or molds that are heat resistant. Most spoilage microorganisms can be destroyed in cream cheese using

the processing periods and temperatures employed in its production. Therefore, it is crucial to prevent the recontamination of cream cheese. However, specific aims of this current study were: (i) to assess shelf life of optimized Cream cheese; (ii) to observe changes in the physico-chemical parameters (such as, moisture, pH, titratable acidity, free fatty acids, tyrosine value) and microbiological parameters (such as, aerobic plate count, coliform count and yeast and mold count) in the optimized Cream cheese samples during their storage at 7 ± 2°C.

Materials and methods

The methodology and formulation for production of Cream cheese involved different stages for the process optimization based on cold pack method as reported by (Krishna and Ghosh, 2019) with some modifications.

Materials

The whole milk and Cream used for manufacturing of short-set cream cheese was procured from Anubhav Dairy, Department of Dairy Processing and Operations (DDPO), Kamdhenu University, Anand. DVS culture used were Delvo Tec DX 33B DSL (mixed culture strain of *Lactococcus lactis* spp. *lactis*, *Lactococcus lactis* spp. *cremoris*, *Lactococcus lactis* spp. *lactis biovar diacetylactis* and *Leuconostoc* spp.) and Delvo fresh – DH 1040 DSL (*Streptococcus thermophilus*) supplied by DSM Nutritional Products India Pvt. Ltd. Laboratory grade lactic acid AR, supplied by Molychem, Mumbai, India, while Rennet (Maxiren®1800 Granulate) and Diacetyl (as a flavoring compound) was procured from DSM Nutritional Products India Pvt. Ltd. Iodized salt manufactured by M/S. Tata Chemicals Ltd., Mumbai was purchased from a local market in Anand.

Manufacture of Cream cheese

Milk was standardized to 12 % fat by addition of cream, homogenized at 65°C, followed by pasteurization at a temperature of 80°C for 30 sec. Then, pasteurized milk was divided into three parts for three treatments. For manufacture of control sample, one part of pasteurized milk was cooled down to 22°C, followed by addition of DVS mesophilic culture at a rate of 1.0U/1000 kg per kg of milk, incubated at 22°C for 16 to 18 h for curd formation. For experimental sample one, second part of milk was cooled down to 45°C, followed by addition of DVS thermophilic culture at a rate of 1.0U/1000 kg of milk. Then, the milk was incubated at 45°C for 3.5 to 4.0 h for curd formation. For second experimental sample, the third part of milk was cooled down to below 10°C, followed by addition of 20 % lactic acid to increase acidity to 0.5 % of lactic acid, heated to 25°C, added with rennet at a rate of 2.0g per 100 kg of milk. Then, milk was heated to 31°C and held at such temperature for 1 h for setting of milk. For all the three experimental samples, after the curd formation, curd was stirred, followed by washing the curd using hot water at a temperature of 76°C and then the curd was hung for 2 h to allow removal of whey. After that, salting and smoothening were done in all the three samples. The cream cheese curd formed using mesophilic culture, thermophilic culture and laboratory grade lactic acid were denoted as MC, TC and SLA samples, respectively. The short-set technique led to 55 per cent of time reduction in case of Cream cheese prepared by thermophilic culture and 70 per cent for that of Cream cheese prepared by direct acidification method. In case of TC and SLA, diacetyl was added at a rate of 0.02 and 0.03 %. All three samples were packaged in reclosable polypropylene (PP) cups, stored in refrigerator at a temperature of 7±2°C for assessment of shelf-life.

Analysis of samples during storage

The optimized short-set cream cheese samples as well the control cream cheese sample were subjected to sensory evaluation, physico-chemical analysis and microbiological quality analysis during storage at 7±2°C at every 3 days interval till the products remained acceptable.

Sensory evaluation of Cream cheese during storage study

The sensory evaluation of Cream cheese samples was done by panel of 12 judges (on the basis of duo-trio test) using 100-point scale during storage study. Details of sensory score card were referred from Singh and Tewari (1990).

Physico-chemical attributes

Moisture content of the product was determined by the gravimetric method (AOAC, 1995). Titratable acidity and pH of the cheese samples were measured by the method recommended in the manual of Association of Official Agricultural Chemists (AOAC, 1980). The pH readings were taken on a digital pH meter (CH-8603, M/s. Mettler Toledo AG, Schwerzenbach). Proteolytic activity expressed in terms of tyrosine value of Cream cheese was estimated by the method given by Arnott et al. (1957). The FFA content of the cheese samples was measured as per the method described by Deeth and Fitz-Gerald (1976) using BDI reagent.

Microbiological analysis

All the three experimental cream cheese samples were analyzed for the Aerobic Plate Count (APC) using standard plate count agar, Coliform count and Yeast and Mold count (YMC) by the methods as described in IS: 5550 (2005). The microbiological counts were expressed as log cfu/g for APC, while it was expressed as cfu/g for coliform count and YMC (FSSAI, 2012).

Statistical analysis

During storage study, the information in the form data (from sensory, physico-chemical and microbial parameters) of Cream cheese samples were examined using Factorial Completely

Randomized Design (Steel and Torrie, 1980). The results were analyzed using Analysis of variance. All tests were checked at 5 % level of significance.

Results and discussion

Changes in Sensory Parameters of Cream Cheese during Storage at 7 ± 2°C

During storage, Cream cheese goes through a several physico-chemical, biochemical and microbial changes such as alterations of lipolytic activity, proteolysis, acidity, pH, aerobic plate count, yeast and mold count, coliform count, etc. Ultimately, changing of these properties affects sensory parameters of Cream cheese. Therefore, accurate judgement of the organoleptic quality of Cream cheese helps to identify defects and make corrections to them. By this way, quality of Cream cheese can be improved as well as shelf-life also can be extended before launching of the product in the commercial market.

All the Cream cheese samples including control (MC) and the experimental samples (TC and SLA) were taken for sensory evaluation at every 3 days' interval. The sensory parameters studied were colour and appearance, flavour, body and texture, spreadability and total score based on 100-point scale as delineated in Table 1. After 18th day, there was drastic deterioration of sensory quality and the flavour scores obtained were below 60 % of the maximum score in all the experimental samples. Therefore, the samples of Cream cheese were analyzed till 18th day of storage at 7±2°C. As indicated in Table 1, colour and appearance score was significantly (P<0.05) reduced starting from Day 0 to the 18th day of storage. There was slight reduction in glossiness of the samples, resulting in reduction in colour and appearance scores during storage.

The extent of decrease in the colour and appearance scores denoted that Cream cheese sample made using thermophilic culture (TC) was comparatively more stable to changes in colour and appearance during storage (from 0 to 18 days) than control (MC) and SLA sample. Thus, indicating the role of starter culture in retaining better appearance of the product throughout the storage period. Such observed effect being more in cream cheese by thermophilic starter culture compared to mesophilic starter.

In the same line, Katsiari et al. (2009) observed that colour and appearance scores of Galotyri-type cheeses made from four different starter culture viz. two mesophilic (MA011 and Probat 222), one thermophilic (CH-1) and one mixed mesophilic/ thermophilic (CHOOZIT MT 1) was decreased during storage.

During storage, the mean flavour score of cream cheese using thermophilic starter was highest followed by control (MC) and SLA sample (Table 1). The flavour scores of the Cream cheese showed a progressive decline during the entire storage period. As can be observed in Table 1, the control Cream cheese sample (MC) was less stable to changes in flavour during storage (from 0 to 18 days) compared to TC and SLA samples. The extensive reduction in flavour scores for the MC sample can be attributed to comparatively higher moisture retention in the product accelerating the growth of micro-organisms during storage.

The average flavour scores remained acceptable (above 60 % of the maximum score) till 15th day of storage for all the products at 7±2°C. However, for the Cream cheese sample made by direct acidification technique (SLA), even though the product remained acceptable, there was prevalence of bitter aftertaste after 9th day of storage that drastically reduced its acceptability. The interaction effect (TxP) was also found to be significant, indicating both treatment as well as storage period had significant (P<0.05) effect on flavour scores of the cream cheese samples.

Similar observations have been reported by Katsiari et al. (2009) for deterioration of flavour scores of Galotyri-type cheeses made from four different starter culture viz. two mesophilic (MA011 and Probat 222), one thermophilic (CH-1) and one mixed mesophilic/thermophilic (CHOOZIT MT 1), during their 15 days storage at 4±1°C.

The changes in the body and texture scores of Cream cheese were affected significantly (P<0.05) by treatment as well as storage period. The interaction T × P remained unaffected statistically, as shown in Table 1. A linear decrease in body and texture scores of Cream cheese samples including control (MC) was observed during 18 days of storage at 7±2°C.

The values denoted that the body and texture of Cream cheese samples made using thermophilic starter culture (TC) deteriorated faster during storage (from 0 to 18 days) compared to control (MC) and SLA sample.

Katsiari et al. (2009) observed that body and texture score of Galotyri-type cheeses made from four different starter culture viz. two mesophilic (MA011 and Probat 222), one thermophilic (CH-1) and one mixed mesophilic/thermophilic (CHOOZIT MT 1) was decreased during their storage at 4±1°C.

As shown in Table 1, the spreadability scores of all the three Cream cheese samples decreased during 18 days of storage at 7±2°C. The Cream cheese sample made by direct acidification technique (SLA) was less stable to spreadability changes during storage compared to control (MC) and TC sample, the sample TC being the most stable.

The changes in the total sensory scores of Cream cheese were affected significantly (P<0.05) by treatment as well as storage period. The interaction T × P remained unaffected statistically, as can be observed in Table 1. The data indicated a significant linear decrease in total sensory scores for all the three products with increase in storage period from 0 to 18 days.

Table 1 reveals that the Cream cheese sample made by direct acidification technique (SLA) was significantly less stable to deteriorations, indicated by the sensory scores, during storage (from 0 to 18 days) compared to control (MC) and TC sample. The bitter aftertaste in the product after 9th day of storage resulted in poor sensory scores for the product. This bitterness could be due to formation of bitter peptides due to uncontrolled breakdown of proteins by residual renned during storage of the product. The hydrolysis of β-casein by rennet is the primary source of cheese bitter peptides (Meng et al. 2021).

Chemical changes in Cream Cheese during Storage at 7 ± 2°C

The changes in moisture content as well as physico-chemical changes during storage at 7±2°C of MC (Control), TC and SLA samples are reported in Table 2 to 6.

Moisture content in Cream cheese is very important parameter to determine extent of changes in sensory properties and growth of microorganisms during storage at 7 ± 2°C. As shown in Table 2, there was continuous decrease in percentage of moisture content of all samples i.e., control Cream cheese (MC), Cream cheese

Table 1 Changes in sensory scores of cream cheese made using mesophilic culture (MC) thermophilic culture (TC) and laboratory grade lactic acid (SLA) during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
				Colour and appearance Score* (Out of 15)				
MC	13.42	13.03	12.63	12.27	11.89	10.93	10.65	12.12[a]
TC	13.38	13.11	12.64	12.11	11.85	11.33	10.90	12.19[a]
SLA	12.52	12.27	11.99	11.52	10.94	10.49	10.05	11.40[b]
Period (P) mean	13.11[a]	12.81[b]	12.42[c]	11.97[d]	11.56[e]	10.92[f]	10.53[g]	
		T			P			T × P
CD (0.05)		0.15			0.23			NS
				Flavour Score* (Out of 50)				
MC	41.90	41.24	39.36	38.01	35.99	31.15	25.53	36.17[b]
TC	45.63	43.68	41.94	40.20	37.91	34.66	28.83	38.98[a]
SLA	39.20	38.08	35.43	30.88	27.73	26.52	23.75	31.66[c]
Period (P) mean	42.24[a]	40.99[b]	38.91[c]	36.36[d]	33.88[e]	30.78[f]	26.04[g]	
		T			P			T × P
CD (0.05)		0.45			0.69			1.19
				Body and texture score* (out of 20)				
MC	16.12	15.90	15.61	15.23	14.66	13.97	13.11	14.94[b]
TC	16.81	16.49	16.18	15.92	15.52	14.65	13.59	15.59[a]
SLA	14.47	14.12	13.81	13.53	13.26	12.86	12.59	13.52[c]
Period (P) mean	15.80[a]	15.50[a]	15.20[b]	14.89[b]	14.48[b]	13.83[c]	13.10[d]	
		T			P			T × P
CD (0.05)		0.27			0.41			NS
				Spreadability Score* (out of 10)				
MC	8.18	7.98	7.65	7.19	6.85	6.54	6.32	7.24[b]
TC	8.30	8.12	7.85	7.54	7.29	7.10	6.63	7.55[a]
SLA	7.49	7.29	7.13	6.93	6.66	6.28	5.71	6.78[c]
Period (P) mean	7.99[a]	7.80[b]	7.54[c]	7.22[d]	6.93[e]	6.64[f]	6.22[g]	
		T			P			T × P
CD (0.05)		0.11			0.15			NS
				Total Score*[#] (Out of 100)				
MC	84.69	83.16	80.24	73.61	74.38	67.59	60.60	74.90[b]
TC	89.11	86.40	83.61	80.78	77.56	72.72	64.96	79.31[a]
SLA	78.68	76.77	73.36	67.87	63.59	61.15	57.10	68.36[c]
Period (P) mean	84.16[a]	82.11[b]	79.07[c]	74.09[d]	71.85[e]	67.16[f]	60.89[g]	
		T			P			T × P
CD (0.05)		1.12			1.72			NS

*All values are average of three replications; # Package score of 5 was added to the total score

made with thermophilic starter culture (TC) and Cream cheese made by direct acidification technique (SLA) during the entire storage period of 18 days at 7±2°C. There was significant (P<0.05) loss of moisture in all Cream cheese samples affected by treatment (T) and storage period (P). The interaction T x P remained unaffected.

It was observed that Cream cheese made by direct acidification technique (SLA) was significantly more stable to changes in moisture content during storage period (from 0 to 18 days) compared to control (MC) and TC sample. The presence of starter bacteria, causing changes in protein structure, in control (MC) and TC samples might have caused higher loss of moisture during storage. Thus, higher moisture loss was observed in control Cream cheese sample (MC) followed closely by TC sample.

Similar trend has been observed in the study made by Perveen et al. (2011), they observed that moisture content in Cream cheese was decreased significantly (P<0.05) during storage at 4±1°C for 28 days. Pappa et al. (2022) also observed that moisture content decreased during storage of soft cheese prepared from goat milk with two different starter culture one being mixture of thermophilic (C-1) and mesophilic culture and another one was mesophilic (C-2).

Both pH and titratable acidity in Cream cheese are very important parameter to determine extent of changes in flavour profile and growth of microorganisms during storage. The changes in titratable acidity and pH in all three samples (MC, TC and SLA) are depicted in Table 3 and Table 4, respectively. The titratable acidity of all the samples i.e., control Cream cheese made using mesophilic starter culture (MC), Cream cheese made using thermophilic culture (TC) and Cream cheese made by direct acidification method (SLA) increased concomitantly and pH decreased during the storage period of 18 days at 7 ± 2°C.

Salman et al. (2022) also observed that titratable acidity increased during storage of soft cheese from cow milk with different LA bacteria viz. *Lactobacillus helveticus* (S_1), *Lactobacillus rhamnosus* (S_2) and *Streptococcus thermophilus* S_{3855} (S_3). Pappa et al. (2022) observed that the pH was decreased during storage of soft cheese prepared from goat milk with two different starter cultures viz. one being mixture of thermophilic (C-1) and mesophilic culture and other was mesophilic (C-2).

As proteolysis of cheese affects both the flavour as well as texture of cheese, tyrosine value was considered to be an important factor during the current study to determine extent of proteolysis of the Cream cheese samples during storage at 7 ± 2°C. It is very much evident from the table 5 that tyrosine values of all the samples including control Cream cheese made using mesophilic starter culture (MC), Cream cheese made using thermophilic culture (TC) and Cream cheese made by direct acidification

Table 2 Changes in moisture content of Cream cheese during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
	Moisture* (%)							
MC	54.91	54.31	53.91	53.28	52.65	52.16	51.98	53.31[a]
TC	52.77	52.43	52.09	51.73	51.39	51.14	51.02	51.80[b]
SLA	51.09	50.69	50.46	50.15	49.84	49.62	49.40	50.18[c]
Period (P) mean	52.92[a]	52.48[b]	52.15[c]	51.72[d]	51.29[e]	50.97[f]	50.08[g]	
	T			P			T × P	
CD (0.05)	0.29			0.30			NS	

*All values are average of three replications

Table 3 Changes in titratable acidity of Cream cheese during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
	Titratable acidity* (%LA)							
MC	0.74	0.77	0.80	0.84	0.88	0.94	0.98	0.85[b]
TC	0.78	0.81	0.85	0.88	0.91	0.95	0.99	0.88[a]
SLA	0.68	0.71	0.74	0.75	0.78	0.81	0.84	0.76[c]
Period (P) mean	0.73[a]	0.76[b]	0.79[c]	0.82[d]	0.86[c]	0.90[f]	0.94[g]	
	T			P			T × P	
CD (0.05)	0.01			0.02			NS	

*All values are average of three replications

method (SLA) increased linearly during the storage period of 18 days.

Gursoy et al. (2010) made set type yoghurt using isolated culture of village type yoghurt i.e. *Lactobacillus delbrueckii* spp. *bulgaricus* (B3) and *Streptococcus thermophilus* (W22) with higher production ability of exopolysaccharide (EPS) and stored at 4°C up to 21th day. Here, they found that tyrosine content of set type yoghurt by wild strains (i.e., sample D, containing 1.5 % of B3 and W22) was higher, followed by sample B (having 1.5 % commercial starter and B3 culture) than other samples *viz*. sample A (having 3 % commercial starter) and sample C (having 1.5 % of both commercial starter and B3 culture). Similar increasing trend for tyrosine value for Mozzarella cheese has also been reported by Monika (2012) during storage at 7°C for 35 days and by Ahmed et al. (2011) on 28th day of storage at 4°C.

The FFA content of all the samples i.e., control Cream cheese made using mesophilic starter culture (MC), Cream cheese made using thermophilic culture (TC) and Cream cheese made by direct acidification method (SLA) increased during the storage period of 18 days at 7 ± 2°C (Table 6). The FFA content of all the three samples differed significantly, depicting the role of different cultures (mesophilic and thermophilic) and lactic acid in formation

Table 4 Changes in pH of Cream cheese during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
				pH*				
MC	4.38	4.35	4.27	4.24	4.21	4.12	4.05	4.23^{b}
TC	4.31	4.25	4.16	4.11	4.05	3.97	3.87	4.10^{c}
SLA	4.61	4.55	4.49	4.46	4.43	4.41	4.39	4.48^{a}
Period (P) mean	4.43^{a}	4.38^{b}	4.31^{c}	4.27^{c}	4.23^{d}	4.17^{e}	4.10^{f}	
		T		P			T × P	
CD (0.05)		0.03		0.04			NS	

*All values are average of three replications

Table 5 Changes in tyrosine value of Cream cheese during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
				Tyrosine value* (mg of tyrosine/ 5 ml of filtrate)				
MC	0.15	0.28	0.39	0.54	0.66	0.77	0.91	0.53^{a}
TC	0.11	0.24	0.36	0.49	0.60	0.68	0.74	0.46^{b}
SLA	0.10	0.19	0.25	0.33	0.51	0.57	0.63	0.37^{c}
Period (P) mean	0.12^{a}	0.24^{b}	0.33^{c}	0.45^{d}	0.59^{e}	0.67^{f}	0.76^{g}	
		T		P			T × P	
CD (0.05)		0.017		0.025			0.044	

*All values are average of three replications

Table 6 Changes in free fatty acid content of Cream cheese during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
				FFA* (meq of KOH/ 100g of Fat)				
MC	0.75	1.13	1.84	2.87	3.74	4.65	4.98	2.85^{a}
TC	0.70	1.12	1.55	2.43	3.30	4.05	4.47	2.52^{b}
SLA	0.54	0.88	1.24	2.11	2.91	3.46	4.05	2.17^{c}
Period (P) mean	0.66^{a}	1.04^{b}	1.54^{c}	2.47^{d}	3.32^{e}	4.05^{f}	4.50^{g}	
		T		P			T × P	
CD (0.05)		0.12		0.18			0.31	

*All values are average of three replications

of free fatty acids. Since, SLA sample was not having any starter bacteria, the FFA content was lower compared to other two samples. Increase in lipolytic activity during storage, that was higher in Cream cheese made by starter cultures, increased FFA content of the products substantially.

Similar increasing trend for total free fatty acid during storage has been reported by Katsiari et al. (2009) in Galotyri-type cheeses made from four different starter culture viz. two mesophilic (MA011 and Probat 222), one thermophilic (CH-1) and one mixed mesophilic/thermophilic (CHOOZIT MT 1). Based on observations reported by Atasoy and Turkoglu (2009), Urfa cheese made without starter bacteria had significantly ($P < 0.05$) higher lipolysis activity than cheese made with mesophilic or thermophilic starter culture. But, no significant ($P > 0.05$) difference was observed among the type of cultures used for making Urfa cheese.

Microbial Changes in Cream Cheese during Storage

During storage at 7±2°C, microbial changes of MC (Control), TC and SLA samples are indicated in Table 7.

The Aerobic Plate Count (APC) in any dairy product sample would consist of added lactic acid bacteria (LAB) along with other adventitious micro-organisms. Higher the LAB count, higher would be the APC values in cheese samples. During the present investigation, the changes in APC of Cream cheese samples were evaluated at an interval of 3 days and expressed as log cfu/g that is presented in Table 7. There was continuous increase in APC of all samples i.e., control Cream cheese made using mesophilic starter culture (MC), Cream cheese made using thermophilic culture (TC) and Cream cheese made by direct acidification method (SLA) sample during the entire storage period of 18 days.

The APC of sample MC and TC were higher since they were added with starter cultures, while sample SLA had no bacterial culture added to it.
Similar observations have been reported by Debnath (2016) for the viable lactic acid bacterial count of control Cream cheese as well as low fat, inulin and phytosterol added Cream cheese with no preservative during storage till 20th day but the counts decreased thereafter, while in cheeses with preservatives, lactic acid bacterial count increased up to 25th day of refrigeration storage.

Even Perveen et al. (2011) observed progressive increase in viable count of lactic acid bacteria in Cream cheese during storage, the count being higher at 21±1°C as compared to 4±1°C.

As per FSSAI (2020) the coliform count of Cream cheese shall not be more than 500 cfu/ g, whereas yeasts and molds count of Cream cheese shall not be more than 250 cfu/g.

The changes in yeast and mold count in all three samples (MC, TC and SLA) are delineated in Table 7. There was a continuous increase in yeast and mold count of all samples i.e., control Cream cheese made using mesophilic starter culture (MC), Cream cheese made using thermophilic culture (TC) and Cream cheese made by

Table 7 Changes in microbiological quality of Cream cheese made using mesophilic culture (MC thermophilic culture (TC) and laboratory grade lactic acid (SLA) during storage at 7±2°C

Cheese sample	Days							Treatment (T) mean
	0	3	6	9	12	15	18	
	Aerobic plate count* (log cfu/g)							
MC	6.12	6.23	6.32	6.42	6.55	6.78	6.96	6.49^{a}
TC	6.11	6.18	6.26	6.35	6.44	6.68	6.76	6.40^{b}
SLA	2.40	2.49	2.59	2.65	2.77	2.93	3.09	2.70^{c}
Period (P) mean	4.87^{a}	4.97^{b}	5.06^{b}	5.14^{c}	5.25^{c}	5.46^{d}	5.60^{e}	
		T		P		T × P		
CD (0.05)		2.34		0.12		NS		
	Yeast and Mold count* (cfu/g)							
MC	0.0	5.67	8.67	15.67	23.67	35.67	49.67	20.00^{a}
TC	0.0	3.33	7.67	13.67	23.67	34.67	46.67	18.52^{b}
SLA	0.0	2.67	6.0	11.67	19.67	29.67	40.67	15.76^{c}
Period (P) mean	0.0	3.89^{a}	7.45^{b}	13.67^{c}	22.34^{d}	33.34^{e}	45.67^{f}	
		T		P		T × P		
CD (0.05)		0.33		0.51		0.88		

*All values are average of three replications

direct acidification method (SLA) sample during the storage period of 18 days.

However, the counts remained well below the acceptable limits for yeast and mold counts as per FSSA regulations for Cream cheese even after 18 days of storage period. No visible mold growth was observed in the products at the end of storage period. Coliforms were absent in 1.0 g Cream cheese samples stored at 7±2°C during the entire storage period. This indicates good, hygienic manufacturing practices and purity of cultures used. Makhal et al. (2015) made Cottage cheese using microGRAD by direct acidification technique to improve shelf life of Cottage cheese. Here, yeast and mold count in the fresh control samples were 0 that increased to 0.60 log cfu/g on 28th day of storage.

Pappa et al. (2022) observed that yeast and mold count were found at end of storage (60 days at 4±1°C) for soft cheese prepared from goat milk with two different starter culture viz. one with mixture of thermophilic (C-1) and mesophilic culture and another one with only mesophilic (C-2).

Conclusion

Acceptable quality Cream cheese can be obtained by short-set technique by using thermophilic starter (TC) and by direct acidification (SLA) with addition of pure diacetyl flavour. The standardized products along with control (MC, made by using mesophilic culture) remained stable till 15 days of storage at 7±2°C temperature when packaged in re-closable polypropylene (PP) cups. However, there remains further scope for improving the flavour as well as body and texture profile of Cream cheese prepared by direct acidification technique. Also, further improvement in shelf-life of the cream cheese samples can be obtained by technological interventions including thermal treatment and natural preservative addition.

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RESEARCH ARTICLE

Optimization of ingredients and processing parameters for the development of Cheddar cheese based beverage

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Abstract: The present study was carried out to develop Cheddar cheese based beverage by optimizing the level of ingredients and the processing parameters to meet the increasing consumer demand for functional foods. Cheddar cheese (5-6 months ripened) was used for the preparation. The effect of varying the Cheddar cheese to water ratios (1:1, 1:2 and 1:3) along with different type of emulsifying salts, TSC (tri-sodium citrate) and STPP (sodium tri-polyphosphate) and processing parameters like cooking time (4 & 6 min) and stages of water addition (2 & 3 times) on the moisture, viscosity and sensory properties of the developed beverage were studied. At cheese to water ratio of 1:3 fat separation was found to occur even after using tri-sodium citrate and sodium tri-polyphosphate as emulsifiers at all processing conditions. Cheese to water ratio of 1:1 resulted in highly viscous product. At cheese to water ratio of 1:2 it was found that the type of emulsifier used, cooking time and stages of water addition had a significant ($p<0.05$) effect on the beverage properties. Acceptable quality cheese beverage was made at cheese: water of 1:2 with sodium tri-polyphosphate as an emulsifier when cooked for 4 minutes with water added at 2 stages. The resultant product showed acceptable moisture content of 71.88$\pm$ 0.17%, viscosity of 2.04 $\pm$ 0.06 Pa s at room temperature with good sensory scores and creamy mouthfeel.

