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RESEARCH ARTICLE

Effect of bakery fat substitution by ghee residue on the quality characteristics of pearl millet based biscuit

Vinay G M[1] (✉) **and A K Singh**[2]

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Abstract: Conventionally bakery products are prepared from wheat and its derivatives. Awareness of the allergy and health complications associated with gluten and refined wheat flour, leads to increased consumption of gluten-reduced or gluten free products. Pearl millet flour is rich in dietary fiber, protein and minerals. Pearl millet has been used in this investigation to partially replace refined wheat flour. In this study, pearl millet flour and refined wheat flour were taken in a ratio of 1:1. Ghee residue (GR) is a by-product that is nutrient dense and possesses flavouring properties. The underutilised product is a source of fat, protein and phospholipid, and may find application in chocolate, burfi, sweets and bakery products. GR is used as a fat replacer in the preparation of biscuits. Fat substituted with GR at 0, 10, 15, 20 and 25% levels. The effect of fat substitution with GR on proximate physical, textural and sensory properties was studied. It reveals the colour (L*) and hardness of the biscuit increased with GR substitution whereas the percentage of fat, redness, browning index and some sensorial properties decreased. Descriptive sensory analysis reveals that biscuits are distinguished from coarseness, browning appearance, toasted and sweet flavour.

Keywords: Biscuits, Fat substitution, Ghee residue, Pearl millet

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Introduction

Bakery and confectionery products have gained attention in the last 2 decades due to changes in lifestyle and food habits. Busy lifestyle, nuclear family, all working members in a family, urbanization and Western influence attract convenience and fast foods. According to IMARC (2023) reports, the global bakery market size reached USD 497.5 billion in 2022 and is expected to increase at 3.7% CAGR during 2023-2028 to reach USD 625.9 billion by 2028. These products are highly valued compared to other products due to their availability in various types, packaged and convenient food consumption and low cost. The whole world witnessed during covid period that the food market never fell. So, it is important to notice that even in emergencies like natural calamities and disasters, products play a very important role in relieving hungriness in people.

Wheat is the main raw ingredient used as a base material in the preparation of a variety of bakery products. Generally, soft wheat flour with low protein (7-11%) is preferred for making biscuits, as hard wheat produces tougher biscuits (Pauly et al. 2013). Soft wheat flour has finer granulation, less starch damage and lower water absorption than hard wheat flour (Barak et al. 2014). Wheat flour contains gluten protein, which contributes to a peculiar viscoelastic property of a dough, which is essential for the formation of protein-starch networks and trapping carbon dioxide (Islam et al. 2019). It is quite difficult to prepare a product without gluten, but it causes allergies to people. The prevalence of gluten allergy limits the consumption of wheat-derived bakery products. There are many alternatives to wheat such as rice, maize, pseudo cereals and millet, which are gluten-free in nature. Millets are considered Nutri cereals as they are nutritionally superior and rich in dietary fibers and many bioactive compounds.

Pearl millet (*Pennisetum glaucum*) is a multipurpose cereal grown for food, feed, and fodder, especially in African and Asian countries (Manwaring et al. 2016). Pearl millet is known as "Nutri-cereal" due