Keywords: Cheddar cheese, Beverage, Emulsifying salts, Sensory, Rheology

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Introduction

Functional foods are the foods which at efficacious levels are found to result in potentially positive effects on health beyond basic nutrition (Granato et al. 2020). The market demand for such foods is increasing continuously. Cheese is one among those products and is considered superior to non-fermented dairy products in terms of nutritional attributes as the microflora present produce simple compounds like lactic acid, amino acids and free fatty acids that are easily assimilable. It is an excellent source of calcium and protein and rich in several vitamins. Cheese is a good source of bioactive peptides and conjugated linoleic acid (CLA) which provide many health benefits. Both of these components are reported to be anticarcinogenic and antihypertensive. Cheese contains friendly bacteria that have been shown to promote immune and intestinal health, in addition to their anti-inflammatory effects (Kanawjia et al. 2018). Cheddar cheese is a hard variety of natural cheese produced by acidification of milk and concentration of milk proteins followed by formation of gel by rennet (Banks, 2002). Natural cheeses are processed into different products by heating and continuous agitation with the addition of emulsifiers (Rafiq & Ghosh, 2017) . Various emulsifying salts such as citrates and phosphates have been used in the preparation of processed products from natural cheese (Kapoor et al. 2007).

The beverage market of India is witnessing an interesting transformation. Earlier the sugary beverages and carbonated drinks were popular among the consumers but these are now giving way to health drinks which can be attributed to the changing consumption pattern. Nowadays consumers demand natural and functional products. The studies are focussed on the preparation of dairy beverages based on cheese whey utilization. A number of healthy milk and whey based beverages are available in the market but no attempts have been made so far to develop cheese based beverages. Cheese based beverages have the potential to fill the gap for those consumers who are looking towards healthier options. The cheese market of India is growing @ 20-25% and seeing the current scenario there is a need to diversify the cheese market in India. One of the ways is to process the natural cheese into beverages. In this study, an attempt was made to develop Cheddar cheese based (CB)

beverage for health conscious consumers and diversify the cheese market.

Materials and methods

Materials

Cheddar cheese (5-6 months ripened) procured from the Experimental Dairy of ICAR- National Dairy Research Institute, Karnal was used for the manufacture of cheese beverage (CB). Common salt was procured from the local market. TSC (Tri-sodium citrate) and STPP (Sodium tri-polyphosphate) were used as emulsifying salts for the preparation of CB.

Method of Manufacture of CB

The cheese beverage was prepared using mid ripened variety (5 to 6 months ripened) of Cheddar cheese. Cheddar cheese was cleaned and grated using the grating machine. Grated Cheddar cheese was taken into cooking vessel and mixed with other ingredients. Different cheese and water (1:1, 1:2, 1:3) ratios were used. The common salt (0.5%) and TSC/ STPP (3%) (ES) were dissolved in calculated amount of hot water and added into the cheese initially during mixing. It was then continuously agitated in order to produce a homogenous mass. The remaining water was added once or twice during cooking and heated to 85 ± 2°C for 4 or 6 minutes with continuous stirring and the surface was scraped off with a steel ladle. Heating was then stopped and the product was transferred to clean & sterile glass bottles, cooled for 2-3 hours at room temperature and later kept in the refrigerator.

Physico-chemical analysis

The pH measurement of the raw material and CB was performed as described by Kevany et al. (2015) with slight modification. Acidity was measured by titration method and moisture content was determined by gravimetric method (IS, SP: 18 Part XI, 1981). Fat, protein and ash content were calculated as per the method AOAC (2005).

Rheological analysis

Rheological measurements were done using Modular compact Rheometer (MCR Model 52, Anton Paar, Austria) with a parallel plate attachment of 50 mm diameter and gap size of 1 mm. A total of 100 shear-rate/ shear-stress data points were noted for each sample at an increasing shear rate range of 1-100 $s^{-1}$with mean point duration of 2 s (Bonfim et al. 2019).

Sensory evaluation

Sensory evaluation was conducted according to the methodology described by Balthazar (2018) with slight modification on a score card of 9 point hedonic scale using 8 semi-trained panellists. The cheese drinks of 10–15 ml of each were presented in polypropylene cups covered with aluminium foils. During each session of evaluation, three samples were presented at a time. These were placed in a random manner with coded numbers (1, 2 and 3). The judged parameters were: colour & appearance, mouthfeel & consistency, taste & flavour and overall acceptability.

Statistical analysis

The statistical analysis was performed using the statistical software SPSS 16.0 (Stat Soft Polska Sp. z o. o., Krakow, Poland). One way analysis of variance (ANOVA) was performed and the significant differences among all the samples were reported according to Duncan's test at $p < 0.05$.

Results and discussion

Proximate analysis of the raw material

The proximate analysis of the Cheddar cheese used as raw material for the preparation of CB was performed and the results are tabulated in Table 1. The moisture, protein, fat and ash were found to be 46.73±0.14%, 24.61± 0.07%, 20.95±0.45 and 4.56± 0.01% respectively. Fat content was found to be lower and moisture was found to be higher than other reported studies for Cheddar cheese (Sukumar De, 2001) which could be due to the difference in type of milk used, the ripening time provided during ageing of cheese, climate or season, the method and location of preparation. The pH and acidity of the cheese sample was found to be 5.47± 0.06 and 0.185± 0.72 % LA respectively.

Effect of level of ingredients and processing parameters on the moisture content

The effect of level of ingredients and processing parameters on the moisture content was evaluated and the results are depicted in Table 2. It was found that at higher cheese and water ratio of 1:3 the product got curdled in case of both the emulsifiers and showed fat separation at all processing conditions. This could be because of higher water addition which resulted in higher water phase and lower fat phase thus could not produce a homogeneous emulsion. At cheese and water ratio of 1:1 in case of both TSC and STPP addition the cooking time was found to have a significant effect ($p<0.05$) on the moisture content however

Table 1 Proximate analysis of Cheddar cheese

Attributes	Cheddar cheese
Moisture (%)	46.73± 0.14
Protein (%)	24.61 + 0.07
Ash (%)	4.56 ± 0.01
Fat (%)	20.95 ± 0.45
pH	5.47 ± 0.06
Acidity (% LA)	0.185 ± 0.72

Results are expressed as Mean ± SD; (n = 3)

the stage of water addition had no significant effect ($p>0.05$). The cooking time of 4 minutes resulted in significantly ($p<0.05$) higher moisture content as compared to cooking time of 6 minutes which could be due to the evaporation of more water in the latter case. However among all the samples at cheese and water ratio of 1:1 the maximum moisture attained was 67.36 % which resulted in thick consistency. At cheese and water ratio of 1:2 it was found that in case of TSC addition at all the processing conditions the product got curdled and resulted in fat separation. This could be due to the decreased emulsification property of tri-sodium citrate at higher water phase and lower fat phase. However in case of STPP, water when added at 2 stages and cooked for both 4 and 6 minutes resulted in good quality product with the former showing high acceptable moisture content of 71.88%. In contrast water when added at 3 stages resulted in fat separation which could be due to the altered emulsion properties due to the combined effect of the varying processing conditions and ingredients (Kapoor and Metzger, 2008).

Effect of level of ingredients and processing parameters on the viscosity

The viscosity of the samples was measured using rheometer and the results are depicted in Table 2. It was found that at higher cheese and water ratio of 1:3 the product got curdled in case of both the emulsifiers and showed fat separation at all processing conditions so the viscosity was not measured. At lower cheese and water ratio of 1:1 the product resulted in high viscosity which was not acceptable for a beverage. In general on increasing the amount of water addition the viscosity decreased. Cooking time was found to have a significant effect ($p<0.05$) on the viscosity and at cheese and water ratio of 1:2 in case of STPP, cooking time of 4 minutes resulted in significantly ($p<0.05$) lower viscosity of 2.04±0.06 Pa s compared to the viscosity at 6 minutes (9.76± 0.09 Pa s). This could also be because of the significant difference ($p<0.05$) in the moisture content of these products. At cheese and water ratio of 1:2 water addition at 2 stages resulted in acceptable quality product whereas water addition at 3 stages

Table 2 Effect of level of ingredients and processing parameters on the moisture and viscosity of CB using TSC & STPP

Emulsifying salt	Cheese: water	Cooking Time (minutes)	Stages of water addition	Moisture (%)	Viscosity (Pa s)
TSC	1:1	4	3	66.67 ± 0.20^{bc}	26.32 ± 0.07^{f}
			2	65.99 ± 0.81^{cd}	44.14 ± 0.14^{d}
		6	3	58.38 ± 0.43^{f}	31.34 ± 0.05^{e}
			2	57.50 ± 0.49^{f}	135.91 ± 0.10^{c}
	1:2	4	3	N	N
			2	N	N
		6	3	N	N
			2	N	N
	1:3	4	3	N	N
			2	N	N
		6	3	N	N
			2	N	N
STPP	1:1	4	3	66.36 ± 0.50^{bd}	5.41 ± 0.20^{h}
			2	65.63 ± 0.31^{d}	145.88 ± 0.20^{c}
		6	3	60.61 ± 1.10^{e}	190.16 ± 0.09^{b}
			2	60.51 ± 0.13^{e}	266.69 ± 0.57^{a}
	1:2	4	3	N	N
			2	71.88 ± 0.17^{a}	2.04 ± 0.06^{i}
		6	3	N	N
			2	66.56 ± 0.05^{bc}	9.76 ± 0.09^{g}
	1:3	4	3	N	N
			2	N	N
		6	3	N	N
			2	N	N

Results are expressed as Mean ± SD; means with different superscripts in a column differ significantly ($p < 0.05$) ($n = 3$) N – sample could not be measured; CB- Cheese Beverage; TSC- Tri-sodium Citrate; STPP- Sodium tri-polyphosphate added @ 3%.

Table 3 Effect of level of ingredients and processing parameters on the sensory properties of CB using TSC & STPP

Emulsifing salt	Cheese:Water	Cooking Time (min)	Stages of water addition	Colour	Taste & Flavour	Mouthfeel & consistency	Overall acceptability
TSC	1:1	4	3	6.62±0.51[a]	6.12± 0.35[a]	5.87± 0.35[bc]	5.62± 0.74[bc]
			2	6.37±0.51[a]	6.25±0.46[a]	5.75±0.46[bc]	5.37± 0.74[bc]
		6	3	6.50±0.35[a]	6.59±0.53[a]	5.75±0.46[bc]	5.62± 0.51[bc]
			2	6.75±0.46[a]	6.58±0.53[a]	6.52±0.53[b]	5.37±0.74[bc]
	1:2	4	3	N	N	N	N
			2	N	N	N	N
		6	3	N	N	N	N
			2	N	N	N	N
	1:3	4	3	N	N	N	N
			2	N	N	N	N
		6	3	N	N	N	N
			2	N	N	N	N
STPP	1:1	4	3	6.75±0.46[a]	6.50±0.75[a]	6.25± 0.88[b]	5.12± 0.64[c]
			2	6.62±0.51[a]	6.25±0.46[a]	5.75± 0.46[bc]	5.62± 0.51[bc]
		6	3	6.75±0.46[a]	6.48±1.06[a]	6.20± 0.92[b]	5.37± 0.91[bc]
			2	6.75±0.46[a]	6.25±0.46[a]	5.75± 0.46[bc]	5.62± 0.51[bc]
	1:2	4	3	N	N	N	N
			2	6.50±0.64[a]	6.50±0.53[a]	7.25 ± 1.03[a]	7.12 ± 0.83[a]
		6	3	N	N	N	N
			2	6.75±0.70[a]	6.25±0.46[a]	6.37±1.68[b]	6.00±0.92[b]
	1:3	4	3	N	N	N	N
			2	N	N	N	N
		6	3	N	N	N	N
			2	N	N	N	N

Results are expressed as Mean ± SD; means with different superscripts in a column differ significantly ($p < 0.05$) ($n = 3$) N– sample could not be measured; CB- Cheese Beverage; TSC- Tri-sodium Citrate; STPP- Sodium tri-polyphosphate added @ 3%.

resulted in fat separation which could be due to the disturbance caused to the formed emulsion. The best combination showing the lowest viscosity (2.04±0.06 Pa s) with good emulsification was observed when the STPP was used along with cheese: water (1:2) cooked for 4 min with water added two times accompanied with continuous stirring.

Effect of processing parameters and level of ingredients on the sensory properties

The effect of processing parameters and level of ingredients on the sensory properties of the product were evaluated and results are depicted in Table 3. On varying the cheese: water, emulsifying salt and the processing conditions it was found that there was no significant effect ($p>0.05$) on the colour and appearance of all the samples. All the samples appeared creamy white to white in colour. Similarly, the taste & flavour was not affected and all samples exhibited similar scores. The flavour resembled mild Cheddar cheese flavour which was liked by the sensory panellists. At cheese and water ratio of 1:1 mouth feel & consistency was similar among all samples. However, at higher cheese and water ratio of 1:2 the scores increased significantly ($p<0.05$). The product produced with STPP using cheese: water (1:2) and cooked for 4 minutes exhibited significantly higher ($p<0.05$) scores of 7.25 ± 1.03 as compared to others. The increased score could be because of the high moisture content and low viscosity of the product at these conditions which resulted in the flowy or thin consistency of the product. The product had a creamy and pleasant mouth feel which was liked by the sensory panellists. The overall acceptability score of 7.12 ± 0.83 was found to be significantly higher ($p<0.05$) in case of product with cheese and water ratio of 1:2 using STTP as emulsifier when cooked for 4 minutes compared to the other samples. Although colour and flavour were similar in all samples and exhibited no significant effect ($p>0.05$) on the overall acceptability the increased acceptability score could be due to the increased mouth feel and consistency score.

Conclusion

In the current study it was found that acceptable quality cheese beverage can be developed from Cheddar cheese aged between 5 to 6 months with addition of water in the ratio 1:2 using sodium tri-polyphosphate as an emulsifier and cooking time of 4 minutes at 85 ± 2°C. The developed beverage may be a better and healthier choice for health conscious consumers who are looking for alternate options for the existing sugary beverages in the market.

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RESEARCH ARTICLE

Development of functional golden milk by incorporating *Curcuma amada* and stevia by Response Surface Methodology

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Abstract: The objective of this study was to develop and optimize golden milk using the incorporation of *Curcuma amada* and stevia into the milk and preserve it using pasteurization. The analysis was carried out by incorporating *Curcuma amada* powder and stevia in the milk and optimizing using the Response surface methodology. A set of thirteen experiments were done in which optimized golden milk with the maximum desirability was produced using 3.43 percent *Curcuma amada* and 0.35 percent stevia, according to the outcomes of the variable optimization. The physicochemical and biochemical characteristics of the milk were also accessed. The antioxidant activity of Functional golden milk is 56.14and total phenolic content was found to be38.04 to 36.95 mg GAE. The product was accessed for its shelf life using a sensory score and found that it was stable for 7 days at 4°C.

Keywords: *Curcuma amada*, stevia, Response surface methodology, Storage life.

Introduction

Today consumers are choosing food products that strengthen their immune systems and encourage good health because they are becoming more and more aware of the significance of diet for human health. In this regard, functional foods are the best dietary choices since they enhance life quality by lowering illnesses linked to nutrition. (Janssen et al. 2021). As a result, the demand for health-promoting and immune-boosting functional foods has risen dramatically. The global functional food industry is anticipated to reach $280.10 billion by 2025. As a result, the main trend is to provide a wide range of food with health-promoting features at a low cost (Alongi and Anese; 2021).

Milk is the most acceptable nutrient-dense food, supplying energy and providing high-quality protein with a range of essential micronutrients (especially magnesium, calcium, phosphorus, potassium, and zinc) among other categories of foods taken regularly (Górska- Warsewicz et al. 2019). Milk-based beverages are proven to be excellent delivery systems for recently identified bioactive food components due to their rich flavor and nutritional content. (Sawale et al. 2020)

Curcuma amada (Mango ginger) is herb or spice used in folk medicine, culinary preparations such as preserves, sauce, pickles, candies, and the production of oleoresin, essential oil, and other products. Its extracts and powders have beneficial qualities like antioxidant, antibacterial, and antifungal properties, which are frequently used to improve the nutritional content and quality of foods (Narayanankutty et al. 2021). Rhizomes of the C. amada have long been used in Indian traditional medical systems such as Unani and Ayurveda to treat various disorders, including cancer, tumors, bronchitis, alexiteric, diuretic, skin diseases, asthma antipyretic, hiccough, and inflammation (Behera et al. 2007). Due to the presence of bioactive compounds such as curcumin, phenol, dimethoxy curcumin, bis-dimethoxy curcumin, terpenoids, and myrcene. C. amada has also shown therapeutic action (Umar et al. 2020). With the evidence that herbal milk is gaining popularity in the market, there are huge opportunities for valueaddition in milk by converting it into functional milk (Golden milk) by incorporating *C. amada*. However, introducing any herb into the milk comes with several technological obstacles, including physio-chemical, color, and flavor incompatibility, which can result in astringency, bitterness due to slow proteolysis, poor thermal stability, and change in the functional property.

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Stevia (*Stevia rebaudiana*) has been used to develop food products as a natural, low-calorie alternative for sugar.Due to the active ingredients in its leaves, the so-called steviol glycosides, stevia is the only plant in the Asteraceae family with sweetening capabilities.Stevia has also been proven in numerous research to offer antidiabetic, anti-obesity, anticancer, antihypertensive, antibacterial, anticaries, and antioxidant health effects.(Schiatti-Sisó et al. 2022)

Keeping in view the therapeutic benefits of *C. amada,* the present study was designed to develop and characterize milk-based functional golden milk incorporated with different percentages of *C. amada* (Mango ginger) powder. Optimization of *C. amada* (Mango ginger) powder level is done by response surface methodology (RSM) because it is a widely used empirical modeling methodology recognized as comprehensive, efficient, and concise methodology. The present study also analyzed golden milk's physicochemical, sensory, and nutritional potential. Furthermore, optimized golden milk was pasteurized to see the effect of heat on the golden milk.

Materials and methods

Milk (Fat, 4.32%, and SNF 8.86%) was collected from the Dairy Farm of Banaras Hindu University. *Curcuma amada* and stevia (Organic India) were procured from the local market of Varanasi.

Preparation of*Curcuma amada powder* (CAP)

The fresh rhizomes of *Curcuma amada* were washed thoroughly with tap water and then placed at room temperature for 2hr to drain the excess water. After which the cleaned *C. amada* rhizomes were sliced into a thin layer and dried in a tray drier (Khera instrument, India) at 45 ± 2°C until moisture content reached below 5%. The dried rhizomes were then grounded and sieved into a fine powder.

Preparation of Functional Golden Milk (FGM)

For the preparation of FGM, RSM trials were conducted to calculate the level of *Curcuma amada* powder (CAP)and stevia. Different concentrations of *Curcuma amada* and stevia were added to the preheated milk (35-40ÚC). Samples were homogenized and filtered out to remove undissolved large particles of CAP. Figure1 illustrates the flow diagram for the preparation of golden milk using CAP. The prepared golden milk was then pasteurized using the HTST method (72 °C for 15 s), cooled, glass bottled, and stored at 5°C.

Optimization of Functional Golden Milk (FGM)

Central composite design (CCD) of the response surface methodology (RSM) technique was adopted to optimize the golden milk, Preliminary trials were conducted to select the levels of independent variables viz. *Curcuma amada*, stevia. Responses viz. sensory evaluation (color and appearance, taste, mouth feel, sweetness, flavor, and overall acceptability) were used to select the levels of independent variables for optimizing the functional golden milk formulation. Design expert software version 8.0.7.1 was used for the optimization of golden milk. A total of 13 experiments were designed according to the experimental design of RSM. A second-order polynomial equation was derived based on the chosen quadratic model as followed:

$$Y = 5Ø7ß + 5Ø7ß_1 X_1 + 5Ø7ß_2 X_2 + 5Ø7ß_{12} X_1 X_2 + 5Ø7ß_{11} X_{12} + 5Ø7ß_{22} X_{22} + õ$$

Where Y is the response variable $5Ø7ß$, $5Ø7ß_1$, $5Ø7ß_2$&$5Ø7ß_{12}$ are the regression coefficient, and X_1, X_2, & X_{12} are the independent variables or the quadratic Interactive effect of chosen variables the factor, is the residual error. The error includes experimental errors and lack of fit chosen for the model. The quality of fit of the chosen model was evaluated by the coefficient of determination (R^2).

Physico-chemical analysis of the optimized functional golden milk

Proximate analysis of optimized functional golden milk

Protein content was measured using the Kjeldahl method and fat content was determined according to the Gerber method. The ash content was determined according to AOAC 923.03. Carbohydrate content was measured according to the phenol sulphuric method described by Jayathilake et al. (2022). Titratable acidity (%) of optimized functional golden milk was determined by Ranagana's (2001) method. Calibrated digital pH meter (Meter Lab, Pacific Laboratory Products; Blackburn, Vic, Melbourne, Australia) was used for pH measurements. Specific gravity and viscosity of the golden milk was measured by Brookfield viscometer (DV Next Rheometer AMETEX, Massachusetts, U.S.A) at 25°C.

Color

Color characteristics of functional golden milk were measured by colorimeter hunter lab (Color Flex EZ Spectrophotometer, Virginia, U.S.A). The following parameters were defined: L″ (lightness; black (0) to white (100)), a″ (red saturation index; +a″=red, -a″=green) and b″(yellow saturation index; +b″=yellow, -b″=blue).

Nutraceutical characteristics of Functional Golden milk

Determination of antioxidant activity

The antioxidant activity of the golden milk was determined using the DPPH inhibition technique, (Jayathilake et al. 2022)

Determination of total phenolic content

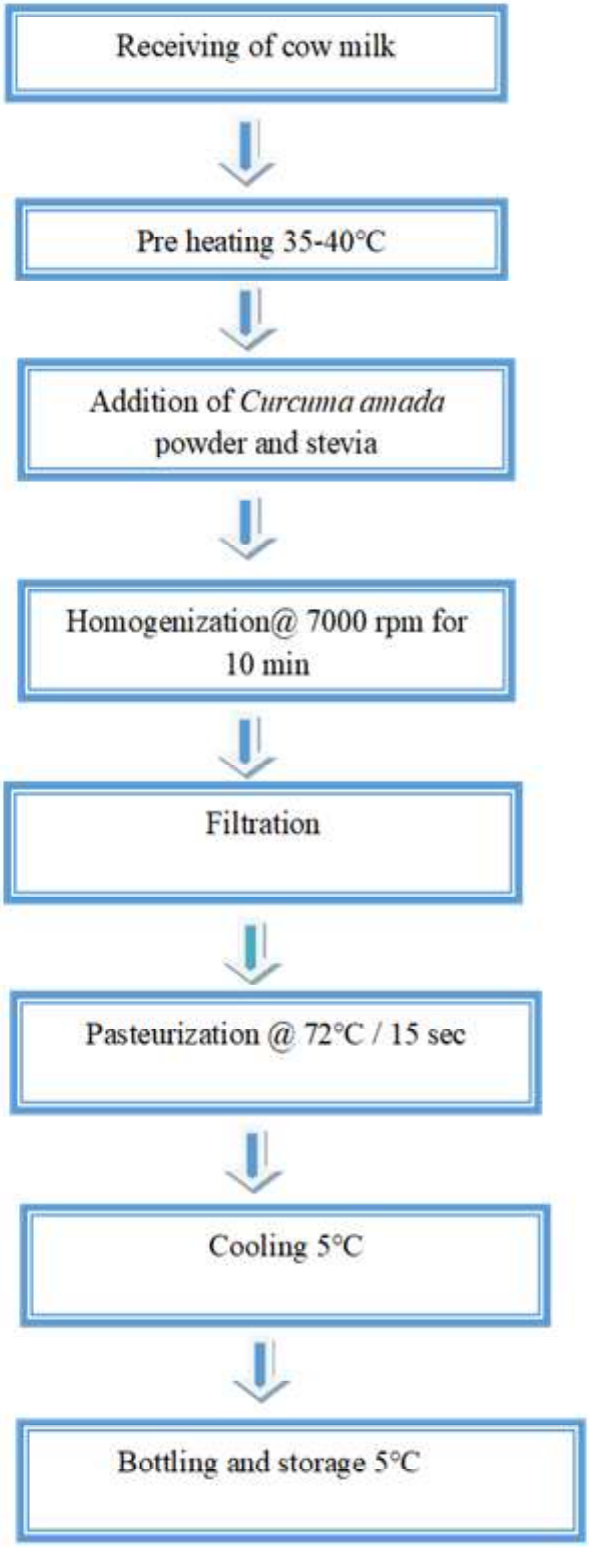

Fig 1 Flow chart of prepared Golden Milk preserved by pasteurization

The total phenolic content was determined by the Folin-Ciocalteu method with minor variations (Jayathilake et al. 2022)

Sensory evaluation

The prepared golden milk was evaluated by a panel of 10 semi-trained judges. Their response for various attributes namely color and appearance, taste, mouth feel, sweetness, flavor, and overall acceptability were recorded using 9 points hedonic scale rating ranging from 'like extremely = 9' to 'neither like nor dislike=5' to dislike extremely=1.

Results and discussion

A central composite design was employed to derive the optimum process conditions for the best composition of the functional golden milk. A set of thirteen experiments were designed to optimize the amounts of *Curcuma amada* and stevia. The experimental design and sensory score are given in Table 1.

Model fitting and interactive effects of process variables on sensory properties of Golden milk

Appearance and Color

Colour and appearance (C&A) is a common and basic sensory parameter that appeals to the consumer and influences the acceptability of any food product. Milk has yellowish color with a nice sweet flavor and no sediment while the color and appearance of flavored milk are determined by the type of flavor and color additives added to it.

The Appearance and Color of different trails of functional golden milk ranged from 5.7 to 8.8. Experiment 10 shows the lowest color, whereas experiment 8 shows the highest. In experiments 10 and 8, *Curcuma amada* and stevia levels were 0.17 percent, 0.35 percent, and 3.00 percent, 0.35 percent, respectively. The data fit the quadratic model below.

Appearance & Color = + 8.49 + 0.93 * A + 0.029 * B – 0.40 * A * B-0.79* A2 – 0.24 * B2

The coefficient of determination (R^2) was 0.9537 for the quadratic model. The adequate precision was 16.266. The "pred R- squared" is in reasonable agreement with the "adj R- squared". The model can be used to navigate the design space. Model F value of 29.48 implied that the model was significant. The response surface plot for the color and appearance as influenced by the level of *Curcuma amada* and stevia is given in Fig 2. It can be observed that with the increase in the level of *Curcuma amada,* the acceptability of color and appearance of functional golden milk increased. Unlikely Sawale et al. (2020) reported that an increased level of addition of herb (0.1 to 0.5%, *Pueraria tuberosa*) into milk resulted in decrease in color and appearance, and mouthfeel scores.

Taste

Taste plays a paramount role in the evaluation of the quality of food products. The taste ranged from 5.00 to 8.89 on a scale of one to ten. Experiment 1 yielded the lowest taste score, whereas experiment 8 yielded the highest flavor score. In experiments 1 and 8, *Curcuma amada* and stevia levels were 5.83 percent,0.35 percent, and 3.00 percent,0.35 percent, respectively. The data fit into the quadratic model below.

Taste = + 8.17 – 0.49 * A – 0.19* B + 0.13 * A * B – 1.45 * A2 – 0.71 * B2

Table1 Experimental design and sensory score of functional golden milk with different levels of *Curcuma amada* and stevia

Run	*Curcuma amada A (%)*	Stevia B (%)	Color and appearance	Taste	Mouth feel	Sweetness	Flavor	Overall acceptability
1	5.83	0.35	8.20	5.00	6.10	5.50	5.5	7.0
2	3.00	0.35	8.67	7.94	8.04	7.20	7.03	6.4
3	3.00	0.35	8.09	8.27	8.70	8.00	7.49	8.5
4	1.00	0.60	6.84	6.19	5.12	6.37	6.25	6.5
5	5.00	0.60	7.98	5.03	5.76	5.90	5.75	6.0
6	3.00	0.35	8.09	7.99	7.58	7.25	7.88	8.5
7	5.00	0.10	8.75	5.38	5.09	5.60	5.83	8.2
8	3.00	0.35	8.80	8.89	8.90	8.67	8.17	8
9	3.00	0.35	8.78	7.78	8.66	8.27	8.15	8.4
10	0.17	0.35	5.70	5.75	5.13	6.61	5.70	7.8
11	3.00	-0.00	7.99	6.96	5.45	5.15	5.25	7.0
12	1.00	0.10	6.02	7.04	5.87	5.08	6.01	8.8
13	3.00	0.70	8.12	6.74	5.11	5.05	5.95	6.8

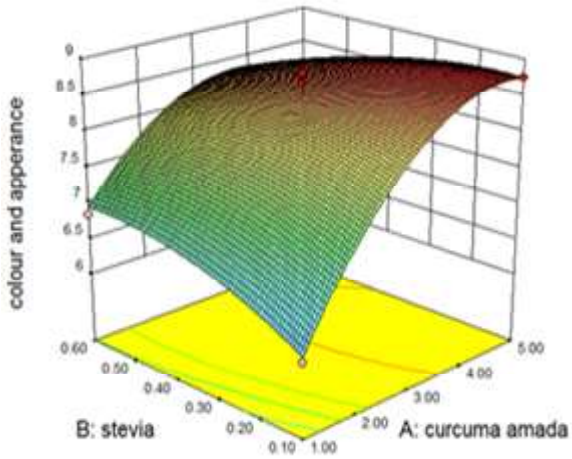

Fig 2. 3D Surface plot showing the interaction of *Curcuma amada* and stevia on the color and the appearance of functional golden milk

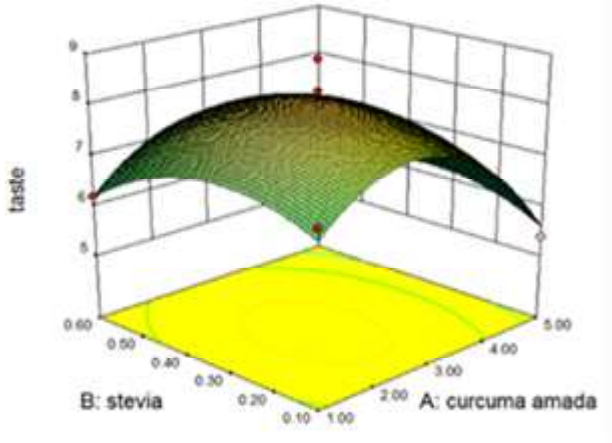

Fig 3. 3D Surface plot showing interaction of *Curcuma amada* and stevia on the taste of functional golden milk

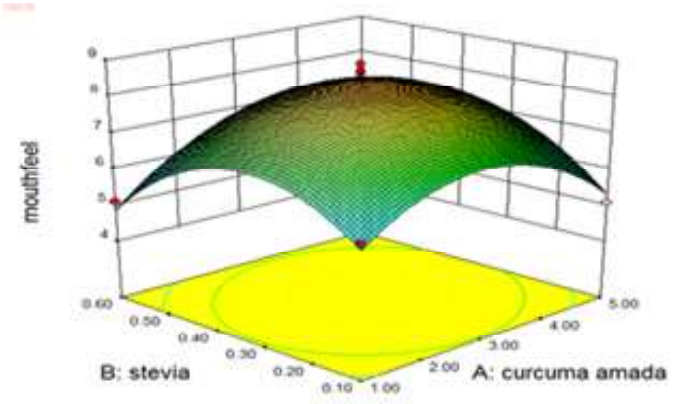

Fig 4. 3D surface plot showing interaction of *Curcuma amada* and Stevia on the mouthfeel of functional golden milk

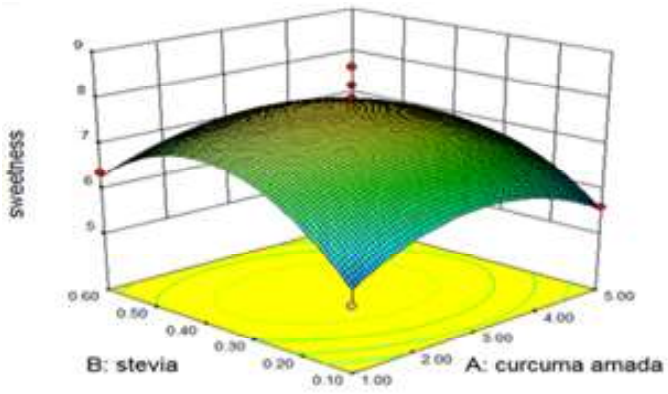

Fig 5. 3D surface plot showing the interaction of *Curcuma amada* and Stevia on the sweetness of functional golden milk

The coefficient of determination (R^2) was 0.9338 for the quadratic model. The adequate precision was 12.095. the pre-R- squared is in reasonable agreement with the Adj R-Squared. The response surface plot for the taste as influenced by the level of *Curcuma amada* and stevia is given in Fig 3. The model can be used to navigate the design space. Model F value of 19.74 implied that the model was significant.

Mouthfeel

Mouthfeel plays an important role in examining the body and texture characteristics. The pressure between the teeth and jaws determines the hardness, chewiness, and gumminess.

The mouthfeel ranged from 5.09 to 8.89 on a scale of one to ten. Experiment 7 yielded the lowest taste score, whereas experiment 8 yielded the highest flavor score. In experiments 7 and 8, *Curcuma amada* and stevia levels were 5.00 percent, 0.10 percent, and 3.00 percent, 0.35 percent, respectively. The data fit into the quadratic model below.

Mouthfeel = + 8.37 + 0.15 * A – 0.070 * B + 0.36 * A * B – 1.38 * A2 – 1.54 * B2

The coefficient of determination (R^2) was 0.9472 for the quadratic model. The adequate precision was 12.095. the pre-R- squared is in reasonable agreement with the Adj R-Squared. The model can be used to navigate the design space. Model F value of 5.09 implied that the model was significant and the lack of fit was insignificant.

The response surface plot for the mouthfeel as influenced by the level of *Curcuma amada* and stevia is given in Fig 4. It can be observed that as the level of *Curcuma amada* increased, the sensory score for mouthfeel also increased.

Sweetness

Sweetness is an attribute of perceived taste. The sweetness varied from 5.05 to 8.67. The minimum sweetness was obtained from experiment no 13 and the maximum sweetness score was

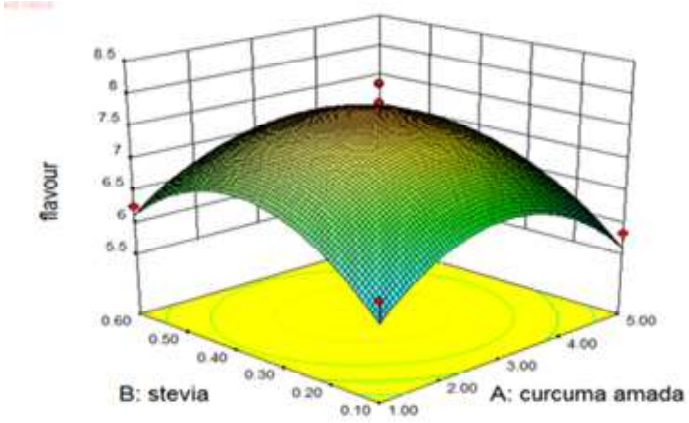

Fig 6. 3D surface plot showing interaction of *Curcuma amada* and stevia on the flavour of functional golden milk

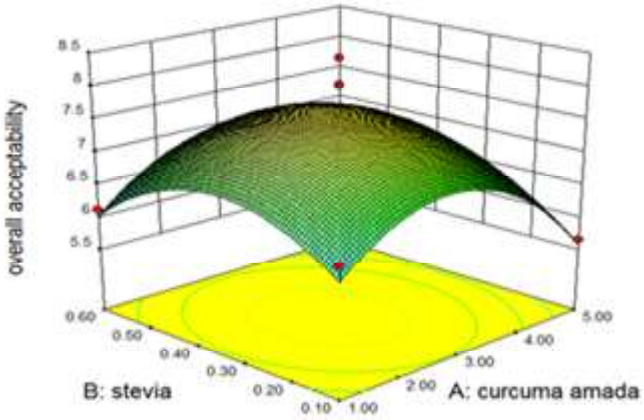

Fig 7. 3D surface plot showing the interaction of *Curcuma amada* and stevia on the overall acceptance of functional golden milk

obtained from experiment no 8. The level of *Curcuma amada* and stevia in experiments no 13 and 8 were 3.00%, 0.70%, and 3.00%,0.35% respectively. The data fitted the following quadratic model.

Sweetness = + 7.88 – 0.19 * A + 0.18 * B – 0.25 * A * B – 0.87 * A2 – 1.35 * B2

The coefficient of determination (R^2) was 0.8753 for the quadratic model. The adequate precision was 7.420. the "Pred R-Squared" is in reasonable agreement with the "Adj R- Squared". The model can be used to navigate the design space. Model F value of 9.83 implied that model was significant. The response surface plot for the sweetness as influenced by the level of *Curcuma amada*, stevia is given in Fig 5. The coefficient of estimation of *Curcuma amada* incorporated in functional milk golden showed that the level of *Curcuma amada* and stevia both have a positive effect on the score of sweetness.

Flavor

Flavor, as an attribute of foods, beverages, and seasonings, has been defined as the sum of perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts.

The flavor intensity ranged from 5.25 to 8.17. Experiment 11 yielded the lowest flavor score, whereas experiment 8 yielded the highest flavor score. In experiments 13 and 8, the levels of *Curcuma amada* and stevia were 3.00 percent, -0.00 percent, and 3.00 percent,0.35 percent, respectively. The data fit into the quadratic model below.526

Flavour = + 7.74 – 0.12 * A + 0.14 * B – 0.080 * A * B – 0.98 * A2 – 0.98* B2

The coefficient of determination (R^2) was 0.9033 for the quadratic model. The adequate precision was 7.391. The pre R^2 is in reasonable agreement with the Adj R-Squared. The model can be used to navigate the design space. Model F value of 13.07 implied that the model was significant.

The response surface plot for the flavor as influenced by the level of *Curcuma amada*, stevia is given in Fig 6. It can be observed that as level of *Curcuma amada* increased, sensory score for flavor also increased. The increase in the level of stevia also depicts increase in score of flavors remarkably.

Overall acceptability

The overall acceptance ranged from 5.25 to 8.42 on a scale of one to ten. Experiment 1 yielded the lowest overall acceptance score, whereas experiment 9 yielded the highest overall acceptability score. In experiments 13 and 8, *Curcuma amada* and stevia levels were 5.83 percent, 0.36 percent, and 3.00 percent, 0.35 percent, respectively. The data fit into the quadratic model below.

Overall acceptability= + 7.70 – 0.25 * A – 0.028 * B + 0.060 * A * B – 1.06 * A2 – 0.78 *B2

The coefficient of determination (R^2) was 0.8630 for the quadratic model. The adequate precision was 7.188. The "Pred R-Squared' is in reasonable agreement with the "Adj R- Squared". The model can be used to navigate the design space. Model F value of 8.82 implied that the model was significant.

The response surface plot for the overall acceptability as influenced by the level of *Curcuma amada*, stevia is given in Fig 7.

The coefficient of estimation of *Curcuma amada* functional golden milk showed that the level of *Curcuma amada* had a positive effect on the score of overall acceptability but stevia

had a slightly negative effect on the score of overall acceptability. In contrast to this result, it was observed that increasing the addition of the herb *Pueraria tuberosa* (0.1 to 0.5%) in milk decreased its flavor score and overall acceptability score. (Sawale et al. 2020)

Analysis of regression of sensory characteristics

Coefficients of determination (R^2) for appearance and color, taste, mouth feel, sweetness, flavor, and overall acceptance of functional golden milk were 0.9537, 0.9338, 0.9472, 0.8753,0.9033, and 0.8630, respectively, according to the analysis of regression as presented in the data of Table 2, and the test for 'lack of fit ' was non-significant, suggesting that the model was accurate enough to predict these sensory features of functional golden milk created with any combination of components lies within the tested level range.

For appearance and color, taste, mouthfeel, sweetness, flavor, and overall acceptance of functional golden milk, the precised adequate values are determined as 16.266, 12.095, 11.086, 7.420,7.391, and 7.188, respectively.

The signal-to-noise ratio is calculated with adequate precision. It is preferable to have a ratio of four or greater. The ratio of all sensory ratings shows that the signal is adequate. The design space may be navigated using this concept.

Optimized product

To discover the best combination of ingredient-based variables, the Design Expert 8.0.6 optimization command was utilized. The responses were chosen based on sensory evaluations that influenced the ultimate product's acceptance. The responses were chosen based on sensory evaluations that influenced the ultimate product's acceptance. Appearance and color, taste, texture, flavor, and overall acceptance were all preserved at their highest levels, while sweetness was kept in check during optimization.

Functional golden milk was prepared using the optimized combination as given in Table 3 and subjected to evaluate its physicochemical properties after preserving the optimized product.

Proximate analysis of functional golden milk

Table 4 shows the ash, total solids, protein, fat, carbohydrates, and energy content of pasteurized functional golden milk. (Palthur et al. 2014) showed somewhat comparable results for total solids, protein, and fat. Milk produced with wheatgrass juice had a calorific value of 64.43 Kcal, which was lower than our data, (Kumar et al. 2017). (Palthur et al. 2014) found a specific gravity of 1.078, which was lower than our results in table 4.

The prepared functional golden milk viscosity was measured at 25 °C using a Brookfield viscometer. The viscosity of functional golden milk was 4.12Cp at 25°C.

Antioxidant activity of functional golden milk

Table 2 Curcuma amada and stevia impact on sensory attributes of functional golden milk: Regression Coefficient and ANOVA of the quadratic model

Factor	Colour& appearance	Taste	Mouth feel	Sweetness	Flavor	Overall acceptance
Intercept	8.486	8.17	8.38	7.88	7.74	7.70
Curcuma amada (A)	0.93	0.49	0.15	-0.19	0.12	-0.25
Stevia(B)	0.029	-0.19	-0.070	0.18	0.14	-0.028
AB	-0.40	0.12	0.36	-0.25	-0.080	0.060
A^2	-0.79	-1.45	-1.38	-0.87	-0.98	-1.06
B^2	-0.24	-0.71	-1.54	-1.35	-0.98	-0.78
R^2	0.9537	0.9338	0.9472	0.8753	0.9033	0.8630
Adequate precision	16.266	12.095	11.086	7.420	7.391	7.188
PRESS	1.10	5.23	4.06	7.94	4.06	3.53
Model F- value	29.48	19.74	25.10	9.83	13.07	8.82
Lack of fit	N S	N S	N S	N S	N S	N S

Table 3 Based on RSM analysis, suggested solutions for the components of functional golden milk, as well as their quantities and expected scores

No.	*Curcuma amada*(A)	Stevia (B)	Colour and appearance	Taste	Mouth feel	Sweetness	Flavour	Overall acceptability	Desirability
1	3.36	0.35	8.62522	8.04072	8.3597	7.81751	7.69236	7.62186	0.782

Table 4 Physiochemical, Proximate and biochemical analysis of the optimized functional Golden milk

Parameters	Optimized Product
L^*	79.01±0.4
a^*	0.61±0.21
b^*	72.95±0.83
Specific Gravity	1.12 @ 25°C
Viscosity	4.12Cp @ 25°C
Total Solids (%)	15.72±0.17
Protein (%)	3.93±0.10
Fat (%)	4.6±0.10
Ash (%)	0.74±0.12
pH	6.3
Acidity (%)	0.10±0.21
Carbohydrates (%)	15.09±0.80
Energy (Kcal)	117.48
Antioxidant (% DPPH activity)	56.14±0.12
Total Phenolic GAE/100 g Content	38.04 ± 0.61mg

Table 5 Mean sensory score of pasteurized functional golden milk

Duration	Color & Appearance	Flavor	Texture	Taste	Sweetness	Overall acceptability
0 DAY	8.5±0.2	7.9±0.5	7.7±0.2	7.9±0.4	7.5±0.2	8.02±0.9
3^{rs} DAY	8.4±0.9	7.9±1.0	7.7±0.4	7.8±0.3	7.5±1.0	7.9±1.1
5th DAY	7.4±0.7	7.1±0.6	7.0±1.0	7.2±0.2	7.0±0.5	6.7±0.3
7th DAY	6.9±0.4	6.5±0.3	5.5±0.06	6.1±0.7	5.3±1.0	5.6±0.5

The antioxidant activity of Functional golden milk is 56.14. Similarly, the antioxidant activity of milk was made by the partial substitute of *Ocimum sanctum* powder using the DPPH technique and discovered 40% DPPH activity. (Palthur et al. 2014)

Total phenolic content of functional golden milk

Functional golden milk has a total phenolic concentration of 38.04 to 36.95 mg GAE (Gallic acid equivalent)/100 g. According to Tyagi et al. 2020, because phenolic components are active hydrogen donors and powerful antioxidants, total phenols and antioxidant activity are interrelated.

Variation in the sensory score during the storage period

The shelf life of a product may be defined as the time period within which it is found to be fit for consumption under defined conditions of storage and distribution. Shelf life depends on several intrinsic (water activity, pH, acidity, buffering power, redox-potential, inhibitory substances, etc.) and extrinsic product parameters (storage temperature, Relative Humidity, or RH, packaging material, etc.) The final optimized functional golden milk was packaged in sterilized bottles. The pasteurized sample was stored at 5 °C, sensory scores were assessed every 2 days. Pasteurized functional golden milk was stable for 7 days at 4°C as given in Table 5.

Conclusions

Based on the results of sensory and Physico-chemical analysis of the Golden milk formulated using different levels of *Curcuma amada* and Stevia, a combination of both the ingredients with desirable attributes were obtained. 3.36 percent *Curcuma amada* powder and 0.35 percent stevia can be used to make high-quality functional golden milk.This functional golden milk obviously has more nutritious characteristics than plain milk because it includes the optimal quantity of antioxidants and total phenolic content.Consequently, *Curcuma amada*'s application may be promoted to all aspects of human health. *Curcuma amada* has a cooling impact on our bodies and can be utilized by persons who live in hotter climates. With the result of this research, it may be concluded that *Curcuma amada* is beneficial and indeed be effectively and feasibly used in milk to make functional golden milk, which is very nutritious, inexpensive, and has several advantages.

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RESEARCH ARTICLE

Effect of non-genetic factors on semen characteristics of Murrah bulls under tropical condition

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Abstract: The objective of this study was to assess the effect of non-genetic factors on semen characteristics of 22479 ejaculates from 139 Murrah bulls during the period of 2017-2020 analyzed under tropical conditions, maintained and recorded at Frozen Semen Bank (FSB), Bassi, Rajasthan Cooperative Dairy Federation, Jaipur, Rajasthan, India as well as in Information Network for Animal Productivity & Health (INAPH) application maintained at National Dairy Development Board (NDDB). Results were recorded as 2.94±0.03 mL for volume, 1234.72±13.51 million/mL for sperm concentration and 68.44±0.25 percentage for motility. Statistical analysis presented a significant difference for all semen parameters with studied non-genetic factors except semen collector for motility traits. Also, seasonal dynamics presented winter as the most suitable season for semen collection under tropical conditions. Season of ejaculates was observed as most influencing factors for all semen characteristics in Murrah bulls. Age of bull showed significantly increased values for all semen traits with the increasing age of bull except motility traits. Data analysis in this regard may be utilized to enhance the fertility rate by increasing the semen quality.

Keywords: Age of Bull, Murrah Buffalo, Semen characteristics

Introduction

Ruminant production systems are very much dependent on the environment than non-ruminant system in tropical region (Bertoni et al. 2021). Buffalo showed adaptability to wider range of climate and excellent feed conversion efficiency making them ideal livestock for tropical countries. Additionally, nutritional make up and therapeutic components of buffalo milk may also serve in enhancing the nutritional security hence, the demand for buffalo milk-based products is increasing in developed and developing countries. Establishment of commercial dairy farms with high productive buffaloes has created a higher demand of quality frozen semen. Semen characteristics are important factors which determine fertility among buffalo population in tropical countries. The quantity and quality characteristics of semen straws produced per ejaculate depend on the volume of the ejaculate, the concentration of spermatozoa in the ejaculate, and the percentage of spermatozoa that are motile. Also, these ejaculate characteristics are known to be affected by environment and genetics (Biniova, 2017). In dairy animals, male fertility has received much less attention compared to female fertility (Butler et al. 2019). Hence, evaluation of semen in context of season, period and age of bull from well managed and genetically superior bulls is importantfor better conception and faster genetic progress in buffalo population. In the present study, effect of non-genetic factors on semen characteristics was assessed to improve the quality of frozen semen of Murrah in semi-arid region.

Materials and methods

A total of 22479 ejaculates related to 139bulls of Murrah buffalo maintained at Frozen Semen Bank (FSB), Bassi, Jaipur, Rajasthan, India were collected over a period of 4 years (2017-2020). The FSB is located in the hot semi-arid region (Koopen climate classification BSh) of India with dry and subtropical in characteristics with long, extremely hot summers and short, mild to warm winters. Bulls used in AI programme satisfy quality norms, semen was collected and processed in accordance with the standard protocols. Bulls were prepared for collection by giving two/three false mounts followed by restraint with the gap of half an hour to one hour depending on the bull. After examination of sperm concentration and initial motility, semen samples were primarily diluted with dilutor maintained at 34°C. Sperm concentration was checked preferably by a digital photometer with auto dilutor. To get a desired 20 million sperm per doses, initial semen volume was extended considering the sperm concentration per mL for estimation of total extended volume (TEV) and sperm concentration per dose (SCPD). After freezing, the semen straws were stored in a liquid nitrogen

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container. Post-thaw motility of semen was examined at 24 hours (after freezing). Preferably, the person involved in evaluation of freshly collected ejaculates did not check the post thaw motility. For a minimum concentration of 20 million per dose, minimum acceptable post thaw motility was 50%. Semen doses below 50% progressive motility were discarded. Datasets were analyzed at Department of Animal Genetics and Breeding, PGIVER, Jaipur by using SPSS software. Parameters *viz*. ejaculation volume, total extended Volume (TEV), motility, sperm concentration and post thaw motility (PTM) were analyzed. Effect of non-genetic factors *viz*. season, period, age of bull, collectors, number of ejaculations for various genotypes were analyzed in the present study. Statistical analysis was carried out using least squares and maximum likelihood analysis method for non-orthogonal data as described by Harvey (Harvey, 1987) using following model.

Yijkl = μ + Pi +Sj+ ck+ eijkl

Yijkl = Observation on the lth individual in ith season, jth period and kth age group

μ = Overall population mean

Pi = Effect of ith season of semen collection

Sj = Effect of jth period of semen collection

ck = Effect of kth age group during semen collection

eijkl = Random error, NID

The statistical significance of various fixed effects in the least squares model was determined by 'F' test. For significant effects, the differences between pairs of levels of effects were tested by Duncan's multiple range test as modified by Kramer, 1957. The differences was considered significant, if

$$(Xi\text{ “ }Xj) = \frac{\sqrt{2}}{Cii+Cjj+Cij} > \sigma_e Zpn_2$$

Where,

Xi and Xj are the least squares means for ith and jth treatment and

Cii, Cjj and Cij are diagonal and off-diagonal elements in the inverse of coefficient matrix in the least squares normal equations,

Zpn2 is ranged value in Duncan's table (0.05) at n2 degrees of freedom,

P is number of means in the range chosen,

σeis standard deviation of error,

n2 is degree of freedom for error.

Results and discussion

The effects of non-genetic factors were analyzed for semen characteristics and results of ANOVA have been presented in Table 1.The overall least square means along with standard error of physical characteristics of Murrah buffalo semen have been shown in Table 2. The overall mean of semen characteristics were estimated as 2.94 ±0.03 mL for ejaculates volume, 68.44±0.25 percentage for initial motility, 1234.27±13.51million per mL for sperm concentration, 39.32±0.61mLfor TEV, 20.71±0.07 for SCPD and 49.71 percentage for PTM.

Table 1 ANOVA mean sum of square for factors affecting semen of Murrah bulls

Source of variation	df	Volume (mL)	Concentration (Million/ml)	Motility (%)	TEV (mL)	SCPD (Million/ml)	PTM (%)
Season of collection	3	251.78***	15728299.15***	8661.98***	84299.68***	180.51***	47.61***
Period of collection	3	404.22***	47195249.64***	2054.16***	161886.28***	173.32***	163.95***
Collection by individual	5	44.07***	2442015.58***	308.83	12564.57***	18.27*	30.73***
Order of ejaculation	2	97.79***	21150340.72***	1885.80***	58205.93***	58.32***	70.85***
Age in Months		53.48*** (3)	24762107.38** (3)	3737.35** (3)	30252.71*** (3)	35.45*** (3)	23.69* (3)
Error		1.76 (22462)	275468.47 (22462)	88.43 (21495)	571.52 (22459)	6.97 (19345)	8.53 (19273)
R^2		0.064	0.062	0.025	0.090	0.009	0.007

*p>0.05; ** p >0.01;*** p>0.001

Table 2 Season, period, collection by individual, order of ejaculation and age of breeding bull, wise Least squares means and standard errors (Mean ±SE) of Semen of Murrah bulls

Effect	Volume (mL)	Concentration (Million/ml)	Motility (%)	TEV (mL)	SCPD (Million/ml)	PTM (%)
Overall	2.94±0.03 (22479)	1234.72±13.51 (22479)	68.44±0.25 (21511)	39.52±0.61 (22476)	20.71±0.07 (19362)	49.71±0.08 (19290) (19290)
Season						
Winter (Jan. - March)	3.03±0.03^{a} (5258)	1313.57±14.84^{a} (5258)	68.35±0.27^{a} (5069)	43.18±0.67^{a} (5259)	20.96±0.08^{a}(4589)	49.62±0.08^{a} (4569)
Summer (April-June)	2.71±0.03^{b} (6180)	1185.97±14.82^{b} (6180)	66.68±0.27^{b} (5800)	35.11±0.67 ^{b}(6177)	20.72±0.08^{b} (4993)	49.62±0.09^{a} (4993)
Rainy(July-September)	2.79±0.03^{c} (7194)	1215.33±14.37^{c} (7194)	69.28±0.26 ^{c}(6879)	37.16±0.65 ^{c}(7193)	20.49±0.07 ^{c}(6256)	49.76±0.08^{ab} (2954)
Autumn (Oct-Dec)	3.22±0.04^{d} (3847)	1223.99±15.90^{c} (3847)	69.47±0.29^{c} (3764)	42.65±0.72^{a} (3847)	20.69±0.08 ^{b}(3524)	49.85±0.09^{b} (3500)
Period						
2017	3.20±0.04^{d} (2838)	1450.27±16.48^{a} (2838)	68.21±0.30 ^{ac}(2771)	50.21±0.75^{a} (2836)	20.71±0.08^{a} (2574)	49.74±0.09^{a} (2574)
2018	2.58±0.03^{b} (7880)	1206.31±14.31^{b} (7880)	67.81±0.26^{a} (7315)	34.01±0.65 ^{b}(7889)	20.84±0.07^{a} (6589)	49.81±0.08^{a} (6568)
2019	2.75±0.03^{c} (6935)	1148.38±15.05^{c} (6935)	69.25±0. 27^{b}(6713)	34.15±0.68 ^{c}(6935)	20.46±0.08 ^{b}(6152)	49.89±0.09^{a} (6146)
2020	3.21±0.04^{a} (4826)	1133.91±16.35^{c} (4826)	68.50±0.30 ^{c}(4713)	39.73±0.74 ^{c}(4826)	20.85±0.08^{a} (4047)	49.41±0.09^{b} (4002)
Collection by Individual						
I	2.770±0.03^{a} (6500)	1210.69±14.18^{ac} (6500)	68.26±0.26 (6216)	36.54±0.64^{a} (6500)	20.82±0.07^{a} (5479)	49.53±0.08^{a} (5433)
II	3.1±0.07^{b} (334)	1332.67±31.20^{b}(3 34)	69.19±0.57 (325)	44.90±1.42 ^{b}(333)	20.58±0.16^{a} (303)	49.85±0.18^{a} (299)
III	3.05±0.04^{b} (1935)	1219.29±16.84^{ab} (1935)	67.81±0.31 (1875)	40.32±0.76 ^{c}(1936)	20.69±0.09^{a} (1671)	49.59±0.10^{a} (1666)
IV	3.00±0.04^{b} (1319)	1265.33±18.92^{b} (1319)	68.34±0.34 (1283)	41.33±0.86 ^{dc}(1319)	20.78±0.10^{a} (1176)	49.81±0.11^{b} (1175)
V	2.92±0.04^{b} (2222)	1170.68±16.64^{c}(2 222)	68.89±0.30 (2114)	37.33±0.75^{a} (2222)	20.61±0.08^{a} (1954)	49.81±0.09^{b} (1954)
VI	2.77±0.03^{a} (10169)	1209.63±13.37^{a}(1 0169)	68.16±0.25 (9699)	36.73±0.61^{a} (10166)	20.81±0.07^{b} (8779)	49.69±0.08^{a} (8763)
Ejaculation order						
1	3.07±0.01^{a} (13471)	1322.14±7.35^{a} (13471)	67.65±0.13^{a}(1307)	42.91±0.33^{a} (13471)	20.79±0.03^{ab} (11601)	49.64±0.04^{a} (11556)
2	2.88±0.02^{b} (8802)	1236.92±7.95 ^{b} (8802)	68.49±0.14 ^{b}(8297)	38.25±0.36 ^{b}(8800)	20.62±0.04 ^{b}(7583)	49.82±0.04^{b} (7559)
3	2.85±0.09^{b} (206)	1145.09±36.91^{c}(2 06)	69.18±0.69 ^{b}(188)	37.42±1.68 ^{b}(205)	20.73±0.20 ^{b}(178)	49.69±0.22^{b} (175)
Age of bull at ejaculation						
<42 Months	2.82±0.04^{a} (5621)	1119.68±15.70^{a} (5621)	69.41±0.29^{a} (5225)	35.46±0.71^{a} (5620)	20.63±0.08 ^{ac}(4820)	49.76±0.09^{ab} (4814)
43-78 months	2.89±0.03^{ab} (10298)	1243.68±14.02^{b}(1 0298)	69.03±0.26^{a} (9974)	39.19±0.64 ^{b}(10297)	20.81±0.07^{bc} (9010)	49.79±0.08^{b} (8962)
79-114 months	2.92±0.04^{b} (3435)	1324.39±15.91^{c}(3 435)	68.37±0.29 ^{b}(3298)	41.50±0.72 ^{c}(3434)	20.62±0.08 ^{c}(2964)	49.70±0.09^{a} (2954)
115 –and more	3.12±0.04^{d} (3125)	1251.11±16.34^{b}(3 125)	66.96±0.30 ^{c}(3015)	41.95±0.74 ^{c}(3125)	20.79±0.08 ^{c}(2568)	49.60±0.09^{a} (2560)

Season of ejaculation

Season of ejaculates was observed as most influencing factors for all semen characteristics in Murrah bulls. Least squares ANOVA showed significant effect of season of ejaculates on volume, motility, sperm concentration, TEV, SCPD and PTMin Murrah buffalo. Winter and autumn season were reported to be comparatively favorable season for all semen parameters in Murrah buffalo under tropical conditions. However, summer being the most unfavorable season for all semen parameters except PTM which may be attributed to the fact that post thaw motility is influenced by thawing rate instead of season of ejaculation. Due to standard practice of thawing and minimum variability for motility effect of season of ejaculation may diminish after thawing. The difference in semen characteristics between seasons might be attributed to variation in ambient temperature and relative humidity. In the present finding lower semen volume, motility and concentration of sperm cell of ejaculates during summer season may be due to climatically stressful environment. Being seasonal breeders, the maximum breeding activity occurs during the winter and autumn season. Similar to our findings, seasonal variation was observed and summer was considered as most unfavorable season for semen volume and sperm cell concentration in Karan Fries bull (Bhakat et al. 2015). Hot humid condition affects the body's normal physiological mechanism. Regulation of body temperature cannot cope with extreme environmental condition (Morrell2020). Summer stress under tropical environments affects normal reproductive function by reducing feed intake, inhibiting release or response to GnRH, FSH and LH. The reduced secretion of thyroxin and further reduction in feed intake may also be a reason for reduction in semen volume and other parameters. Thermal stresses causes testicular degeneration and hence lower the semen output (D'Andre et al. 2017).Similar to the present findings, summer season was unfavorable season and it might be due to seasonal alteration of fatty acid composition and cholesterol concentration (Orgal et al. 2012).

Period of ejaculation

Period of ejaculates had significant effect on semen parameters and it showed erratic trend over the period from 2017-19 and increased in the year of 2020. The significant difference in semen parameters between years may be observed due to changes in feed, climatic condition, management practices and techniques.

Age of bull at semen ejaculation

A significant effect of age of bull was observed on all semen parameters with an increasing trend for volume and TEV traits. Similar to other parameters of present findings, sperm concentration showed reducing trend over the age but reduction in sperm concentration was estimated in oldest age group (>115 Months). High ejaculated semen volume and concentration was observed in older age of Murrah buffalo. The total ejaculation volume was observed to be significantly enhanced with the increasing age in buffalo. Contrary to the volume, sperm concentration and TEV in reference to the age of bull, initial and post thaw motility was higher in younger bull as compared to old age of bull (<78 months of age).Murrah buffalo showed highest semen motility (69.41) in youngest age group bull (<42 months of age). These results are in agreement with those of most studies, which reported that an increase in semen production with age of bull (Boujenane and Boussaq 2013).The similar pattern was observed by Prastowo et al. (2014) in Bali cattle. It seems that ejaculate volume is increased with increasing testicular development since the size of testes increase for at least five years after puberty. In general, scrotal circumference, scrotal shape and testicular size increase with age (Ahirwar et al. 2018).Similar to this finding, low semen concentration associated with young bulls compared with older bulls corroborates with the findings of Murphy et al. (2018). Sonar et al. (2016) also reported the variation in semen motility due to age in Gir bull.

Semen collection by individual

A highly significant ($P<0.001$) variability in semen characteristics was observed for the factor of semen collectors except semen motility may be hypothesized due to the systematic training of the individual engaged in semen collection, and may be considered as an important factor in Murrah buffalo for higher quality semen parameters. Collector expertise along with the ability to judge the sexual behavior may enhance the semen quality and needs further exploration.

Order of ejaculates

Order of ejaculates had significant effect on all semen traits with highest semen parameters were observed in first ejaculation. A significant reduction in the semen quality over the orders of ejaculates and it may be a useful strategy for grading the quality of Frozen Semen Doses FSDs on the basis of ejaculates order. Similar to this finding, significant variation in sperm cell was also reported by Bhave et al. (2020) in buffalo. This may be primarily due to lower semen production with the collection of multiple ejaculates on the same day.

Conclusion

Non genetic factors need attention for quality semen production especially in buffalo bulls of developing tropical countries like India. Seasonal dynamics presented winter and autumn as comparatively better to summer season in tropical environment. A significant effect of age of the bull son better semen quality parameters as well as for initial and post thaw motility traits was observed. However, semen traits for volume, concentration, TEV and SCPD was better in old age bulls than young bulls. Individual

semen collector effect showed highly significant effect on semen quality and needs proper attention.

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RESEARCH ARTICLE

Y-chromosome diversity in dairy bulls of Tamil Nadu

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Abstract: The Y-chromosome specific microsatellite studies are of particular interest as they could reveal the paternal lineage and domestication pattern among the population distributed over geographical area. As few bulls are used against large number of cows using artificial insemination approach, it is necessary to study the Y-chromosome microsatellite markers to understand the Y-chromosome diversity, paternal lineage and molecular variation among population. The present study included 431 bulls comprising *Bos taurus, Bos indicus* and crossbreds from various frozen semen stations of Tamil Nadu. The microsatellites loci *viz. UMN0103*, UMN0307, BM861, UMN0504, INRA189, INRA124, DDX3Y and UMN2008 were screened. The overall mean number of different alleles, number of effective alleles, Shannon's information index, diversity and unbiased diversity were 2.18 ± 0.16, 1.37 ± 0.06, 0.35 ± 0.04, 0.20 ± 0.02 and 0.21 ± 0.02 respectively. The analysis of molecular variance revealed that the variation within populations accounted for 66 per cent of total variation.

Keywords: Genetic diversity, Microsatellite, Paternal lineage, Y-chromosome

Introduction

Y-chromosome-specific microsatellites are of particular interest as they are haploid and paternally inherited. As there is no recombination in the Y-specific region, which makes up around 95% of the Y chromosome, the Y chromosome is inherited "en bloc" as a haplotype. Y chromosome polymorphisms have been utilized in the analysis of domesticated bovine breeds, showing new perspectives in the paternal origin and also the development of a breed (Edwards et al. 2000; Gotherstrom et al. 2005; Li et al. 2007; Kantanen et al. 2009; Ganguly et al. 2020). Y chromosome polymorphisms have been utilized in the analysis of domesticated bovine breeds, showing new perspectives in the paternal origin and also the development of a breed (Edwards et al. 2000; Gotherstrom et al. 2005; Li et al. 2007; Kantanen et al. 2009).Y chromosome is an efficient indicator for the demographic events namely domestication, migration, population expansions and population bottlenecks (Edwards et al. 2000; Ginja et al. 2010)The microsatellites residing in Y-chromosome aids in understanding and distinguishing taurine, indicine and crossbred patrilines and their introgression (Edwards et al. 2000; Giovambattista et al. 2000; Hanotte et al. 2000; Li et al. 2007).

With effective implementation of artificial insemination programme across the country, the boss taurus breeds *viz.* Jersey and Holstein Friesian have been used for extensively for crossbreeding, and upgrading Bos indicus cattle breeds. This necessitates to understand the paternal lineage and Y-chromosome diversity. As the studies pertaining to paternal lineage of cattle in India are scanty this study was undertaken to evaluate Ibos taurus, Bos indicus and crossbred population of Tamil Nadu.

Materials and methods

Diluted frozen semen samples were collected from 417 bulls of Jersey (78), Holstein Friesian (16), Crossbred Jersey (253), Crossbred Holstein Friesian (36), Kangayam (12), Red Sindhi (18) and Umblachery (4) from various frozen semen stations (Exotic Cattle Breeding Farm (ECBF), Eachenkottai; Nucleus Jersey and Stud Farm (NJF), Udhagamandalam; District Livestock Farm (DLF), Udhagamandalam and District Livestock Farm (DLF), Hosur) of Tamil Nadu. The genomic DNA was extracted from 0.5 mL of semen (0.25 mL per semen straw) by Phenol Chloroform extraction method. The isolated genomic DNA was subjected to horizontal gel electrophoresis for quality check, optical density (260/280 nm) and concentrations were calculated by using spectrophotometer (NanoDrop OneC of Thermo Scientific, USA).

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The samples with $OD_{260/280}$ ratio of 1.7 to 1.9 were considered and used further in the study.

Seven STRs namely UMN0103, UMN0307, BM861, UMN0504, INRA189, INRA124, DDX3Y and UMN2008 located Y-chromosome were screened. The primers for amplifying microsatellite loci were fluorescent-labelled with dyes *viz.* TET, FAM, ATO550 and ATO565. The details of primers, fluorescent labels and annealing temperature are furnished in the Table 1. The standardized PCR thermocycling protocol is as follow: comprised initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 sec; annealing at 58°C and 60°C temperature for different microsatellites for 30 sec; extension at 72°C for 30 sec and final extension at 72°C for 10 min.

The PCR products were subjected to assay by multiplexing of the PCR product based on allelic range and fluorescent label, followed by capillary electrophoresis on ABI 3730 instruments (Applied Biosystems). Sizing of microsatellite fragments in the chromatogram was performed by the Peak Scanner v2.0. and GeneMapper® v6.1 software. The STR allelic data were used to ascertain frequency, Shannon's information index, unbiased diversity, Nei's genetic distance, AMOVA were analyzed using GenAIEx 6.5. The pairwise Nei's genetic distances were represented with Multidimensional scaling in two-dimensional space using SPSS v21 software package.

Results and discussion

The markers considered in this study were highly polymorphic except UMN0504. Single genotype 144 bp of UMN0504 was observed in previous study by Ginja et al. (2009) which included 13 Portuguese, three Portugal and five Brahman cattle breeds. However, Ozsensoy et al. (2014) reported three genotypes *viz.* 106, 144 and 146 bp of UMN0504 in Turkish cattle breeds. The haploid allelic frequencies of microsatellite loci are furnished in Table 2.

BM861 locus displayed two major genotypes (156 and 158 bp) of which 156 bp was observed to be fixed in *Bos indicus*. This result is in concordance with the reports by Li et al. (2009), Ginja et al. (2009), Perez-pardal et al. (2011) and Edwards et al. (2011). However, this locus was previously reported to be polymorphic in cattle breeds of Ethiopia (Li et al. 2007), Portuguese (Ginja et al. 2009), Creoles (Ginja et al. 2010), Europe (Perez-pardal et al. 2011), Turkey (Ozsensoy et al. 2014), Polish (Prusak et al. 2015) and *Bos indicus* (Ganguly et al. 2020) with genotypes 156, 158, 160 and 164 bp. The presence of 156 bp genotype in crossbred of our study might be result of using *Bos indicus* bulls for crossbreeding.

The 247 bp genotype of DDX3Y was observed to be fixed in *Bos indicus*; while it was a major genotype in Holstein Friesian, Jersey, crossbred Holstein Friesian and crossbred Jersey. But previous reports indicated that, the genotypes 245 and 249 bp were exclusively found in *Bos indicus* and *Bos taurus* breeds respectively (Ginja et al. 2009; Ginja et al. 2010; Ganguly et al. 2020).

As expected, genotype 88 bp was observed in *Bos indicus* breeds while presence of the same genotype in crossbred was unlooked. Upon karyotyping samples of crossbred bulls possessing 88 bp genotypes revealed acrocentric Y-chromosome in one sample. The underlying reason might be due to fortuitous mating or

Table 1 Details of primers used for amplification of Y-chromosome specific microsatellites

S. No	Microsatellite	Repeat motif	Primer sequence (5'-3')	FL	AT(°C)
Panel 1					
1	BM861	$(GT)_6C(TG)_{10}$	Forward: TTG AGC CAC CTG GAA AGC	TET	60°C
			Reverse: CAA GCG GTT GGT TCA GAT G		
2	DDX3Y	$(TA)_9(TC)_9$	Forward: TGA ACC ACT AGG GAG GTC ATC	FAM	60°C
			Reverse: TTC CAA TTT AGC TGT GGT TAT CTG		
3	INRA124	$(GT)_4A(TG)_9$	Forward: GAT CTT TGC AAC TGG TTT G	FAM	60°C
			Reverse: CAG GAC ACA GGT CTG ACA ATG		
4	INRA189	$(TG)_{22}$	Forward: TAC ACG CAT GTC CTT GTT TCG G	FAM	60°C
			Reverse: CTC TGC ATC TGT CCT GGA CTG G		
Panel 2					
5	UMN0103	$(CA)_{22}$	Forward: ACA CAG AGT ATT CAC CTG AG	TET	58°C
			Reverse: ATT TAC CTG GGT CAA AGC AC		
6	UMN0307	$(CA)_{18}$	Forward: GAT ACA GCT GAG TGA CTA AC	ATO550	58°C
			Reverse: GTG CAG ACA TCT GAG CTG TG		
7	UMN0504	$(CT)_2GT(CT)_3(GT)_2$	Forward: AGG CCA TCT GCA TAG TGA AG	FAM	58°C
			Reverse: TGC TGG ACT GCT CAT CTC TG		
8	UMN2008	$(CA)_2GA(CA)_{11}G(CA)_3(TG)_{17}$	Forward: CAA GCA TAT CAG TGG CCT GG	ATO565	58°C
			Reverse: GCT GCA AGG AAA CTA TTT CA		

AT: Annealing temperature (°C); FL: Fluorescent Label

artificial insemination with bulls having *Bos indicus* lineage. The frequency of 104 bp genotype and 100 bp were previously reported in Turkish (Ozsensoy et al. 2014), Creole (Ginja et al. 2010), Portuguese (Ginja et al. 2009), European (Perez-pardal et al. 2011) cattle breeds. However, in this study, these genotypes were exclusive to Holstein Friesian, Jersey, crossbred Holstein Friesian and crossbred Jersey and thus testifies the taurine paternal lineage.

Supported by previous finding of Perez-pardal et al. (2011), microsatellite UMN0103 displayed two loci in *Bos indicus* breeds as expected while single locus in Holstein Friesian, Jersey, crossbred Holstein Friesian and crossbred Jersey. The two loci were separated and named as UMN0103a and UMN0103b for ease of analysis. The two allelic combinations found in this study *viz.* 116/124 bp and 114/124 bp were supported by findings of Ginja et al. (2009), Ginja et al. (2010), Ganguly et al. (2020).

Table 2 Haploid allele frequencies of Y-chromosome specific microsatellite markers in various genetic groups of cattle

Locus	Allele size (bp)	Jersey (n=75)	Holstein Friesian (n=16)	Crossbred Jersey (n=264)	Crossbred Holstein Friesian (n=29)	Kangayam (n=12)	Red Sindhi (n=18)
BM861	152	-	-	0.004	-	-	-
	154	-	-	0.004	-	-	-
	156	0.080	-	0.452	0.069	1.000	1.000
	158	0.920	1.000	0.540	0.931	-	-
DDX3Y	247	0.773	0.813	0.818	0.862	1.000	1.000
	249	0.227	0.187	0.182	0.138	-	-
INRA	88	-	-	0.049	-	1.000	1.000
189	100	-	0.250	0.012	0.517	-	-
	104	0.840	0.688	0.871	0.483	-	-
	106	0.160	0.062	0.068	-	-	-
UMN010	114	-	-	0.023	-	0.417	0.833
3a	116	-	-	0.133	0.069	0.500	0.167
	124	0.067	1.000	0.394	0.897	-	-
	126	-	-	0.011	-	-	-
	128	0.933	-	0.439	0.034	0.083	
UMN010	124	0.067	1.000	0.545	0.966	0.917	1.000
3b	126	-	-	0.012	-	-	-
	128	0.933	-	0.443	0.034	0.083	-
UMN030	145	-	-	0.004	-	-	-
7	147	0.013	-	0.004	-	-	-
	149	0.013	-	-	-	-	-
	151	0.014	-	0.030	-	-	-
	153	0.960	-	0.792	-	0.167	0.889
	155	-	-	0.008	-	0.833	0.111
	157	-	-	0.010	-	-	-
	159	-	1.000	0.152	1.000	-	-
UMN050	144	1.000	1.000	1.000	1.000	1.000	1.000
4							
INRA124	130	0.040		0.155		0.583	0.556
	132	0.960	1.000	0.845	1.000	0.417	0.444
UMN200	134	0.067	-	0.030	-	-	-
8a	136	-	-	0.008	-	-	-
	140	0.933	1.000	0.932	1.000	0.667	0.889
	144	-	-	0.011	-	-	-
	146	-	-	0.019	-	0.250	0.111
	148	-	-	-	-	0.083	-
UMN200	136	-	-	0.008	-	-	-
8b	140	0.853	0.813	0.515	0.690	0.084	0.223
	144	0.040	-	0.034	-	-	-
	146	0.014	0.187	0.125	0.034	0.583	0.444
	148	0.093	-	0.318	0.276	0.333	0.333

However, the two loci of UMN0103 observed in crossbred cattle (crossbred Holstein Friesian and crossbred Jersey) might be due to deployment of bulls with indicine paternal lineage for crossbreeding programme.

Among two genotypes 130 and 132 bp observed in INRA124, former was major genotype in *Bos indicus* breeds; while the latter was major genotype in *Bos taurus* and crossbred cattle. However, 130 bp genotype was reported to be exclusive to *Bos indicus* and North-Ethiopian cattle breeds (Ginja et al. 2009; Ginja et al. 2010; Prusak et al. 2015; Ganguly et al. 2020). In the same studies mentioned, genotype 132 was observed in bulls with taurine lineage.

UMN2008 displayed two loci as expected, because of its location in pseudo autosomal region of Y-chromosome (Stafuzza et al. 2009). The two loci were separated and renamed as UMN2008a and UMN2008b for ease of analyses. The genotypes were observed in combination *viz.* 140/148, 140/144, 140/146, 134/140, 136/136 bp and 140/140. The genotypes identified are in concordance with previously reported genotypes by Alyethodi et al. (2016).

The overall mean number of different alleles, number of effective alleles, Shannon's information index, diversity and unbiased diversity across the breeding sires of six genetic groups maintained in the organized farms of Tamil Nadu were 2.18 ± 0.16, 1.37 ± 0.06, 0.35 ± 0.04, 0.20 ± 0.02 and 0.21 ± 0.02 respectively.

Y-chromosome based genetic studies on populations which included both *Bos taurus* and *Bos indicus* breeds revealed overall mean diversity index of 0.75 (Li et al. 2007), 0.20 (Ginja et al. 2009), 0.42 (Edwards et al. 2011) and 0.11 (Prusak et al. 2015). Though diversity index varies with respect to the population under study, the diversity of cattle population considered in this study was substantially low (0.21 ± 0.02). Ganguly et al. (2020) reported diversity index of 0.485 and zero in Red Sindhi and Kangayam cattle breeds respectively. However, in this study substantial amount of diversity was observed in Kangayam (0.36 ± 0.08) and Red Sindhi (0.19 ± 0.78) breeds. Studies reported relatively zero (Ginja et al. 2009; Ginja et al. 2010) and 0.021 (Perez-pardal et al. 2011) diversity in Holstein Friesian breed. Similar diversity index was observed in Holstein Friesian population in this study (0.11 ± 0.06). Diversity in Jersey breed was observed to be 0.16 ± 0.34, which is in concordance with the report by Ginja et al. (2009).

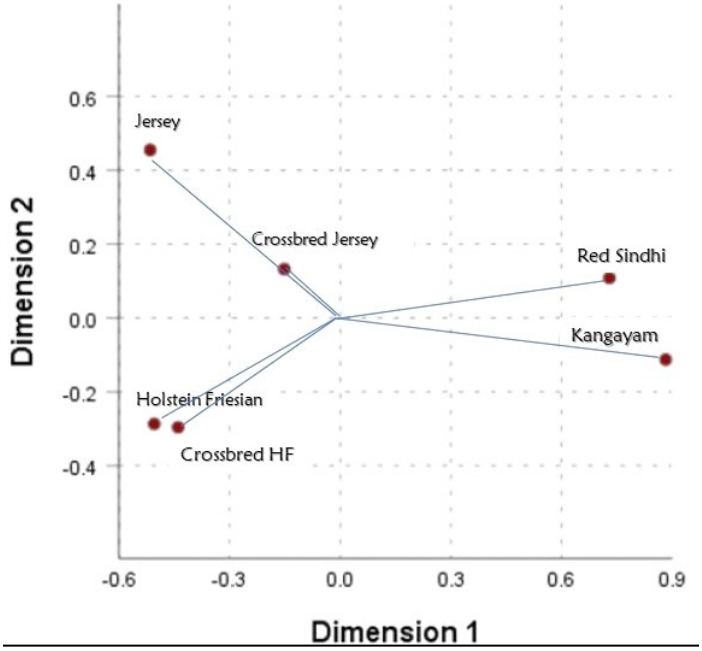

Fig. 1 Multi-dimensional scaling plot for various genetic groups based on Nei's genetic distances estimated from microsatellite data

Table 3 Nei's genetic identity (above diagonal) and genetic distance (below diagonal) based on microsatellite alleles across various genetic groups

Genetic group	Jersey	Holstein Friesian	Crossbred Jersey	Crossbred Holstein Friesian	Kangayam	Red Sindhi
Jersey	-	0.680	0.915	0.671	0.443	0.515
Holstein Friesian	0.386	-	0.810	0.985	0.495	0.516
Crossbred Jersey	0.089	0.211	-	0.811	0.648	0.712
Crossbred Holstein Friesian	0.399	0.015	0.210	-	0.522	0.542
Kangayam	0.815	0.704	0.434	0.649	-	0.898
Red Sindhi	0.664	0.662	0.340	0.613	0.107	-

Analysis of molecular variance revealed that the variation within populations accounted for 66 per cent, whereas the variation among populations accounted for 34 per cent of total genetic variation. The mutli-dimensional scaling plot depicting the relative genetic distances between populations considered in this study is presented in Figure 1. Nei's genetic distances of Holstein Friesian, crossbred Holstein Friesian, Jersey, crossbred Jersey, Kangayam and Red Sindhi cattle were established based on the allelic frequency of the microsatellite loci (Table 3) revealed shortest genetic distance (0.107) between Holstein Friesian and crossbred Holstein Friesian depicting common paternal lineage between them. The longest genetic distance was observed between Jersey and Kangayam (0.815) representing colossal distance between *Bos indicus* and *Bos taurus* paternal lineage. The shorter distances between Holstein Friesian and crossbred Holstein Friesian, Jersey and crossbred Jersey, and Kangayam and Red Sindhi indicated different clusters with common Y-chromosomal microsatellite allelic composition.

Conclusions

To conclude, the UMN0504 locus was monomorphic with 144 bp genotype. The genotypes 156, 247 and 130 bp of BM861, DDX3Y and INRA124 respectively, were observed in higher frequencies in Kangayam and Red Sindhi. The UMN0103 and UMN2008 microsatellites showed two loci each. The presence of two loci for UMN0103 was specific to *Bos indicus* lineage; while UMN0103 was confined to single locus in *Bos taurus* lineage. The 114 and 116 bp genotypes of UMN0103 were observed in combination with 124, 126 and 128 bp genotypes. 88 bp genotype of INRA189, a *Bos indicus* specific genotype was also observed in crossbred Jersey along with Kangyama and Red Sindhi. Based on microsatellite allelic composition the study revealed three distinct clusters; with the *Bos indicus* breeds (Kangayam and Red Sindhi) in one, Holstein Friesian and its crossbred forming the other, and Jersey and its crossbred grouped in another; showing common paternal lineage among them.

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RESEARCH ARTICLE

Effect of varying levels of fibre and starch in calf starters containing finely ground maize grain on the performance of pre-ruminant calves

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Abstract: The nutrient composition and particle size of calf starter play a major role in shaping appropriate rumen development and starter intake and average daily gain in pre-ruminant dairy calves. Hence, a feeding trial was conducted to assess the effect of feeding calf starters containing finely ground maize grain with varying levels of fibre and starch on the performance of pre-ruminant dairy calves fed no roughage. Three iso-nitrogenous calf starters containing different levels of finely ground maize grain and soybean hulls (maize:soybean hulls 40:10; 30:20; 20:30) were evaluated through a feeding trial using 18 Holstein Friesian crossbred female calves (average body weight 34.19 ± 0.94 kg). The calves were divided into three groups of 6 calves each in a completely randomized design. Milk was offered to calves twice daily from the fourth day of age up to 63 day of age as per the step up/step down milk feeding schedule. In addition, calves in each group were offered respective calf starters individually *ad libitum* from 3 to 63 day of age. Daily starter intake and fortnightly body weight of calves were recorded during the trial. The calves were monitored for rumination from the third day of age until rumination was noticed. Blood samples were collected from calves on 63 day of age for estimation of various biochemical constituents. The level of starch and fibre in calf starters containing finely ground maize grain (57.72% of particles with a size of 1.18 mm above) had no influence on overall calf starter dry matter intake (401.89 to 424.68 g/d), average daily gain (0.660 to 0.700 kg/d), feed conversion efficiency, feed cost/kg gain, initiation of rumination and blood biochemical constituents in pre-ruminant dairy calves. Maximum pre-weaning average daily gain (700 g/d) can be achieved in Holstein Friesian crossbred female calves through feeding milk by step up/step down method and calf starter containing finely ground maize grain and soybean hulls in the ratio of 20:30 without offering any roughage.

Keywords: Calf starter, Fibre, Maize grain, Performance, Pre-ruminant calves, Starch

Introduction

Pre-weaning average daily gain (ADG) in calves is considered as the most important factor that affects first lactation performance in cows (Soberon et al. 2012). Supply of adequate nutrients from both liquid and solid feeds and maintaining ADG above 0.5 kg/day can increase first lactation performance of cows (Gelsinger et al. 2016).

The growth of pre-ruminant dairy calves depends on both milk and solid feed intake. Most of the necessary nutrients to pre-ruminant calves is initially supplied from milk. The nutrients requirements increase tremendously with advancing age in the fast-growing pre-ruminant calves. Therefore, in addition to milk, solid feed intake also equally important to the pre-ruminant calves to get adequate nutrients (Gelsinger et al. 2016).

Early rumen development of pre-ruminant calves is important to maximize solid feed intake and the ADG. The overall rumen development depends on the development of rumen epithelium, rumen muscularization and rumen volume. Fermentation of solid feed in the rumen produces volatile fatty acids. Among the volatile fatty acids produced, butyrate and propionate mainly stimulate development of rumen epithelium. Greater rumen epithelial development is achieved through feeding of concentrates (Heinrichs, 2005). Cereal grains rich in starch are main components of concentrates that are fermented to volatile fatty acids which stimulate rumen epithelium development. Cereal grains are frequently ground to reduce particle size and then incorporated into calf starters. Grain particle size and processing affect the

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fermentation rate of starch in the rumen (Suarez-Mena et al. 2016a). However, the optimum particle size distribution of grain for incorporation into calf starters may also be affected by level of inclusion of grain and other dietary factors.

Studies revealed that fibre content and source of fibre in calf starter may affect the starter intake and performance of pre-weaning calves either positively or negatively (Porter et al. 2007; Hill et al. 2008b) possibly through influencing rumen muscularization and capacity. It was also established that calves can be reared without roughage during pre-weaning period (Göncü et al. 2010). Soybean hulls have been used as non-forage fibre source in calf starters (Hill et al. 2008a). Therefore, this study was aimed to evaluate effect of varying levels of fibre and starch in calf starters through changing the levels of finely ground maize grain (FGMG) and soybean hulls on performance of pre-ruminant calves fed no roughage.

Materials and methods

A feeding trial was conducted using pre-ruminant dairy calves at Haveli Dairy Farm, Mulugu, Siddipet, Telangana. Laboratory analysis of collected samples was done at Department of Animal Nutrition, College of Veterinary Science, P.V. Narsimha Rao Telangana Veterinary University, Rajendranagar, Hyderabad.

Particle size distribution of maize grain

The mean particle size distribution (%) of FGMG was determined according to the procedure described by Lammers et al. (1996). A specific quantity of ground maize sample was taken on the upper screen of 7 screens of British Standard System (B.S.S) (410/1969) 7, 10, 14, 25, 36, 52 and 72 and allowed to rotate on Rotop sieve shaker (Secor India Standard Test Sieves, Scientific Engineering Corporation, Delhi).

Experimental diets and distribution of calves

Three iso-nitrogenous calf starters containing 19% crude protein (CP) with varying levels of fibre and starch were prepared by keeping FGMG and soybean hulls content in the ratio of 40:10 (F_1), 30:20 (F_2) and 20:30 (F_3), respectively with minor adjustment on the content of other ingredients (Table 1). Eighteen Holstein Friesian (HF) crossbred female calves (average body weight, 34.19 ± 0.94) were randomly assigned to three treatment groups of 6 calves each in a completely randomized design. The calves were reared for 61 days from 3 to 63 day of age to evaluate the above mentioned experimental rations.

Housing, feeding and management

The calves were separated from the dam immediately after birth and 2 litres of colostrum was fed through bottle as soon as possible. About two hours after birth, the calves were shifted to well ventilated calf shed having elevated individual pens covered with rubber mattresses as bedding. Another 2 l of colostrum was bottle fed on first day of birth. The calves were fed with colostrum @ 4 l per day up to 3 days of birth in two feedings. Milk (treated with 125 mg of tetracycline hydrochloride/l) was offered in separate buckets to calves twice daily from fourth day of birth onwards up to 63 day of age at 6.00 am and 6.00 pm following step up/step down feeding schedule. Milk feeding was increased weekly from 4 l/d during first week to 7 l/d at fourth week. Then, the milk offered was gradually decreased from 7 l/d during sixth week to 4 l/d at ninth week.

In addition to milk feeding, the calves in each group were offered respective calf starters individually *ad libitum* from 3 to 63 day of age at 8.00 am. Clean drinking water was always available to each calf in separate bucket. The amount of calf starter offered and residues if any were recorded daily to arrive at calf starter intake. Daily representative samples of calf starter offered and residues if any (next day) were collected in separate polythene bags for estimation of dry matter (DM).

The body weight of calves was recorded initially on 3 day of age and thereafter at fortnightly intervals (18, 33, 48 and 63 day of age) in the morning before offering water, milk and starter using electronic digital balance. Fortnightly and overall DM intake, ADG and feed conversion efficiency and cost economics were calculated as per the standard procedures.

Initiation of rumination

All calves under experiment were observed for rumination from 3 day of age and the day of initiation of rumination from birth was recorded for each calf.

Collection of blood

Blood samples were collected from the individual calves on 63 day of age aseptically from the jugular vein of calves into clean sterilized glass tubes and kept in slanted position at room temperature for separation of serum. The collected serum samples were centrifuged at 3000 rpm for 5 minutes and the supernatant serum was transferred to 5 ml Eppendorf tubes and stored at -20°C for estimation of various biochemical constituents.

Chemical analysis

The DM, total ash (TA), CP, ether extract (EE) and crude fibre (CF) content of calf starters were determined as per the procedures described in AOAC (2012). Nitrogen free extract (NFE) was obtained by subtracting the sum of CP, EE, CF and TA percentage on DM basis from 100. Organic matter (OM) was obtained by deducting the per cent TA on DM basis from 100.

Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and cellulose content of calf starters were determined as per the method described by Van Soest et al. (1991).

The NDF was estimated by refluxing the sample with neutral detergent solution containing 50 µl of heat stable α-amylase (A3306 dietary fiber kit, Sigma Chemical Co., St. Louis, MO). The hemicellulose was calculated by deducting per cent ADF from per cent NDF. Starch in the calf starters was estimated by following the method of Clegg (1956).

Analysis of blood biochemical constituents

Glucose in serum was estimated based on enzymatic method (Trinder, 1969) using a commercial kit (Coral Clinical Systems, Goa). Total protein in serum was determined by biuret method using a commercial kit (Coral Clinical Systems, Goa) as per the method of Reinhold (1953). Bromocresol green (BCG) method (Doumas et al. 1971) was used to estimate the albumin content of serum using a commercial kit supplied by Coral Clinical Systems, Goa. Globulin concentration in the serum was calculated by subtracting the albumin from total protein. Abumin:globulin was calculated by dividing serum albumin with globulin. The blood urea nitrogen (BUN) in serum was determined by enzymatic method (Bretaudiere et al. 1976) using the kit supplied by Accurex Biomedical Private Limited, Boisar, Maharastra. Precision Xtra (Abbott Precision Xtra Ketone Test Strip, New York, USA) calf-side test was used for determination of BHBA in freshly collected whole blood from ear vein of calves on 63 day of age.

Statistical analysis

The results obtained from the feeding trial were subjected to statistical analysis through software (Version 15.0; SPSS) by applying the one-way analysis of variance through the generalized linear model. The treatment means were ranked using Duncan's multiple range test with a significance at $P < 0.05$ (Duncan, 1955).

Results and discussion

Particle size distribution of finely ground maize grain and the chemical composition of calf starters

The FGMG had 57.72% particles with a size of 1.18 mm and above (Figure 1) suggesting that the ground maize had moderate amount of fine particles. The calf starters used in the feeding trial were formulated to be isonitrogenous and hence contained similar levels of CP (19.30 to 19.33%; Table 2). All the proximate principles and cell wall constituents except CP differed among calf starters (Table 2) because of variations in the content of maize and soybean hulls in the calf starters. As the level of soybean hulls increased and maize content decreased, the NDF increased and starch decreased proportionately in the calf starters. The NDF content of calf starters varied from 18.33 to 29.89% (Table 2) which was higher than the NRC (2001) recommended value (NDF, 12.8%). In most of the earlier studies, the NDF content of calf starters was maintained below 20% (Hill et al. 2008a; Hill et al. 2008b; Bateman et al. 2009; Omidi-Mirzaei et al. 2015; Maktabi et al. 2016; Daneshvar et al. 2017). However, in some of the previous studies, the calf starters contained more than 20% NDF (Hill et al. 2009; Göncü et al. 2010; Rezapour et al. 2016). The starch content of calf starters in previous studies ranged from 25 to 42.3% (Suarez et al. 2007; Hill et al. 2008a; Hill et al. 2008b; Suarez-Mena

Table 1 Ingredient composition (%) of calf starters used for the feeding trial

Ingredients (kg/100kg)	F_1	F_2	F_3
Maize grain	40.0	30.0	20.0
Wheat bran	12.0	12.7	13.4
Molasses (cane)	8.0	8.0	8.0
Protected soybean meal	12.5	12.5	12.5
Soybean meal	12.4	11.85	11.3
Soybean hulls	10.0	20.0	30.0
Sodium bicarbonate	0.7	0.7	0.7
Dicalcium phosphate	0.43	0.5	0.55
Protected fat (Calcium soap)	1.0	1.0	1.0
Yeast (*Saccharomyces cerevisiae*)	0.3	0.3	0.3
Trace mineral and vitamin mixture*	0.3	0.3	0.3
Limestone powder	1.57	1.35	1.15
Toxin binder	0.5	0.5	0.5
Salt	0.3	0.3	0.3
Total	100	100	100

*Values per kg: Cu 6.750 g; Mn 18 g; Zn 33.750 g; Co 0.540g; Se 0.200g; Vitamin A 0.500 MIU; Vitamin D_3 0.500 MIU; Vitamin E 22.50 g and Biotin 0.500 g.

F_1: Finely ground maize grain and soybean hulls in the ratio of 40:10

F_2: Finely ground maize grain and soybean hulls in the ratio of 30:20

F_3: Finely ground maize grain and soybean hulls in the ratio of 20:30

et al. 2016b). In the present study, the starch content of calf starters varied from 18.71 to 32.37% (Table 2).

Milk intake

Even though, the amount of milk (l/d) fed was similar in the first and fourth fortnights, the fortnightly average milk intake of calves expressed as per cent of body weight was low at fourth fortnight (Table 3) because of increase in body weight of calves from the first week to ninth week. However, there was no significant difference in milk intake among calves fed different calf starters at different fortnights during the experimental period. The milk offered to calves by following step up/step down method was relatively higher in this study if compared to the conventional method (@ 10% of body weight). As the calf starter intake is limited during the first 4 weeks of age (Table 4), *ad libitum* to close to *ad libitum* milk feeding is beneficial to the calf in terms of resistance to disease, lifetime performance and calf welfare (Lorenz, 2021).

Dry matter intake

Even though the level of fibre and starch varied among calf starters, the fortnightly and overall average DM intake (Table 4; 401.89 to 424.68 g/d) was comparable among calves fed different calf starters. Similarly, starter intake did not differ significantly in pre-ruminant Holstein calves fed high-fibre (29.03%) or low-fibre (16.90%) diets (Porter et al. 2007). Further, high levels of soybean hulls with varying levels of fibre and starch in the pelleted starter containing large amount of fine particles (more than 93% of particles had particles <1.180 mm) did not influence on starter intake during the pre-weaning period in calves (Hill et al. 2008a). However, the starter DM intake in calves in the present study was relatively less as compared to those observed in earlier studies (Hill et al. 2008a; Göncü et al. 2010; Omidi-Mirzaei et al. 2015) which might be due to relatively higher milk intake.

Fig. 1 Finely ground maize grain

Table 2 Chemical composition (% DM) of calf starters containing finely ground maize grain

Constituent (%)	F_1	F_2	F_3
Dry matter	91.83	92.32	92.32
Organic matter	91.71	91.21	91.17
Total ash	8.29	8.79	8.83
Crude protein	19.33	19.30	19.33
Ether extract	3.72	3.66	3.55
Crude fibre	7.36	11.39	14.69
Nitrogen free extract	61.30	56.86	53.60
Starch	32.37	25.39	18.71
Neutral detergent fibre	18.33	23.64	29.89
Acid detergent fibre	10.25	14.53	18.76
Hemicellulose	8.08	9.11	11.13
Cellulose	8.52	11.92	16.23
Lignin	1.17	1.31	1.55
Calcium	1.35	1.31	1.25
Phosphorus	0.79	0.68	0.69

Each value is the average of duplicate analysis

F_1: Finely ground maize grain and soybean hulls in the ratio of 40:10

F_2: Finely ground maize grain and soybean hulls in the ratio of 30:20

F_3: Finely ground maize grain and soybean hulls in the ratio of 20:30

Fortnightly body weight changes and average daily gain

Step up/step down milk feeding and *ad libitum* calf starter feeding from 3 day of age ensure more bioavailable nutrient supply from milk and necessary solid feed supply for rumen development during the initial period. With increasing calf starter intake in response to rumen development, the nutrient supply from milk is decreased by reducing the milk offered gradually until weaning.

The varying levels of fibre and starch in the calf starters did not significantly (P>0.05) affect the fortnightly body weight changes and ADG in calves throughout the experimental period (Tables 5 and 6). The overall ADG (kg/d; Table 6) was 0.69, 0.66 and 0.70 in calves fed calf starters F_1, F_2 and F_3, respectively. However, in the second fortnight, the average starter DM intake and ADG were 75.84 and 64.8 g and 50 and 90 g higher in F_2 and F_3 calves (Tables 4 and 6), respectively than F_1 calves. It appears that the high level of inclusion of FGMG (40%) in calf starter F_1 could have increased volatile fatty acids production, lowered rumen pH and adversely affected the health of rumen papillae thereby reducing DM intake and ADG. During fourth fortnight, the calves under F_1, on an average consumed 149.69 and 61.04 g more calf starter DM and recorded 50 and 60 g more ADG than calves fed F_2 and F_3 calf starters, respectively which suggested that the inclusion of high level of maize grain (40%) in calf starters may be beneficial at 8 to 9 weeks of age.

Table 3 Fortnightly average milk intake (% body weight) in pre-ruminant dairy calves fed different calf starters containing finely ground maize grain

Fortnight	F_1	F_2	F_3	SEM	P value
1	13.67±0.52	13.65±0.71	13.59±0.73	0.358	0.996
2	15.54±0.59	15.52±0.76	15.05±0.71	0.381	0.855
3	12.14±0.50	12.16±0.58	11.75±0.54	0.301	0.841
4	6.74±0.26	6.85±0.32	6.64±0.28	0.159	0.883

Table 4 Effect of varying levels of fibre and starch in calf starters containing finely ground maize grain on fortnightly and overall average dry matter intake (g/d) in pre-ruminant dairy calves

Fortnight	F_1	F_2	F_3	SEM	P value
1	39.67±12.65	77.00±22.66	39.49±5.53	9.35	0.172
2	150.96±36.63	226.80±48.28	215.76±43.37	24.71	0.425
3	431.50±97.46	381.04±87.96	419.31±78.75	48.22	0.916
4	1063.88±100.01	914.19±153.64	1002.84±116.40	69.61	0.703
Overall	424.68±57.31	401.89±76.80	423.38±56.20	34.88	0.961

Table 5 Effect of varying levels of fibre and starch in calf starters containing finely ground maize grain on fortnightly body weight (kg) changes in pre-ruminant dairy calves

Fortnight	F_1	F_2	F_3	SEM	P value
Initial weight	33.95±1.53	34.65±1.68	33.97±1.94	0.94	0.949
1	39.75±1.64	39.55±2.10	40.62±2.01	1.05	0.918
2	48.50±2.37	49.08±2.22	50.77±2.58	1.32	0.789
3	61.25±2.96	60.75±3.19	62.85±3.18	1.70	0.884
4	76.32±2.80	75.00±3.31	77.05±3.39	1.74	0.899

Table 6 Effect of varying levels of fibre and starch in calf starters containing finely ground maize grain on fortnightly and overall average daily gain (kg/d) in pre-ruminant dairy calves

Fortnight	F_1	F_2	F_3	SEM	P value
1	0.39±0.05	0.33±0.05	0.44±0.05	0.032	0.340
2	0.58±0.07	0.63±0.04	0.67±0.09	0.041	0.695
3	0.85±0.08	0.77±0.07	0.80±0.05	0.040	0.783
4	1.00±0.04	0.95±0.05	0.94±0.06	0.029	0.697
Overall	0.69±0.39	0.66±0.03	0.70±0.04	0.021	0.694

The increased level of fibre in the calf starter through non-forage fibre sources did not influence ADG significantly in most of the previous studies (Porter et al. 2007; Hill et al. 2008a; Hill et al. 2008b) which is in accordance with observations of present study. Maktabi et al. (2016) recorded 582 g/d ADG in pre-ruminant male calves fed starter with no fibre source (NDF, 14.1%), while the ADG (g/d) was 657 and 605 in calves fed starter containing 10% (NDF 17.1%) and 20% (19.9%) beet pulp as non-forage fibre source. However, the pre-weaning ADG in the calves of the present investigation was comparatively higher than the earlier studies (Porter et al. 2007; Hill et al. 2008a; Hill et al. 2008b; Göncü et al. 2010; Daneshvar et al. 2017) which could be attributed to higher milk intake of calves in the present study due to step up/ step down method of milk feeding and longer pre-weaning period.

Feed conversion efficiency

The calves, in general, exhibited better feed conversion efficiency (calculated considering starter intake and ADG) during the initial period of the study as compared to the final period (Table 7) which could be attributed to a higher intake of milk during the first few weeks of life. Increased calf starter intake with advancing age might have contributed for poor efficiency towards the end of the pre-weaning period. The higher feed conversion efficiency ($P<0.05$) observed in F_2 calves than F_1 and F_3 calves in the first fortnight might be due to inefficient utilization of milk. However, relatively lower feed efficiency (9.8%) observed in F_2 calves during the fourth fortnight could be attributed to relatively lower calf starter DM intake. The overall feed conversion efficiency in F_1, F_2 and F_3 claves was 0.61, 0.60 and 0.59, respectively and was not influenced by varying levels of fibre and starch in the calf starters.

More or less similar feed efficiency was observed in some of the earlier experiments with pre-ruminant calves (Hill et al. 2009; Omidi-Mirzaei et al. 2015; Maktabi et al. 2016) and the feed efficiency was not influenced due to varying levels of fibre in calf starters in pre-ruminant calves (Maktabi et al. 2016). However, extremely high (Göncü et al. 2010) or low (Fokkink et al. 2011; Daneshvar et al. 2017) feed efficiency was observed in other studies involving pre-ruminant calves. The difference in feed efficiency between studies could be attributed to variations in milk/milk replacer feeding, weaning age, type of calf starter, breed and environment.

Table 7 Effect of varying levels of fibre and starch in calf starters containing finely ground maize grain on fortnightly and overall feed conversion efficiency in pre-ruminant dairy calves

Fortnight	F_1	F_2	F_3	SEM	P value
1	0.10±0.03[b]	0.24±0.04[a]	0.09±0.02[b]	0.023	0.009
2	0.28±0.07	0.35±0.06	0.32±0.04	0.034	0.698
3	0.50±0.08	0.47±0.85	0.51±0.06	0.042	0.950
4	1.12±0.09	1.01±0.14	1.12±0.11	0.066	0.746
Overall	0.61±0.06	0.60±0.08	0.59±0.04	0.037	0.975

Table 8 Cost economics of feeding calf starters with varying levels of fibre and starch containing finely ground maize grain in pre-ruminant dairy calves

Parameter	F_1	F_2	F_3	SEM	P value
Feed intake/day (kg)	0.456±0.06	0.420±0.08	0.448±0.06	0.037	0.927
Cost of feed/kg (Rs.)	22.71	22.80	22.87	-	-
Cost of feed/day (Rs.)	10.38±1.42	9.58±1.87	10.25±1.40	0.863	0.929
Cost of feed/kg gain (Rs.)	14.84±1.75	14.07±2.10	14.25±1.13	0.931	0.946

Table 9 Effect of varying levels of fibre and starch in calf starters containing finely ground maize grain on blood biochemical profile in pre-ruminant dairy calves

Constituent	F_1	F_2	F_3	SEM	P value
Glucose (mg/dl)	92.01±1.80	89.47±1.38	87.48±1.30	0.933	0.139
Total protein (g/dl)	7.16±0.09	7.18±0.06	7.22±0.09	0.046	0.881
Albumin(g/dl)	3.95±0.07	3.99±0.02	3.98±0.04	0.029	0.874
Globulin(g/dl)	3.21±0.12	3.20±0.07	3.24±0.10	0.056	0.949
Albumin:globulin	1.24±0.06	1.25±0.03	1.23±0.04	0.276	0.972
Blood urea nitrogen (mg/dl)	13.92±0.58	13.40±0.32	13.43±0.19	0.226	0.602
β- hydroxy butyrate (mmol/l)	0.38±0.02	0.37±0.03	0.30±0.03	0.016	0.09

Cost economics

There was no much variation in cost of the ground maize grain and soybean hulls which varied among calf starters. Hence, the cost of the calf starters was more or less similar among treatments (Table 8). The feed cost per kg gain was 59 paise less in calves fed calf starter F_3 as compared to those calves fed calf starter F_1 which could be attributed to relatively better body weight gain and feed conversion efficiency. The primary objective of pre-weaning calf nutrition is to achieve maximum ADG. Even though, the lowest cost per kg gain was recorded in calves fed calf starter F_2, the body weight gain in this group was relatively lower and therefore, the advantage on feed cost/kg gain may not be favourably considered.

Initiation of rumination

Initiation of rumination in pre-ruminant calves indicates the beginning of rumen development. The average days required for initiation of rumination in the present study were 17.17±1.72, 17.33±1.02 and 18.67±1.25 in F1, F2 and F3 groups of calves and the differences among the treatments were not significant. The varying fibre levels (NDF, 18.33 to 29.89%) in the calf starters did not affect initiation of rumination in pre-ruminant calves. Similarly, Porter et al. (2007) also reported that fibre levels (NDF, 16.90 vs 29.06%) in calf starters did not affect initiation of rumination in pre-ruminant Holstein calves.

Blood biochemical profile

Calf starters containing varying levels of fibre and starch fed to calves did not significantly ($P>0.05$) influence serum glucose, total protein, albumin, globulin, albumin: globulin and BUN and blood BHBA levels (Table 9) suggesting that the level of starch or NDF in calf starters may not influence blood biochemical constituents.

The BUN in the blood of pre-weaned calves is indicative of initiation of urea recycling and absorption during rumen development (Hayashi et al. 2006). The BUN levels observed in the calves of the present study at 63 day of age indicated the possibility of a higher level of ammonia in the rumen due to degradation of starter proteins.

The blood BHBA (mmol/l) in calves fed calf starters F_1, F_2 and F_3 at 63 day of age was 0.38, 037 and 0.30, respectively and was not significantly different among treatments. However, BHBA concentration was 21% higher in calves fed calf starter (F1) containing 40% maize than calves fed calf starters containing 20% maize. Increase in blood BHBA was observed earlier with increasing grain intake in calves (Quigley et al. 1991).

Conclusion

Varying levels of starch and fibre in calf starters containing FGMG (57.72% of particles with a size of 1.18 mm above) had no influence on overall starter DM intake, ADG, feed conversion efficiency, initiation of rumination, faecal score and blood biochemical constituents in pre-weaning dairy calves. Feeding milk by step up/step down method and calf starter containing finely ground maize grain and soybean hulls in the ratio of 20:30 without offering any roughage can be recommended to achieve maximum pre-weaning average daily gain (700 g/d) in HF cross bred female calves.

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RESEARCH ARTICLE

Prostasomes as interlocutor for zygotic epigenome health: role of prostasome secretome in maintenance of zygotic epigenome

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Abstract: Prostasomes as transgenerational epigenetic toolbox has not been established, its role in transferring small RNA, lncRNA as paternal messages is yet to be explored. The present study was undertaken to establish role of prostasomes in modulating testicular and vaginal environmental cues such as protein and RNA messages through to zygote and thereby acting as interlocutor of paternal genome. The results from the study found out prostasomes fusion with spermatozoa takes place at the time of maturation and capacitation wherein it causes downregulation of ROS (Protein and mRNA) in terms of decreased NADPH and NOS activity significantly ($p<0.01$), it was also found to modulate mitochondrial membrane potential significantly ($p<0.01$). Prostasome supplementation lead to increased survival of spermatozoa in sp-TALP medium and was found to be superior to cryopreserved and fresh semen when used in routine in vitro fertilization (IVF) experiment. The study further envisages to establish prostasomes as repository of novel biomarkers for infertility in male and female and as diagnostic tool in in vitro fertilization and somatic cell nuclear transfer protocol and thereby establishing prostasomes as one of the fundamental vesicles influencing transgenerational transfer of RNA and protein to zygotic genome thereby influencing zygotic genome health.

Keywords: Zygotic genome, ROS, transgenerational, prostasome, small RNA, Secretome.

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Introduction

Prostasomes are extracellular vesicles that fuse with sperm cells in the acidic environment of the vagina thus modifying the composition of the spermatozoan membranes. (Kravets et al. 2000) reported that prostasomes harbor numerous enzyme systems, many small signaling molecules and neuroendocrine markers, thus these vesicles may play a complex role in regulation of sperm viability and in facilitation of the fertilization process. Besides, the prostasomes have been implicated in several other reproductive functions, such as the improvement of sperm motility, capacitation, and acrosome integrity and acrosome reaction of sperm as well as coagulation and liquefaction of seminal fluid in humans (Wasylewska and Wasylewski, 2007). Role of mitochondrial DNA (mtDNA) methylation and demethylation with respect to sperm health is still unknown. The genome health of healthy spermatozoon is maintained by seminal plasma and its secreted vesicles through its crosstalk with mtDNA by its secretome comprising of ncRNA, eRNA and other putative RNA enzymes which contributes to mitochondrial genome and seminal plasma exosomes (Epididymosomes and Prostasomes). The role of ncRNA, tRNA and other RNA enzymes has not been elucidated *vis –a-vis* zygotic genome health. There is dearth of literature w.r.t. RNA enzymes both de novo in spermatozoa and Extracellular Vesicles (EV, Prostasomes and Epididymosomes), low mitochondrial membrane potential and high ROS production have been detected in spermatozoa from infertile patients (Wang X et al. 2003) only one report has been noted regarding long range genome silencing in spermatozoa (Bohacek J et al. 2020). Epididymosomes (vesicles that fuse with sperm during epididymal transit) carry RNA payloads matching those of mature sperm and can deliver RNAs to immature sperm in vitro (Nejabati, HR et al. 2021) the role of Prostasomes which contributes to about 20% of total RNA and small RNA content of spermatozoon has not been studied.

The present study was undertaken to establish role of prostasomes in modulating spermatozoa health both morphologically and biochemically. The study envisaged to study how prostasome which fuses with spermatozoa transfer small RNA (ncRNA, eRNA, lncRNA) to spermatozoa and how ROS affects these small RNA to be transferred to spermatozoa and

then to zygote impinging on "zygotic genome health" after fertilization. The study found out that supplementation of Prostasome during maturation of spermatozoa significantly reduced ROS which might affect mitochondrial DNA (mtDNA) mtDNA as a loosely packaged structure, therefore, it is more easily damaged by ROS than the nuclear genome (Yakes F.M and Van Houten B 1997). Point mutations, rearrangement and/or decreased content of mtDNA are all features correlated with sperm dysfunctions and infertility (Luo S.M et al. 2013, Rosati A.J., et al. 2020). Conversely, a low mtDNA copy number has been suggested as an indicator of good-quality sperm (May-Panloup P et al. 2003); thus, its manipulation may be a powerful therapeutic strategy to decrease aging-associated mtDNA mutations (Jiang M et al. 2017). Interestingly, supplementation of prostasomes in sp-TALP improved maturation of spermatozoa and had favorable impact on motility, ATP production, ROS production. It reduced ROS production in vitro significantly ($P<0.001$), the effect of reduced ROS production on epigenetic processes are not fully described in spermatozoa the field of epigenome health is unexplored in spermatozoa.

The present study was undertaken to establish that prostasomes which carry payload of small RNA and other discrete RNA enzymes are the factors which modulate spermatozoan epigenome and envisages to elucidate prostasome secretome and its potential role in spermatozoon reprogramming thereby elucidating role of prostasome secretome in paternal epigenome mediated transgenerational transfer and metabolic heredity.

Material and methods

Sephadex G-200 was purchased from GE Healthcare, Uppsala, Sweden. FITC-conjugated CD26 antibody (orb13855) and Caveolin-1 antibody (orb15247) were purchased from Biorbyt Ltd., 5 Orwell Furlong, Cowley Road, Cambridge, Cambridgeshire, CB4 0WY, United Kingdom. Cholesterol Assay Kit (E2CH-100) was purchased from BioAssay Systems, 3191 Corporate Place, Hayward, CA 94545, USA. Phospholipid assay kit (MAK122) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Bovine Reactive Oxygen Species (ROS) ELISA Kit (Cat. No: MBS029376) was purchased from MyBioSource, San Diego, California, United States. Protein Estimation Kit by Biuret method, 250 reactions (KT19) was obtained from Geneilabs.

Isolation of the Prostasomes

Prostasomes were isolated from semen of both Karan Fries and Sahiwal bulls according to the protocol of (Siciliano et al. 2008) with slight modifications. Briefly, eight to ten ejaculates (mass activity > or = +++) were collected from Sahiwal and KF bulls (n=6 from each breed) and 20 mL of seminal plasma were pooled together separately and processed soon for prostasomes isolation. Semen was centrifuged for 10 min at 1,000 g in order to separate spermatozoa and possible other cells from the seminal plasma, and further ultracentrifuged at 10,000 g for 20 min to pellet possible cells and cell debris. The supernatant was subsequently subjected to another ultracentrifugation for 2 h at 100,000 g to pellet the prostasomes. The prostasomes were resuspended in a Tris-HCl buffer (30 mM, pH 7.6) made isotonic with 130 mM NaCl. The suspensions were purified by Sephadex G-200 (GE Healthcare, Uppsala, Sweden) chromatography, to separate them from an amorphous substance at 6 mL/h, and 2 mL fractions were collected. The eluant was the isotonic Tris-HCl buffer, and the eluate was monitored at 260 and 280 nm. Those fractions with elevated absorbances at 260/280 nm were collected and analysed for aminopeptidase activity, a marker enzyme for prostasomes (Laurell et al. 1982). Those fractions with relatively high aminopeptidase activity were pooled and ultracentrifuged at 100,000g for 2h. The pellet representing the prostasomes was resuspended in the isotonic Tris-HCl buffer and adjusted to a protein concentration of 0, 0.5, 1, 1.5, and 2 mg/mL using a Protein Assay kit (Biuret method-based kit), for standardization of sperm protein ($20x10^6$ sperms) to prostasome protein ratio isolated prostasomes were subjected to chemical composition evaluation. Isolated prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids, in both Karan Fries and Sahiwal bull semen (Table 1). The variations in individual constituent of the prostasomes were not significant ($P>0.05$) during different seasons in both Karan Fries as well as Sahiwal bulls.

Estimation of ROS and its effect on motility

Standardization of dose dependent response of prostasomes on sperm motility

To standardize the dose of prostasomes to be used for assessing sperm motility, 20 million immobilized sperms of both Karan Fries and Sahiwal bulls were incubated with different concentrations of prostasomes viz. 0, 0.5, 1, 1.5 and 2 mg/ml in sp-TALP for 1h at 37ÚC, 5% CO_2 in CO_2 incubator. After incubation, Neubauer's chamber of hemocytometer was charged with 0.1 µL of the above sample containing around 2000 spermatozoa. Number of motile sperms passing across a border line of RBC counting chamber was counted for 1 min. Both Sahiwal and Karan Fries sperms showed a dose dependent change in motility. Sperm motility increased significantly ($P<0.01$) up to 1 mg/ml of prostasomes followed by a non-significant ($P>0.05$) elevation up to 2mg/ml

Table 1 Chemical composition of prostasomes isolated from pooled seminal plasma of Karan Fries and Sahiwal bulls

Composition Breed	Protein (g%)	Cholesterol (mg%)	Phospholipids (mg%)
Karan Fries	2.44±0.06	21.99±0.17	11.67±0.27
Sahiwal	2.85±0.05	24.51±0.32	12.66±0.29

prostasomes concentration (Table 2). Since the sperm motility did not vary significantly (P>0.05) from 1 to 2 mg/ml concentration of prostasomes, 1mg/ml concentration of prostasomes was selected for further experiments. The optimum sperm protein: prostasome protein ratio, obtained as 1:2 (the combination of $20x10^6$ sperms/mL and prostasomes protein concentration 1mg/ml) was subsequently used to study the functional parameters of spermatozoa

Acrosomal Integrity Test by Giemsa's Stain

Staining was carried out as per the procedure described by (Chowdhury et al. 2014). The stock Giemsa's stain was prepared as per manufacturers' protocol. Briefly, to study the effect of prostasomes on acrosome integrity, the spermatozoa and prostasomes were incubated together in sp-TALP media. Initially, motile sperms were obtained from fresh semen by swim up technique. Their concentration was adjusted to $20x10^6$ sperms/mL in one mL of sp-TALP medium. Cryopreserved semen ($20x10^6$ sperms in 0.25 mL of extended semen) was added to 0.75 mL of sp-TALP. The test (prostasomes supplemented) and control (not supplemented with prostasomes) samples were incubated for 1 hour in CO_2 incubator with 5% CO_2 at 38ÏC. Then, 3 µL of Sorenson Phosphate Buffer (SPB) was put into clean grease free pre-warmed slide, to which 30 µL of sp-TALP from test and control samples (before and after incubation) was mixed and a smear was drawn and air dried. The slides were put into 5% formaldehyde solution for fixing at 37 ÚC for 30 min (Campbell et al. 1960). The slides were removed and washed and was counted for acrosomal integrity (membrane fragility and phospholipid) by staining with working geimsa solution under oil immersion (100x).

Estimation of Cholesterol Content in Prostasomes

Purified prostasomes were used for estimation of their cholesterol content. Cholesterol content was estimated by using ELISA kit (E2CH-100) procured from BioAssay Systems, 3191 Corporate Place, Hayward, CA 94545, USA. The range of linear detection was 1 to 100 mg/dL cholesterol. Briefly, 50 µL-diluted standards were transferred into wells of a clear 96-wells plate. 50 µL diluted prostasome samples were transferred in separate wells. For each reaction well, 55 µL Assay Buffer was mixed with 1 µL Enzyme Mix and 1 µL Dye Reagent. 50 µL of this Working Reagent was added to each standard and sample well. Plate was tapped to mix it well. The plate was incubated for 30 min at room temperature. The O.D. values were measured at 570 nm using micro scan MS-5608A plate reader. Standard curve was obtained by plotting the absorbance (vertical axis) of the standards against their concentration (horizontal axis) using 4- parameter logistic regression. The concentration of the samples was determined from the plotted standard curve.

Estimation of Phospholipids Content in Prostasomes

Purified prostasomes were used for the estimation of their phospholipids content. Phospholipids content was estimated by using Phospholipid assay kit (MAK122), which was purchased from Sigma-Aldrich, St. Louis, MO, USA. The range of linear detection was 3–200 mM. Briefly, the standards were prepared by adding 24 µL of the 2 mM Phosphatidylcholine standard to 216 µL of water to prepare a 200 µM standard working solution. Thereafter, 0, 30, 60, and 100 µL of the 200 µM standard working solution was transferred into tubes. Water was added to each tube to bring the volume to 100 µL, generating 0 (blank), 60, 120, and 200 µM standards. Transfer 20 µL of standards into separate wells of 96 well plate. The samples were estimated in 20 µL aliquots of each sample into two separate wells of a 96 wells plate. Thereafter, 80 µL of the appropriate Reaction Mix was required for each reaction (well). Reaction Mixes were allowed to equilibrate to room temperature. 80 µL of the appropriate reaction mix was added to each well and mixed well. Then, the plate was incubated for 30 min at room temperature in darkness. The absorbance of the samples and standards was measured at 570 nm.

Prostasomes supplementation and its effect on the concentration of reactive oxygen species (ROS)

Fresh semen (mass activity > or = +++) was initially subjected to swim up procedure. Motile spermatozoa were obtained by swim up procedure and concentration was adjusted to $20x10^6$ in one mL of sp-TALP media. Cryopreserved straws ($20x10^6$ sperms per straw) were directly used in the study. Entire content of a straw (0.25 mL) was added to 0.75 mL of sp-TALP (so that final volume was 1mL). The prostasomes supplemented and control sperm samples obtained from various semen types were incubated in

Table 2 Effect of different concentrations of prostasomes on motility of immobilised sperms of Sahiwal and KF bulls

Prostasomes (mg/ml)	Sahiwal		Karan Fries	
	No. of sperms motile	% Motility	No. of sperms motile	% Motility
0	5.0 ± 1.5^a	0.25	6.0 ± 0.6^a	0.30
0.5	24.0 ± 3.1^b	1.20	23.3 ± 3.2^b	1.16
1	45.7 ± 1.8^c	2.25	43.3 ± 1.9^c	2.16
1.5	49.7 ± 1.5^c	2.48	49.7 ± 0.9^c	2.48
2	52.0 ± 1.2^c	2.60	51.3 ± 1.5^c	2.56

Mean ± S.E. values with different superscripts differ significantly (P<0.05) within a group.

sp-TALP for one hour at 37 ÚC and 5% CO_2. ROS concentration in the sp-TALP was estimated at zero, 20, 40, and 60 minutes by ROS estimation ELISA kit (Cat. No: MBS029376) as per manufacturers protocol. The sensitivity of this kit was 5.0 IU/mL. The detection range of this kit was 31.2 - 1000 IU/mL

Mitochondrial membrane potential of spermatozoa (Anti apoptotic effect of prostasomes)

It was carried out in terms of mitochondrial membrane potential of spermatozoa. Motile sperms were obtained from fresh semen by swim up technique. The concentration was adjusted to 20x 10^6 sperms/mL in 1 mL of sp-TALP medium. Cryopreserved semen (20x10^6 sperms in 0.25 mL of extended semen) was added to 0.75 mL of sp-TALP. The test (prostasomes supplemented) and control (not supplemented with prostasomes) samples were incubated for 1 h in CO_2 incubator with 5% CO_2 at 37ÚC. Mitochondrial membrane potential of spermatozoa was determined before and after incubation with prostasomes. Mitochondrial membrane potential was determined according to the kit protocol (Mitochondrial Permeability Transition Detection Kits, MitoPT™ JC-1 100 Test Kit – catalog no. 924).

Statistical analysis

Comparison of different semen parameter values of semen samples having similar mass activity was performed by univariate multiple analysis of variance (Bonferroni's multiple comparison test). Effect of different concentrations of prostasomes on motility of immobilised sperms of Sahiwal and KF bulls was analyzed by one-way ANOVA (Bonferroni's multiple comparison test). The effects of prostasomes supplementation as well as time of incubation on ROS production by spermatozoa, ATP concentration, calcium signaling, mitochondrial membrane potential, acrosome integrity, percent viability, and matrix metalloproteinase activity of spermatozoa was analyzed by one-way ANOVA (Bonferroni's multiple comparison test). Promotive effect of prostasomes on progressive motility of immobilized spermatozoa was compared to control using student's t test. Differences were considered significant at least at level $P<0.05$. SPSS 16.0 software was used for the statistical analysis. Microsoft excel worksheet was used for the preparation of graphs.

Results and discussion

Isolation of Prostasomes

Isolated prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids, in both KF and Sahiwal bull semen. The variations in individual constituent of the prostasomes were not significant ($P>0.05$) during different seasons in both Karan Fries as well as Sahiwal bulls (suppl table 1a, b) the prostasomes isolated conformed to molecular composition and lipid composition as per (Frenette G et al. 2002), and was established for first time in KF and Sahiwal bulls at National Dairy Research Institute (NDRI), Karnal, India.

Characterization of the Prostasomes

Prostasomes are microvesicles, with their membranes containing Caveolin-1 and CD26 antigens (the surface markers), and exhibit antibacterial and antioxidant properties. These features of prostasomes were used in the present study for their characterization.

Identification of surface markers (Caveolin-1 and CD26)

FITC-conjugated Caveolin-1 and CD26 antibodies binding with prostasomes were used for identification of Caveolin-1 and CD26 antigens on prostasomes in this study. Prostasomes immunostained positively for FITC-conjugated Caveolin-1

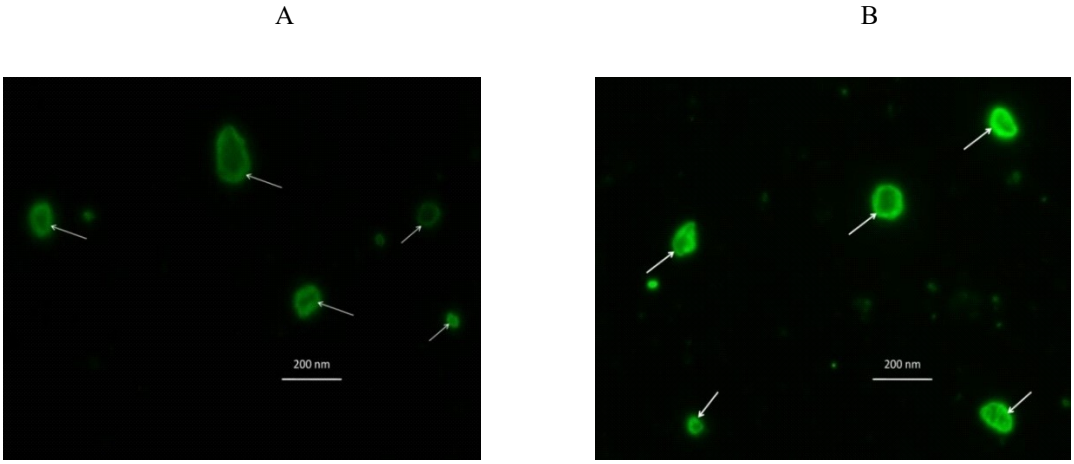

Fig. 1 Fluorescent micrograph of binding of (A) FITC-conjugated Caveolin-1 antibodies (B) FITC-conjugated CD 26 antibodies with the prostasomes membrane (indicated by arrows).

antibodies and FITC-conjugated CD 26 antibodies as depicted in Figure 1.

Effect of prostasomes on progressive motility of immobilized spermatozoa

Unpaired t-test analysis revealed that prostasomes significantly ($P<0.01$) promoted the recovery of motile spermatozoa in swim up media as compared to the control in both the breeds. The average recovery of motile spermatozoa in case of Karan Fries and Sahiwal fresh semen supplemented with prostasomes were 49.5±0.79 and 49.83±0.83 motility per min respectively, while the respective values in case of Karan Fries and Sahiwal cryopreserved semen were 33.22±1.03 and 34.05±0.49 motility per min. Thus, the recovery of motile spermatozoa in treatment group was more prominent in case of fresh semen of Karan Fries (2.47%) and Sahiwal bulls (2.49%) as compared to the cryopreserved semen of the Karan Fries (1.65%) and Sahiwal bulls (1.70%). The average values have been given in (Table 3) and depicted in (**Fig. 2)** Seminal plasma is a mixture of secretions from testes, epididymis and other accessory sex glands and its composition varies among animal species. For example, human and mouse seminal plasma contains secretions mainly from seminal vesicles (70–80% of the volume), and less from the prostate (20%), epididymides – testes (5%) and bulbourethral

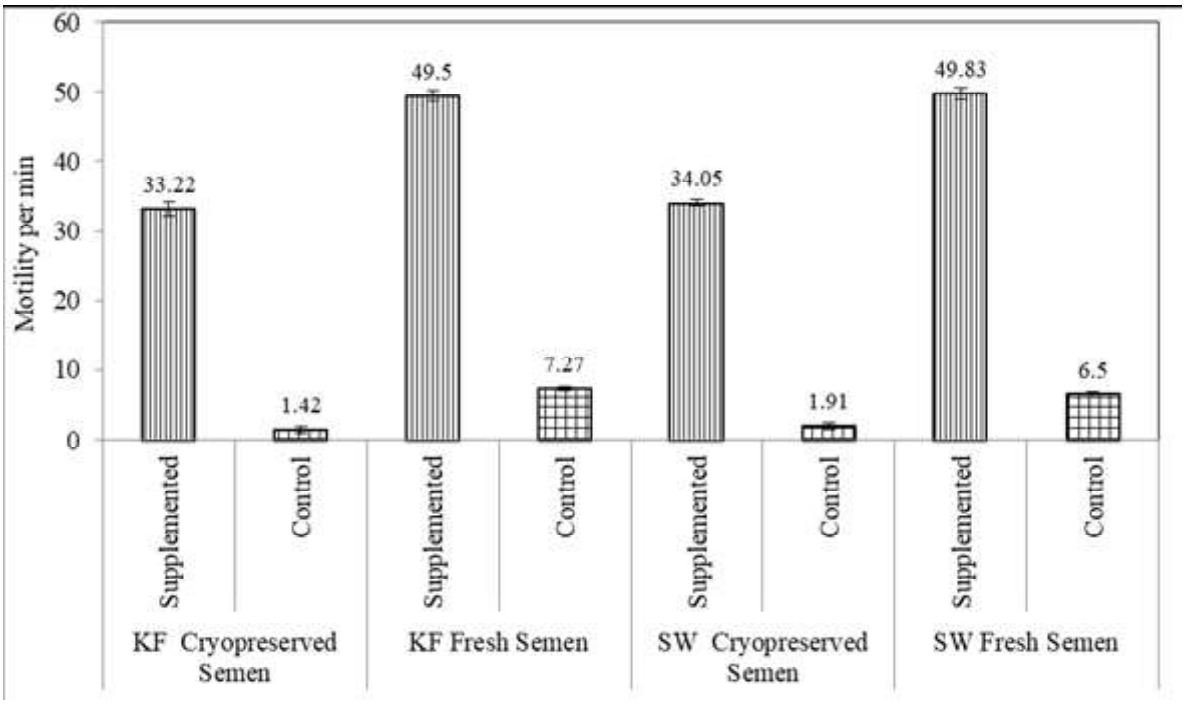

Fig. 2 Promotive effect of prostasomes on progressive motility of immobilised spermatozoa obtained from fresh and cryopreserved semen of Karan Fries (KF) and Sahiwal (SW) bulls

Table 3 Promotive effect of prostasomes on progressive motility of immobilised spermatozoa (motility per min) of fresh and cryopreserved semen of Karan Fries and Sahiwal bulls

Semen type	Treatment	Progressive Motility	
		Mean ± S.E.	Percent motility
Karan Fries Cryopreserved Semen	Supplemented	33.22±1.03[a]	1.65
	Control	1.42±0.34[b]	0.07
Karan Fries Fresh Semen	Supplemented	49.5±0.79[a]	2.47
	Control	7.27±0.48[b]	0.36
Sahiwal Cryopreserved Semen	Supplemented	34.05±0.49[a]	1.70
	Control	1.91±0.45[b]	0.09
Sahiwal Fresh Semen	Supplemented	49.83±0.83[a]	2.49
	Control	6.5±0.29[b]	0.32

For each semen type, values having different superscripts (small letters) vary significantly ($P<0.01$) within columns.

glands (<1%) (McGraw et al. 2015). Isolated prostasomes were subjected to chemical composition evaluation, the isolated prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids, in both KF and Sahiwal bull semen. The variations in individual constituent of the prostasomes were not significant (P>0.05).

Prostasomes supplementation and its effect on the concentration of reactive oxygen species (ROS)

ROS production was highest (P<0.01) in hot humid followed by hot dry and winter months respectively in Karan Fries as well as Sahiwal bulls in both cryopreserved and fresh semen samples. When the semen was supplemented with prostasomes (1mg/ml) and incubated for 60 min, the ROS concentration decreased significantly (P<0.01) with increase in incubation time. The decline initiated as early as 20min (P<0.01) in all three seasons in both the breeds. In KF cryopreserved semen the concentration averaged 48.91±2.16 IU/ml (0 min), 26.45±1.57 IU/ml (20 min), 20.50±1.19 IU/ml (40 min), and 18.33±0.31 IU/ml at 60 min (Table 4 and Figure 3). Similar type of response, but of significantly (P<0.01) lower magnitude, was seen in fresh semen obtained from Karan Fries bulls (Table 4 and Fig. 3). The values averaged 17.47±1.14 IU/ml (within 0 min), 6.82±0.48 IU/ml (20 min), 4.52±0.24 IU/ml (40 min), and 3.29±0.19 IU/ml (60 min).

The univariate multiple ANOVA revealed that ROS production was significantly (P<0.01) reduced by different factors namely breed, season, type of semen, prostasomes treatment, and time of incubation. It was significantly higher in prostasome supplemented KF (P<0.01) as compared to prostasome supplemented Sahiwal semen. The concentration was significantly elevated (P<0.01) in cryopreserved than fresh semen in both the breeds. The highest (P<0.01) concentration was observed in hot humid followed by hot dry and winter months. Incubation of sperms with prostasomes significantly lowered the ROS production (P<0.01), and by the end of 1h, the concentration was found to be lowest in both the breeds. The decline in ROS production was high in fresh semen of both breeds the ROS measured the production of nitric oxide (NOS) and nascent oxygen (0_2^-) produced during the time period of 0-60 min. The ROS was assayed by ELISA utilizing Kit (Cat. No: MBS029376)

Table 4 Effect of Prostasomes on *in vitro* ROS production (IU/ml) by Karan Fries sperms cryopreserved during different seasons

Season	Treatment	Time (min)			
		0	20	40	60
Hot dry	Supplemented	50.24±3.17^{bA}	26.01±0.56^{aA}	19.46±0.43^{aA}	18.17±0.59^{aA}
	Control	49.78±2.92^{aA}	46.52±2.70^{aB}	43.81±2.39^{aB}	42.78±2.69^{aB}
Hot humid	Supplemented	54.24±2.44^{bA}	31.01±3.19^{aA}	23.14±3.42^{aA}	18.90±0.53^{aA}
	Control	55.41±3.02^{aA}	52.52±2.78^{aB}	50.24±2.59^{aB}	49.44±3.14^{aB}
Winter	Supplemented	42.24±1.67^{bA}	22.36±0.64^{aA}	18.89±0.19^{aA}	17.93±0.48^{aA}
	Control	43.11±1.78^{bA}	40.52±1.02^{abB}	37.81±1.43^{abB}	35.78±0.78^{aB}
Overall Mean	Supplemented	48.91±2.16^{bA}	26.45±1.57^{aA}	20.50±1.19^{aA}	18.33±0.31^{aA}
	Control	49.43±2.21^{aA}	46.52±2.08^{aB}	43.96±2.11^{aB}	42.67±2.31^{aB}

For each season, values having different superscripts (small letters) vary significantly (P<0.01) within rows, while values having different superscripts (capital letters) vary significantly (P<0.01) within columns.

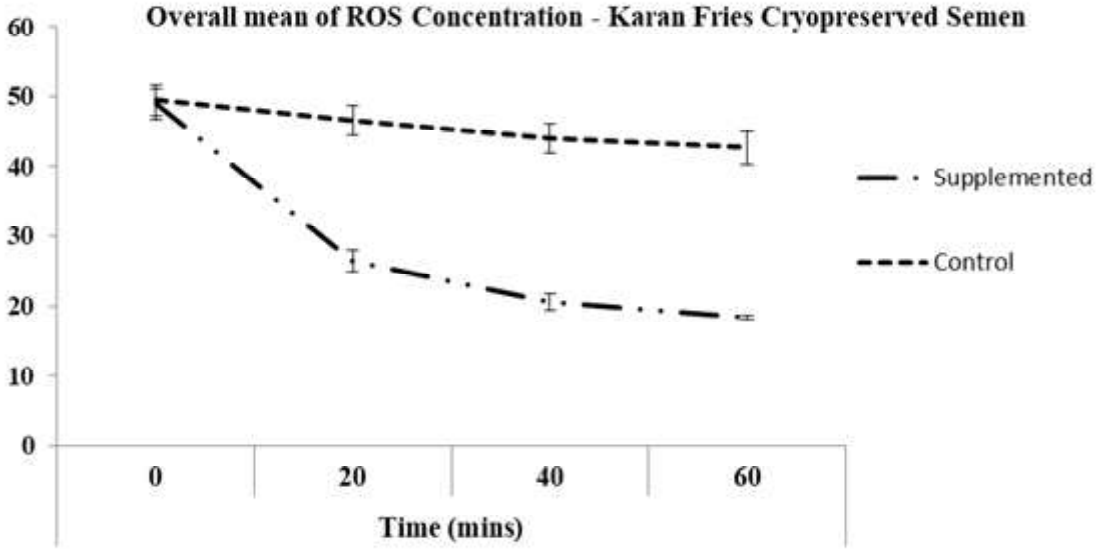

Fig. 3 Prostasomes effect on ROS production by Karan Fries cryopreserved spermatozoa

as per manufacturers instruction. The significant drop in ROS production (Table 5, Fig 4) is due to lowered NADPH levels both in cryopreserved and Fresh semen, the ROS decline is predictive of spermatozoa health and further zygote health.

The ROS decline noticed in fresh and cryopreserved semen may be selected for insemination and in vitro fertilization (IVF) as the prostasome was used in FERT-TALP (in vitro fertilization media) was shown to promote maturation of spermatozoa and leads to significant release of pronuclei in in vitro fertilized oocyte providing evidence that prostasomes are major vehicles for intergenerational transfer of RNA to spermatozoa. The reduced ROS levels were due to scavenging of nascent oxygen species (0_2^-) by electron transport chain through reduction of NADPH, present study is in corroboration with (Saez et al. 2000) who reported decreased in ROS production of seminal PMN cells and seminal plasma. The prostasome fuses with spermatozoa membrane forming spermatozoal seath surrounding mtDNA and tail piece the mechanism through which prostasome delivers the payload (small RNA and Protein) to spermatozoa is still unclear. The role of this nascent RNA and small RNA pool in prostasomes and epididymosmes are yet to defined, the mode of fusion of EV's especially prostasomes to spermatozoa and thereafter delivery of small RNA's needs to be addressed. The decline in ROS found after supplementation with Prostasome may be an indicator of epigenome health of spermatozoa can also influence zygotic health.

Effect of prostasomes on acrosome integrity of spermatozoa

To evaluate the effects of prostasomes on acrosome integrity, both fresh (mass activity > or = +++) as well as cryopreserved sperms ($20x10^6$) were incubated with prostasomes (1mg/ml) and without prostasomes for 1 h in an atmosphere of 5% CO_2 and 38°C temperature. Thereafter, acrosomal status of the spermatozoa was assessed by Giemsa's staining method. The results are expressed in (Table 6) and (Fig. 5).

In case of prostasomes supplemented Karan Fries cryopreserved semen sample, the decrease in mean percent acrosome integrity

Table 5 Effect of Prostasomes on *in vitro* ROS production (IU/ml) by Karan Fries sperms obtained from fresh semen during different seasons

Season	Treatment	Time (mins)			
		0	20	40	60
Hot dry	Supplemented	17.74 ± 0.77^{bA}	6.95 ± 0.55^{aA}	4.78 ± 0.13^{aA}	3.60 ± 0.17^{aA}
	Control	18.31 ± 1.03^{aA}	17.05 ± 0.95^{aB}	16.89 ± 1.21^{aB}	16.46 ± 1.35^{aB}
Hot humid	Supplemented	20.94 ± 0.56^{cA}	8.17 ± 0.43^{bA}	5.07 ± 0.35^{aA}	3.67 ± 0.14^{aA}
	Control	20.78 ± 0.64^{bA}	18.60 ± 0.63^{abB}	17.46 ± 0.62^{aB}	17.56 ± 0.35^{aB}
Winter	Supplemented	13.75 ± 1.29^{bA}	5.48 ± 0.71^{aA}	3.71 ± 0.15^{aA}	2.60 ± 0.17^{aA}
	Control	14.64 ± 0.71^{aA}	14.07 ± 0.63^{aB}	13.33 ± 0.89^{aB}	13.46 ± 0.86^{aB}
Overall Mean	Supplemented	17.47 ± 1.14^{cA}	6.82 ± 0.48^{bA}	4.52 ± 0.24^{abA}	3.29 ± 0.19^{aA}
	Control	17.91 ± 0.98^{aA}	16.80 ± 0.79^{aB}	15.85 ± 0.81^{aB}	15.82 ± 0.77^{aB}

For each season, values having different superscripts (small letters) vary significantly ($P<0.01$) within rows, while values having different superscripts (capital letters) vary significantly ($P<0.01$) within columns.

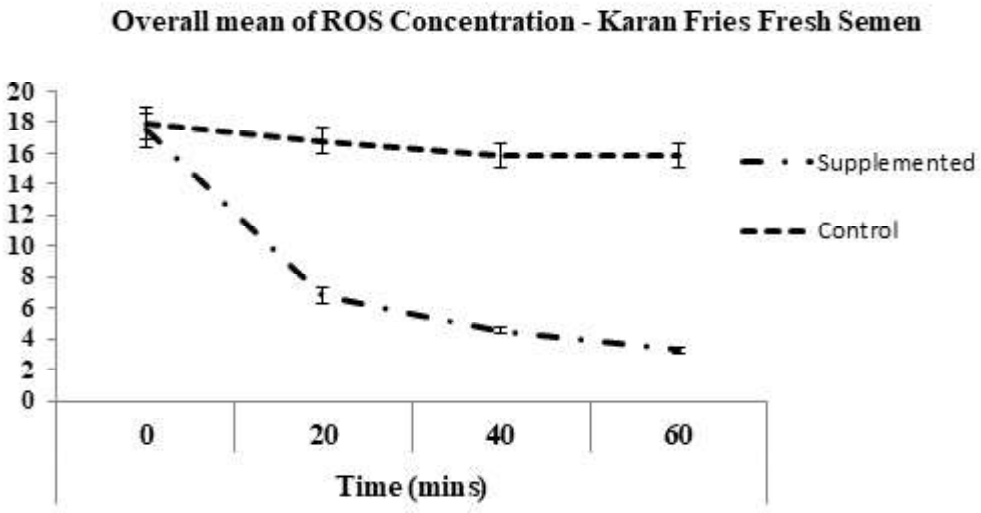

Fig. 4 Prostasomes effect on ROS production by Karan Fries fresh spermatozoa

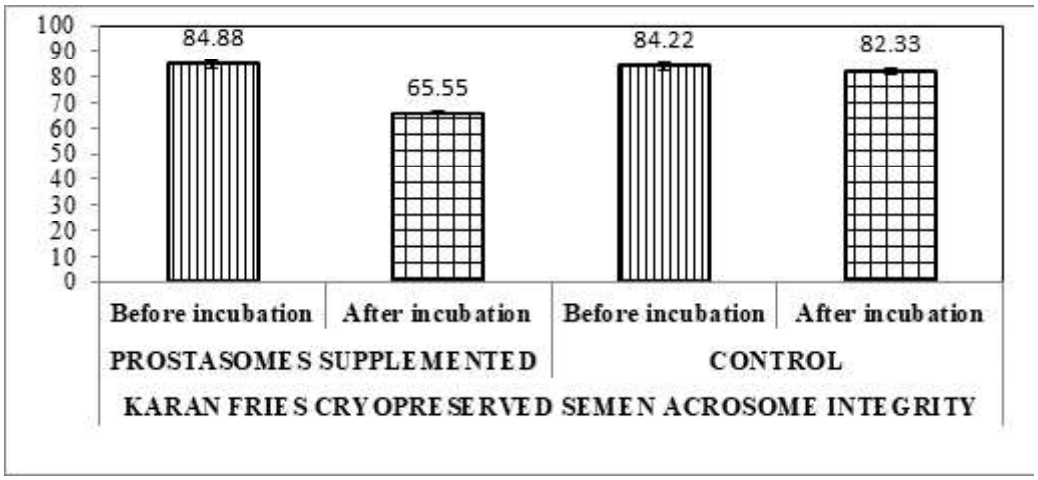

Fig. 5 Prostasomes effect on acrosome integrity of spermatozoa obtained from cryopreserved semen of KF bulls

Table 6 Mean ± SE values of acrosome integrity in KF cryopreserved and fresh semen

Season	Treatment	Cryopreserved		Fresh	
		Incubation Period			
		Before	After	Before	After
Hot dry	Supplemented	85.00±2.30^{bA}	81.0±0.50^{bA}	97.66±0.88^{bA}	85.33±2.02^{bA}
	Control	84.0±2.08^{bA}	64.33±1.45^{aB}	91.00±0.59^{bA}	73.66±2.33^{aB}
Hot Humid	Supplemented	79.33±1.76^{bA}	73.66±0.88^{bA}	95.66±0.33^{bA}	88.33±0.66^{bA}
	Control	80.33±1.45^{bA}	61.66±1.41^{aB}	90.00±0.55^{bA}	73.66±0.85^{aB}
Winter	Supplemented	90.33±1.20^{bA}	86.0±0.57^{bA}	94.66±0.66^{bA}	83.00±1.52^{bA}
	Control	88.33±1.66^{bA}	70.66±0.66^{aB}	91.00±0.60^{bA}	72.66±2.18^{aB}
Overall Mean	Supplemented	84.88± 1.82^{bA}	82.33± 1.11^{bA}	96.0± 0.55^{bA}	85.55± 1.08^{bA}
	Control	84.22± 1.45^{bA}	65.55± 1.47^{aB}	91.66± 0.33^{bA}	73.33± 0.97^{aB}

For each season, values having different superscripts (small letters) vary significantly (P<0.01) within rows, while values having different superscripts (capital letters) vary significantly (P<0.01) within columns.

from 84.88±1.82% to 82.33± 1.11% after one hour of incubation was not significant (P>0.05). However, acrosome integrity decreased significantly (P<0.01) from 84.22±1.45% to 65.55±1.47% after 1h of incubation in control semen samples (incubated with swim up media only). Similar type of results was obtained in case of KF fresh semen, and Sahiwal semen both cryopreserved and fresh as depicted. The present study found out that the destabilization of acrosomal membrane had cholesterol efflux during capacitation when in vagina and its phospholipid showed decline from 0hr through to 60min as was the trend for ROS and motility, thus establishing that prostasomes and other EV's plays a role in both capacitation and acrosome reaction, the study is in consonance with Soderquist et al. 1996. Secretome from the epididymis and accessory sex glands contain energy substrates, ions, proteins, RNAs and lipids, among others components, which could be found freely dissolved and in solution or encapsulated in extracellular vesicles or exosomes (Vojtech et al. 2014). Therefore, it seems obvious to think that the exosomes released by epididymis and accessory sex glands could play a role in the communication between seminal plasma and sperm. There is increasing evidence showing that exosomes detected in different body fluids participate in intercellular communication, through the selective incorporation of their cargo into the target cell (Keerthikumar et al. 2016). This functional involvement has been proposed for exosomes released by the epididymides (epididymosomes), which are capable of modifying the lipid composition of the sperm membrane, thus contributing to the acquisition of sperm motility potential (Sullivan and Saez 2013). The present study contributes on the shortcomings of keerthikumar et al. 2016 and Sullivan and Saez 2013 by finding that prostasomes may be one of the significant partners contributing to seminal plasma its small RNA component which causes decreased NOS concentration and thereby favoring zygotic genome activation (ZGA). Additionally, many studies have shown the role of prostate-derived exosomes (prostasomes) in the stimulation of sperm motility while avoiding at the same time premature capacitation and spontaneous acrosome reaction (Aalberts et al. 2014), the present study is in consonance with Aalberts et al. 2014 with regards to acrosome reaction and capacitation and found that both the parameters were

upregulated with shortened time for capacitation in KF and sahiwal breeds. Of note, exosomes contained in seminal plasma have been reported to increase the expression of immune- and inflammatory response-related genes in porcine endometrial tissue, reaching similar levels as those observed in the endometrium from naturally mated pigs (Bai et al. 2018). This suggests that communication between seminal plasma and the female reproductive tract could also be through the interaction with seminal exosomes.

Role of Prostasomes as interlocutor of Zygotic Genome Health

Prostasomes deposits and transfer RNA payload to spermatozoa during its transit from seminiferous tubule through to epididymis and prostate they deposit DNA, RNA and proteins to spermatozoa which may be transgenerationally transferred, it has been reported that spermatozoa inherits tRNA Fragments (Sharma U et al. 2016), spiRNA, small RNA, lncRNA, ncRNA forming sperm transcriptome, it remains unresolved as to how environmental cues from testicular, oviductal, vaginal, epididymis and prostate is transferred through to spermatozoa and then to zygote modulating spermatozoan and zygotic genome, whether this is epigenetically regulated by deposition of histone ubiquitins (H2Aub) molecules and coordinated by epigenetic marks for histone demethylase and DNA demethylase as early as 2 cell zygote at the before start of minor ZGA in both mouse and buffalo (*Bubalus bubalis*). The study proposes to further on the findings by establishing role of Paternal environment in transgenerational transfer of spermatozoal DNA as broader scope of embryonic genome health.

The present study establishes prostasome as interlocutor of epigenetic transfer of proteins and small RNA to spermatozoa as a phenomenon associated with transgenerational transfer of testicular epigenetic modifications. It also emphasizes on spermatozoon environmental cues like ROS and mtDNA modification especially small RNA mediated enhancer like control of zygotic genome as major mechanism governing zygotic genome health with Prostasomes as one of the fundamental vesicles influencing transgenerational transfer of RNA and protein to zygotic genome thereby influencing zygotic genome health.

Conclusions

Prostasomes have been identified as extracellular vesicular bodies which has effect on spermatozoon maturation. Its role in maintaining embryo epigenome health has not been studied. The present study establishes prostasomes as major extracellular vesicle body affecting spermatozoon fertilizing ability through transfer of small RNA molecules or "gemmules" thereby contributing to fertilizing ability, transgenerational transfer of epigenome and genomic molecules to zygote thereby acting as interlocutor of zygotic genome health. The prostasomes and to some extent epididymosomes maintains zygotic genome health through modulation of spermatozoan genomic and epigenomic payload thereby maintaining spermatozoon fertilizing ability and further influencing zygotic genome health. The study demonstrates that prostasomes when supplemented in sp-TALP medium was superior to normal fresh semen and cryopreserved semen in routine in vitro fertilization (IVF) experiment as they transfer "gemmules" or RNA transcripts which influences the zyogotic (zygotic) health via epigenomic transfer of RNA transcript transgenerationally indicating towards the future scope about utilizing prostasomes for augmenting and increasing the efficacy of IVF and SCNT methodologies especially as the prostosomes and to some extent epididymosomes in combination can be used in IVF and SCNT protocols to generate high quality, superior blastocysts, zygote and fetus as they influence the zygotic genome health. The prostasomes as the extra vesicular organelles loaded with RNA transcript (spiRNA, eRNA, tRNA, lncRNA) has not been studied to modulate the epitranscriptome of zygote, with the advent of scRNAseq and Hi-C technologies the role of these payloads in modulating "zygotic genome health" in routine IVF and SCNT technologies will be defined. We have previously defined the role of GLUT1 and HSP70.1 in regulating Zygotic Genome Activation (ZGA) in buffalo (*Bubalus bubalis*) in routine IVF, The role of prostasomes in regulating ZGA and paternal genome contribution to zygotic genome activation and zygotic genome health has to be elucidated. The potential of combination of Prostasome and epididymosome as supplement in sp-TALP and SCNT protocols has to be defined as paternal genome unification is highly error prone and subjected to intense methylation and RNA enzymes processing it would be of interest to define and identify the biomarkers form Prostasomes which contribute to zygotic genome health.

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RESEARCH ARTICLE

Divulging the magnitude of socio-economic empowerment of dairy women in East district of Sikkim

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Abstract: Farm women are responsible for sustainable food and livelihood security. A study was undertaken to measure the socio-economic empowerment of dairy farm women in East district of Sikkim with sampling size of 200 on the year 2021. An index was prepared for measuring the empowerment scores of farm women. The criteria for selection of farm women were based on possession of at least one milch cattle. The study revealed that, 36.50 percent of the respondents possessed high empowerment scores, while 35.00 percent of the respondents possessed medium empowerment scores and only 28.50 percent possessed low empowerment score. There was a significant difference in the empowerment scores of socio-economic variables like, age, education, marital status, milk production per day (in lit), social status, farming experience in dairying, annual income through dairying and occupation. Young age category was having the highest empowerment scores with 81.48±1.16. Graduate and above category also possessed high empowerment scores with 87.56±1.96. In the case of social status, Other Backward Caste had high empowerment score with 81.46±2.68. Respondents whose occupation was government service+ dairy farming possessed high empowerment scores with 89.10±2.37 while, medium annual income category had the highest empowerment scores with 76.80. Variables like, age, education, marital status, farming experience in dairying, family size, occupation had a significant association with the empowerment score. The study suggests suitable reform in extension service of the state which will mobilize the potential of dairy farm women to adopt sustainable dairy technology and create a means for additional income.

Keywords: Farm women, Sustainable, Socio-economic empowerment**Introduction**

Empowerment refers to the authority given to the under privileged individual or a community in terms of social, economic, political aspect which enables them to make their own decisions and live life according to their own choices. Dairy farming is one of the ways to empower farm women by providing means of extra income for sustainable livelihood. Women's participation in economic activities increased after practising small scale dairy farming (Yasmin and Ikemoto, 2015). Sharma et al. (2015) have reported that women engaged in scientific dairy practices and milk production practices, showed increase in their income through the sale of milk and milk products. Small scale dairy farming women were able to make decision regarding household and personal care, gained self-confidence, and self-esteem, increased social participation and overall increased the empowerment of women in the area (Islam et al. 2019). Economic empowerment of farm women increased as a result of their increase in income and there was increase in social empowerment as the social status was enhanced after joining the dairy cooperative (Dash et al. 2020). Many studies have shown, dairy farming as a tool for achieving progress in empowerment of farm women. The objective of this paper is to measure the magnitude of socio-economic empowerment of the women dairy farmer in East Sikkim. Therefore, an ex-post facto study was conducted to measure the socio-economic empowerment among women dairy farmers and to view the magnitude of association of socio-economic variables on the empowerment scores in the state of Sikkim.

Materials and methods

The study was conducted in East district of Sikkim, where four blocks namely Nandok, Ranka, Rakdong Tintek and Khamdong was selected randomly. Under each block five villages each was randomly selected. Ten respondents each from the selected village possessing at least one dairy cattle were taken in to consideration.

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The total respondents were 200. Collection of data was proceeded through personal interview of prepared index for measuring the socio-economic empowerment.

For index development, five major dimensions, social, economic, freedom to mobility, technical knowledge possession and decision-making factor were used. Under each dimension items/ statements were selected based on secondary sources. For validity purpose, items/statements along with dimensions were sent for judges rating via google form to 150 judges. Scientist, subject matter specialist, assistant professor and expert in the field of women empowerment were selected as judges. Out of 150 judges 58 responses were taken into consideration. They were requested to allot appropriate weightage to the five dimensions, out of 100. The weighted scores were presented in (Table1). Under each dimension statements were provided with 3-point continuum i.e. "Most relevant", "Relevant" and "Irrelevant" for which scores provided were 3, 2, 1. The statements were also sent to the same judges in google form format via email to suggest the degree of relevancy of statements. Out of 150 judges, 58 responses were received and the means of the dimensions were calculated and weightage was taken. The relevancy weightage (RW)was calculated by using the given formula and the statement having more than 0.7 relevancy value were selected for index preparation. Total of 44 statements were taken in consideration under the five dimensions of empowerment

$$RW = \frac{\text{Most relevant responses} \times 3 + \text{Relevant responses} \times 2 + \text{Not relevant responses} \times 1}{\text{Maximum possible scores}}$$

The response of selected items/statements were provided with nominal values (Yes, 1 and No, 0) for measurement of empowerment. Weighted average method was applied to determine the empowerment scores. Weighted score for each dimension was calculated by multiplying the percentage scores of each dimension by their respective weights. Socio-economic empowerment index (SEI), was obtained by adding the weighted score of each of the dimension of a respondent and then divided by 100. Statistical tools like, ANOVA, Post hoc test and Chi-square test were used.

Results and discussion

It showed, post hoc analysis in empowerment score among various socio-economic variable of the respondent. Age ($p<0.05$) was having a statistical difference in the empowerment scores between the categories. Similar results were reported by (Batool and Jadoon, 2018). The empowerment scores of young aged and middle aged categories showed significant difference with that of old aged category. Younger generation were more empowered that the older generation due to technological advancement and educational reform. Education ($p<0.05$) showed significant difference in the empowerment scores between the level of education. Similar results were reported by (Das et al. 2019, Shanti and Murty, 2019). As the level of education increased, so did the empowerment scores of the respondents. Marital status of the respondents ($p<0.05$) displayed a significant difference in empowerment scores. Similar findings were observed by (Redzuan et al. 2010). The table also showed that married and unmarried women were possessing a higher empowerment score than widow. Widow were often neglected and viewed as outcast in the society. Milk production per day (in lit) ($p<0.05$) showed a significant difference in empowerment scores between the categories. The respondent belonging to medium milk production categories possessed higher empowerment scores. Social status ($p<0.05$) displayed a significant difference in empowerment scores between the categories. Other Backward Caste were more empowered than other communities. The social structure of OBC community were favouring empowerment of women in their community by encouraging them to actively participate in social activities, while the Scheduled Caste and Scheduled Tribe community were still having a traditional outlook toward empowerment of women. Farming experience in dairying ($p<0.05$) exhibited significant difference in empowerment scores between the group. The low experience category was more empowered as the respondents under this category were possessing knowledge about the advantage of dairy business and new technology in the field, while other categories were still following traditional dairy practices. Herd size ($p>0.05$) was having non significant difference in empowerment scores. Annual income ($p<0.05$) showed a significant difference in empowerment scores. Similar results were reported by (Shanti and Murty, 2019). The medium income and low income categories were more empowerment than high income category. The respondents under high income category were bound to maintain their high social status, leading to various restrictions in the society. Occupation ($p<0.05$) displayed a significant difference in their empowerment scores between the categories. The respondents under the govt service+ dairy farming category were more empowered that the other categories, as the respondents were employed and had exposure to various developmental activities.

The finding indicates the distribution of dairy farm women according to the empowerment scores and socio-economic variables. Age: It can be seen that, 21.00 percent of total respondents who fell under middle age group and 13.00 percent of total respondents who fell under young age group were possessing high empowerment scores as compared 2.50 percent of total respondents who fell under old age group. The table also

Table1 Weightage score given by the judges

Weightage to dimensions	Scores (out of 100)
(W1) Social dimension (SD)	19.67
(W2) Economic dimension (ED)	23.05
(W3) Freedom to mobility dimension (FMD)	24.57
(W4) Technical knowledge dimension (TKD)	16.06
(W4) Decision making dimension (DMD)	16.65

showed that 44 respondents, of total respondent under old age category were having low empowerment scores, which showed that old aged category and empowerment were having negative relation. Education: It can be seen that,18.50 percent of total respondents who fell under illiterate category were having low

Table 2 Difference in empowerment scores of dairy farm women with respect to socio-economic variables using ANOVA, post hoc test (n=200)

Sl.no	Variables	Categories	Empowerment Scores
			Mean ± SEM
1.	Age	Young (up to 35)	$81.48^{a}_{\pm}$ 1.46
		Middle (36-50)	$79.70^{a}_{\pm}$1.63
		Old (>50)	$63.35^{b}_{\pm}$ 1.71
2.	Education	Illiterate	$61.33^{a}_{\pm}$ 1.80
		Primary	$74.57^{b}_{\pm}$1.90
		Secondary	$80.64^{bc}_{\pm}$ 1.96
		Higher sec.	$81.99^{cd}_{\pm}$ 2.26
		Graduate and above	$87.56^{d}_{\pm}$1.96
3.	Marital status	Married	$74.81^{a}_{\pm}$ 1.20
		Unmarried	$75.30^{a}_{\pm}$ 2.50
		Widow	$61.74^{b}_{\pm}$ 0.92
4.	Milk production/day (in litres)	Low (<7 lit)	$74.62^{ab}_{\pm}$ 1.41
		Medium (7 -13.50lit)	$76.55^{b}_{\pm}$ 1.84
		High (>13.50lit)	$67.83^{a}_{\pm}$ 4.09
5.	Social status	General	$75.20^{ab}_{\pm}$1.62
		OBC	$81.46^{b}_{\pm}$2.62
		SC	$68.75^{a}_{\pm}$3.80
		ST	$73.51^{a}_{\pm}$1.10
6.	Farming Experience in dairying (In years)	Low (<13.08)	$79.87^{a}_{\pm}$ 1.41
		Medium (13.08-28.40)	$74.25^{b}_{\pm}$ 2.15
		High (>28.40)	$67.25^{c}_{\pm}$ 1.11
7.	Herd Size	Small (<3)	$71.43_{\pm}$1.52
		Medium (3-5)	$72.27_{\pm}$2.30
		Large (>5)	$68.37_{\pm}$4.83
8.	Annual income through dairying (in Rs)	Low (<0.99 lakhs)	$73.78^{a}_{\pm}$1.47
		Medium (0.99-4.19lakhs)	$76.80^{a}_{\pm}$1.60
		High (>4.19 lakhs)	$60.39^{b}_{\pm}$8.60
9.	Occupation	Dairy farming	$68.07^{a}_{\pm}$ 1.65
		Crop+ Dairy farming	$68.17^{a}_{\pm}$ 3.35
		Labour work + Crop+ Dairy farming	$76.70^{a}_{\pm}$ 2.18
		Govt service+ Dairy farming	$89.10^{b}_{\pm}$ 2.37

(Different superscript indicates significant difference at 5 percent level of significance)

empowerment scores, while respondents having primary and above education level were having high empowerment scores. Marital status: It can be seen that; 71 married respondents were highly empowered than unmarried and widow. Family type: It can be observed that, 25.50 percent and 23.50 percent of total respondents who fell under nuclear family type were having medium and high empowerment scores as compared to 9.50 percent and 13.00 percent of total respondents who fell under joint family. Family size: It can be observed that, 22.50 percent of total respondents who resided in large sized farming possessed high empowerment scores as compared to 14.00 percent of total respondents who fell under small family size. Milk production per day: It can be seen that, 23.50 percent and 23.00 percent of total respondents who fell under low milk production category were possessing medium and high empowerment scores, respectively. Social status: it can be observed, (17.00% of total

Table 3 Distribution of dairy farm women according to empowerment scores and socio-economic variable

Sl.no	Variables	Categories	Empowerment score			Total
			Low	Medium	High	
1.	Age	Young (up to 35)	3 (1.50)	25 (12.50)	26 (13.00)	54 (27.00)
		Middle (36-50)	10 (5.00)	24 (12.00)	42 (21.00)	76 (38.00)
		Old (>50)	44 (22.00)	21 (10.50)	5 (2.50)	70 (35.00)
2.	Education	Illiterate (0)	37 (18.50)	16 (8.00)	2 (1.00)	55 (27.50)
		Primary (1)	14 (7.00)	23 (11.50)	21 (10.50)	58 (29.00)
		Secondary (2)	6 (3.00)	13 (6.50)	23 (11.50)	42 (21.00)
		Higher sec. (3)	0 (0.00)	10 (5.00)	14 (7.00)	24 (12.00)
		Graduate and above (4)	0 (0.00)	8 (4.00)	13 (6.50)	21 (10.50)
3.	Marital status	Married (1)	49 (24.50)	59 (29.50)	71 (35.50)	179 (89.50)
		Unmarried (2)	2 (1.00)	11 (5.50)	2 (1.00)	15 (7.50)
		Widow (3)	6 (3.00)	0 (0.00)	0 (0.00)	6 (3.00)
4.	Family type	Nuclear (0)	36 (18.00)	51 (25.50)	47 (23.50)	134 (67.00)
		Joint (1)	21 (10.50)	19 (9.50)	26 (13.00)	66 (33.00)
5.	Family Size	Small Family (up to 4)	15 (7.50)	36 (18.00)	28 (14.00)	79 (39.50)
		Large Family (>4)	42 (21.00)	34 (17.00)	45 (22.50)	121 (60.50)
6.	Milk production/ day (in litres)	Low (<7 lit)	38 (19.00)	47 (23.50)	46 (23.00)	131 (65.50)
		Medium (7 -13.5 lit)	13 (6.50)	13 (6.50)	24 (12.00)	50 (25.00)
		High (>13.5lit)	6 (3.00)	10 (5.00)	3 (1.50)	19 (9.50)
7.	Social status	General	24 (12.00)	32 (16.00)	34 (17.00)	90 (45.00)
		OBC	4 (2.00)	5 (2.50)	10 (5.00)	19 (9.50)

		SC	12 (6.00)	3 (1.50)	9 (4.50)	24 (12.00)
		ST	17 (8.50)	30 (15.00)	20 (10.00)	67 (33.50)
8.	Farming Experience in dairying (In years)	Low (<13.08)	11 (5.50)	33 (16.50)	42 (21.00)	86 (43.00)
		Medium (13.08-28.40)	14 (7.00)	16 (8.00)	21 (10.50)	51 (25.50)
		High (>28.40)	32 (16.00)	21 (10.50)	10 (5.00)	63 (31.50)
9.	Landholding	Marginal (<1ha)	53 (26.50)	66 (33.00)	71 (35.50)	190 (95.00)
		Small (1-2ha)	4 (2.00)	4 (2.00)	2 (1.00)	10 (5.00)
10.	Herd Size	Small (<3)	34 (17.00)	45 (22.50)	47 (23.50)	126 (63.00)
		Medium (3-5)	17 (8.50)	18 (9.00)	19 (9.50)	54 (27.00)
		Large (>5)	6 (3.00)	7 (3.50)	7 (3.50)	20 (10.00)
11.	Annual income through dairying (in Rs)	Low (<0.99 lakhs)	35 (17.50)	44 (22.00)	38 (19.00)	117 (58.50)
		Medium (0.99-4.19lakhs)	18 (9.00)	24 (12.00)	34 (17.00)	76 (38.00)
		High (>4.19 lakhs)	4 (2.00)	2 (1.00)	1 (0.50)	7 (3.50)
12.	Occupation	Dairy farming	41 (20.50)	39 (19.50)	35 (17.50)	115 (57.50)
		Crop+ Dairy farming	10 (5.00)	11 (5.50)	7 (3.50)	28 (14.00)
		Labour work + Crop+ Dairy farming	6 (3.00)	16 (8.00)	22 (11.00)	44 (22.00)
		Govt service+ Dairy farming	0 (0.00)	4 (2.00)	9 (4.50)	13 (6.50)

(Figures in parenthesis indicates percentage)

respondents) falling under general category was having high empowerment scores. On the other hand, highest segment of ST respondents (15.00% of the total respondents) fell in medium category. It can be interesting to say that, a greater number of respondents (10 respondents) from OBC category fell in high empowerment category than that of low and medium empowerment category combined. Farming experience in dairying (in years) showed that 21.00 percent and 10.50 percent of total respondents who fell under low and medium farming experience category, respectively, were possessing high empowerment scores whereas 16.00 percent of total respondents who fell under high experience category were possessing low empowerment scores. Land holding: it can be observed that 66 and 71 respondents, falling under marginal land holding category were possessing medium and high empowerment scores, respectively, whereas only 4 and 2 respondents, falling under small land holding category were possessing medium and high empowerment score, respectively. Herd size: it can be observed that 45 and 47 respondents, belonging to small herd size category were having medium and high empowerment scores, respectively, followed by 18 and 19 respondents belonging to medium herd size category respectively. Under the large herd size category 7 respondents each were possessing medium and high empowerment scores. Annual income through dairying: from the table 2, it was observed that a significant portion i.e., 19.00 percent of total respondents falling under medium income category were possessing high empowerment scores while 17.00 percent of total respondents falling under the low income category were having high empowerment scores. It can be interesting to say that 0.50 percent of total respondents falling under the high income group were having low empowerment scores. Occupation: Table 2, exhibited that 4.50 percent of total respondents belonging to government

service + dairy farming category were possessing high empowerment scores, followed by 11.00 percent of total respondents belonging to "labour work +Crop +dairy farming" category were also having high empowerment scores. It can be seen that higher segment i.e. (20.50%) of total respondents belonging to dairy farming category were possessing low empowerment scores, while 5.00 percent of total respondents falling under "Crop+dairy" farming category were possessing low empowerment scores. It was also observed that under "government service + dairy farming" none of the respondents were possessing low empowerment scores.

The chi-square analysis showed that age ($p<0.01$) was having a significant relation with empowerment score at 1 percent level of significance. The findings are in line with the study of (Khan et al. 2008; Sheikh et al. 2016). Education ($p<0.01$) was significantly associated with empowerment scores at 1 percent level of significance. Similar findings have been reported by (Bharathamma et al. 2006; Kaushal and Singh, 2010; Sheikh et al. 2016). Education and empowerment have a direct relationship (Thapa and Gurung, 2010). Marital status($p<0.01$) had a significant association with empowerment score at 1 percent level of significance. Results are in line with the finding of (Sheikh et al. 2016) who reported marital status had a negative but significant relationship with empowerment. Farming experience in dairying ($p<0.01$) was also having a significant relation with empowerment score at 1 percent level of significance. Variables like family size ($p<0.05$) and occupation ($p<0.05$) were having a significant relationship with the empowerment scores at 5.00 percent level of significance. Kaushal and Singh (2010) and Damodar et al. (2016) reported that family occupation had a significant relation with empowerment. Family type, social status, land holding, annual income through dairying, milk production per day, herd size showed a non-significant relationship with the empowerment scores. Das et al. (2019) observed in their study that family type and social status had a non-significant relation with the empowerment scores. Since, most of the parameter under the study shows significant difference with the empowerment. It can be further strengthened by enhancement of institutionalised dairy intervention approaches in the state.

Block wise variation in the empowerment scores showed that there was no significant difference in the empowerment scores between the blocks. Dairy entrepreneurship programs can be organized in each block for the upliftment of women empowerment. Dairy campaign with the help of stakeholders can be organized in the blocks. Appropriate rewards can be provided to the farm women for encouraging dairy entrepreneurship.

It showed the distribution of respondents according to their overall socio-economic empowerment scores, 36.50 percent of the respondents possessed high empowerment scores, 35.00 percent possessed medium scores while 28.50 percent possessed low empowerment scores in the study area. Since, majority of

Table 4 Association of socio-economic variables with the empowerment scores

Sl. no	Socio-economic variables	Empowerment scoresChi-square value
1.	Age	73.84**
2.	Education	73.63**
3.	Marital Status	25.55**
4.	Family type	1.69^{NS}
5.	Family size	8.36*
6.	Milk production/ day (in lit)	7.17^{NS}
7.	Social status	12.53^{NS}
8.	Farming experience in dairying (in years)	30.21**
9.	Land holding	1.35^{NS}
10.	Annual Income through dairying (in Rs)	6.16^{NS}
11.	Herd size	0.41^{NS}
12.	Occupation	18.15*

*** Significant (p<0.01), * Significant (p<0.05)*

Table 5 Block wise variation in empowerment scores

Sl.no	Block	Empowerment scores
1.	Nandok	70.24 ± 2.48
2.	Ranka	71.62 ± 2.48
3.	Rakdong Tintek	70.47 ± 2.48
4.	Khamdong	73.05 ± 2.48

Table 6 Distribution of respondents according to empowerment scores

Sl. no	Empowerment scores	Frequency	%
1.	Low (<59.74)	57	28.50
2.	Medium (59.74-80.74)	70	35.00
3.	High (>80.74)	73	36.50

the respondents have high to medium level of empowerment, reinforcement of appropriate strategies will strengthen the level of empowerment in the state. Encouraging the formation of SHGs and women dairy cooperatives among the young and educated farm women. Promotion of micro enterprise among farm women through hands on training on value additions and management skills. Exposure to innovative dairy practices. Stakeholders like KVKs, NGOs, state department can provide livestock assets for small herd size farmers. Target dairy schemes can be provided to the ST/SCs farm women. Training on capacity building, leadership development and confidence building measure can be organized by the state to elevate farm women empowerment.

Conclusion

The socio-economic empowerment of the majority of farm women was found to be moderate to high level. There was a significant difference in the empowerment scores of socio-economic variables like, age, education, marital status, milk production per day (in lit), social status, farming experience in dairying, annual income through dairying and occupation. Variables like, age, education, marital status, farming experience in dairying, family size, occupation had a significant association with the empowerment score. Thus, empowerment of farm women in the state can be enhanced by targeting the young aged, educated and married women by providing capacity building and knowledge through training on different advanced dairy technologies.

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SHORT COMMUNICATION

Effect of dietary supplementation of Shatavari on productive performance and fertility of crossbred cows

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Abstract: The aim of the present study was to demonstrate the effect of dietary supplementation of Shatavari (*Asparagus racemosus*) on milk yield, raw milk constituent indices and fertility of crossbred cows. Twenty healthy lactating crossbred cows with a close date of calving were randomly divided into two equal groups for control group (CG; n = 10) and supplemented group: Shatavari root powder 50 gm/cow/d (SG; n = 12) from 7 days post partum (dpp) until 90 dpp. Statistically analyzed data revealed that significantly (P<0.005) higher fortnightly milk yield from 45 to 90 dpp was found in the SG (10.735 to 12.396 kg/day) compared to the CG (9.802 to 10.541 kg/day). Differences in the fortnightly raw milk composition related to percent of milk fat, milk protein and milk lactose of both CG and SG cows were non-significant (P>0.005), but only fortnightly percent of milk solid not-fat from 45 to 90 dpp in SG cows (9.254 to 9.446%) than over to CG cows (8.877 to 8.999%) and percent of milk total solids from 60 to 90 dpp cows in SG (13.078 to 13.727%) than that cows in CG (12.617 to 12.648%) were significantly (P<0.005) higher. Occurrence of first post partum estrus interval was reduced significantly (P<0.005) in supplemented Shatavari group (SG: 52 dpp) compared to control group (CG: 77 dpp) cows. In this study, it was observed that the overall conception rate was higher (P<0.005) in SG: 60% (cows pregnant: 6/10) than CONT-group: 20% (cows pregnant: 2/10). The results of this study indicated that dietary supplementation of Shatavari (*Asparagus racemosus*) increased the milk yield, raw milk solid not-fat, milk total solids and improved fertility of crossbred cows.

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Productivity of dairy cows in India is extremely poor due to various factors including nutrient deficient, malnutrition, infertility, chronic diseases, stress, etc, which all impact negatively on the dairy industry's economics (Choudhary et al. 2020). Herbal medicine has a long history in Indian culture and these plants have been used for centuries since they are safe, inexpensive and widely available. They also have no side effects or residual effects in the body or on milk (Krishna et al. 2005; Sawant et al. 2016). Herbal feed boost nutrients utilization efficiency and stimulate the milk secreting tissues in the mammary glands, which helps to make dairy cows more prolific and fertile (Bakshi and Wadhwa, 2000). Supplementation of herbal feed additives as Shatavari (*Asparagus racemosus*) root increases mammary and adrenal gland weight and releases pituitary ACTH (Adreno Cortico Tropic Hormone) due to well developed lobulo-alveolar tissues in mammary gland by a direct action through pituitary or pituitary-adrenal axis, resulting in secretion of prolactin and ACTH (Behera et al. 2013). Peri-parturient secretion of prolactin is essential for maximal synthesis of milk in the post partum period. Prolactin plays a critical role in mammary cell differentiation, a key biochemical steps involved in lactogenesis. Oral administration of roots of *Asparagus racemosus* increased the milk yield in cows, buffaloes and goats (Kumar et al. 2008). In addition, *Asparagus racemosus* have been scientifically validated as reproductive system tonic and anti-stress (Kumar et al. 2008). *Asparagus racemosus* has rejuvenative properties and it stimulates the epithelial cell division resulting in early healing of the uterine wall and leads to early uterine involution and consequently an early initiation of the estrous cycle in supplemented groups (Pandey et al. 2005). According to Tsegaw and Singh (2019), supplementation of Shatavari in feed has advanced the age at puberty and age at first service. Keeping in view these benefits, supplementation of *Asparagus racemosus* herb was selected as supportive management intervention to improve the milk yield, raw milk constituent indices and fertility performance of crossbred dairy cows.

In February 2019, this study was conducted on lactating crossbred (H.F. × Sahiwal) cows maintained at Dairy Farm, Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, during the summer season with total duration of the experiment as 90 days post partum (dpp). Twenty healthy lactating crossbreed Sahiwal cows with the beginning date of calving were randomly allotted into two homogeneous groups: control group (CG; n = 10; Milk yield = 9.41±0.35 kg/d; Body weight = 410.91±16.16 kg) and Supplemented group (SG; n = 10; Milk yield = 9.62±0.44 kg/d; Body weight = 410.02±17.56 kg). The experimental groups received a basal diet twice daily containing wheat straw (*Triticum aestivum*) *ad lib*, green lucerne (*Medicago sativa*) as a green fodder and concentrate mixture in a mixed-ration. Diets of lactating cows were formulated as per the nutritional requirements of NRC (2001). Only the SG-cows were orally supplemented with Shatavari root powder (50 gm/cow/d) during the entire experimental period, which lasted from 7 days after calving to 90 dpp. Cows were milked twice daily by hand milking at 05:00 AM and 04:00 PM. Yields of milk were measured using an automatically Electric digital scale weighing balance machine and individual cow's milk yields (kg/d) were recorded every day, however, individual raw milk samples (100 mL) were collected in clean and dry plastic bottles every 15 days interval (dpp on 0, 15, 30, 45, 60, 75 and 90) at 5.00 AM and again at 4.00 PM using a volumetric milk meter. Milk samples were analyzed in duplicates by EKOMILK ultra milk analyzer to work out the percent of milk fat, milk protein, milk lactose, milk solid not-fat (MSNF) and milk total solids (TS) in the dairy laboratory of the Department of Dairy Science and Food Technology.

Detection of estrus was performed twice daily, in the morning and evening, for at least 30 minutes. During the reproductive performance the following parameters were evaluated:

Estrus response = number of cows showing estrus ÷ number of total cows treated × 100

Conception rate = number of cows conceived ÷ number of total cows mated × 100

In addition, during the remainder of the day, any cows that showed estrus behaviour were reported to the inseminators by the farm workers. Artificial insemination (AI) was performed by well-trained inseminators and frozen semen was distributed by the Dairy Farm, Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi.

Statistically, data related to milk yield, raw milk compositions and fertility were analyzed using the one-way analysis of variance (IBM SPSS software, version 21) for a completely randomized design. Significant differences ($P<0.005$) among treatment, within the experiment, were analyzed using Duncan's multiple range test.

The results (Table 1) showed that the fortnightly milk yield were similar from 0 to 30 dpp in cows of both groups CG (9.422 to 9.650 kg/d) and SG (9.634 to 10.153 kg/d). However, after 30 to 90 dpp in SG cows which provides additional Shatavari root powder were significantly ($P<0.005$) higher in fortnightly milk yield (10.735 to 12.396 kg/d) than CG cows (9.802 to 10.541 kg/d) respectively. the present finding is in agreement with Rawat et al. (2017) and Bhinda et al. (2020) where supplementation of Shatavari resulted in significant increase in milk yield of crossbred cows as compared to control group and Tanwar et al. (2008) observed that the daily and average milk production were significant increased in buffaloes and cows supplemented with 50 gm powder of Shatavari roots. Shatavari feeding could either stimulate the milk secreting tissues in the mammary glands, which helps to improve milk production in dairy cows (Bakshi and Wadhwa, 2000).

During experimental period from 0 to 90 dpp, there was no significant ($P>0.005$) difference in percent of milk fat, milk protein and milk lactose between the CG and SG cows. Whereas, after 30 dpp of the experimental lactation period, the MSNF percent was significantly ($P<0.005$) increased in SG cows (9.254 to 9.446%) than CG cows (8.877 to 8.999%) from 45 to 90 dpp. Statistically analyzed data revealed that fortnightly milk total solids percent was also significantly ($P<0.005$) higher from 60 to 90 dpp cows in SG (13.078 to 13.727%) when compared with cows in CG (12.617 to 12.648%), which corresponds with results of Kumar et al. (2014) who found that in Karan Fries crossbred cow's milk fat, protein and lactose content was not influenced, however milk solid not-fat and total solids were significantly higher in Shatavari supplemented group. However, milk fat and total solid contents were increased in *Asparagus racemosus* supplemented group as compared to un-supplemented group, whereas other composition values were unaffected in crossbred cows reported by Kumawat et al. (2017).

The effects of Shatavari (*Asparagus racemosus*) root powder supplementation on fertility rate in crossbred cows of both groups are shown in Table 2. In the current investigation, dietary supplementation of Shatavari root powder reduced the first post partum estrus interval from calving to first AI by about 24 days which was noted in SG cows. Variation in the interval from calving to first AI from 52 to 77 dpp was less ($P<0.005$) for cows of SC than that for cows of CG during the experimental period. The overall conception rate at first AI was 20% and 60% in cows of CG and SG. In this study, the overall conception rate at first AI was higher ($P<0.005$) for cows in SG i.e. 60% compared to the cows in CG i.e 20%. Our results also corroborate with that of Barhane and Singh, (2002) who reported that supplementation of Shatavari post partum alone led to 100% estrus and 75% conception in treatment group as compared to 50% in control crossbred cow within 90 days of calving. In comparison to the

Table 1: Effect of Shatavari (*Asparagus racemosus*) on fortnightly milk yield and raw milk constituent indices of crossbred cows

Parameters	Groups		SEM	P-value
	CG	SG		
MY (kg)				
0 d	9.422	9.634	0.187	0.586
15 d	9.532	9.788	0.399	0.758
30 d	9.650	10.153	0.270	0.366
45 d	9.802[a]	10.735[b]	0.184	<0.005
60 d	10.008[a]	11.540[b]	0.207	<0.005
75 d	10.284[a]	11.921[b]	0.226	<0.005
90 d	10.541[a]	12.396[b]	0.266	<0.005
MF %				
0 d	3.710	3.745	0.012	0.183
15 d	3.733	3.774	0.016	0.218
30 d	3.781	3.835	0.023	0.258
45 d	3.751	3.802	0.020	0.221
60 d	3.805	3.878	0.018	0.047
75 d	3.85	3.968	0.026	0.025
90 d	3.828	3.922	0.024	0.056
MP %				
0 d	3.359	3.404	0.013	0.105
15 d	3.425	3.471	0.017	0.202
30 d	3.485	3.445	0.025	0.455
45 d	3.478	3.509	0.008	0.081
60 d	3.408	3.444	0.011	0.136
75 d	3.439	3.537	0.013	0.024
90 d	3.465	3.506	0.008	0.009
ML %				
0 d	4.312	4.431	0.081	0.478
15 d	4.378	4.800	0.050	0.000
30 d	4.472	4.784	0.040	0.000
45 d	4.578	4.802	0.030	0.000
60 d	4.621	4.710	0.026	0.099
75 d	4.648	4.482	0.037	0.023
90 d	4.808	4.877	0.025	0.183
MSNF %				
0 d	8.649	8.687	0.008	0.015
15 d	8.778	8.976	0.031	0.000
30 d	8.838	8.912	0.014	0.008
45 d	8.877[a]	9.254[b]	0.044	<0.005
60 d	8.898[a]	9.048[b]	0.020	<0.005
75 d	8.920[a]	9.549[b]	0.073	<0.005
90 d	8.999[a]	9.446[b]	0.052	<0.005
MTS %				
0 d	12.496	12.579	0.012	0.000
15 d	12.728	12.827	0.014	0.000
30 d	12.804	12.781	0.010	0.294
45 d	12.667	12.887	0.028	0.000
60 d	12.617[a]	13.078[b]	0.057	<0.005
75 d	12.762[a]	13.358[b]	0.069	<0.005
90 d	12.648[a]	13.727[b]	0.125	<0.005

a,bMean values for each experiment within a row with different superscript letters differ significantly (P<0.005); MY = Milk yield; MF = Milk fat; MP = Milk protein; ML = Milk lactose; MSNF = Milk solid not-fat; MTS = Milk total solids; d = Day; CG = Control group; SG = Supplemented group; SEM = Standard error the mean

Table 2: Effect of Shatavari (*Asparagus racemosus*) on fertility rate of crossbred cows

Groups	No. of cows treated	Conception rate	
		Interval from calving to first AI D	At first AI % of cows pregnant
CG	10	77.50	(2/10) 20
SG(50 gm/cow/d)	10	52.83*	(6/10) 60*
Overall	20	65.16	(8/20) 40

*P<0.005, AI = Artificial insemination, D = Day, CG = Control group, SG = Supplemented group

control group, the cows who were supplemented with *Asparagus racemosus* root powder @200 mg/kg live body weight during post partum, resulted in a significant reduction in the first post partum estrus interval, days of service period, services per conception and rate of uterine involution reported by (Kumar et al. 2011). Tsegaw and Singh (2019) found that in the *Asparagus racemosus* supplemented group, puberty was reached earlier and the age of first service was lower than in the control group.

Conclusion

The results of this study indicated that dietary supplementation of Shatavari root powder increased the milk yield, raw milk solid not-fat, milk total solids along with improved fertility over cows with non-supplemented group.

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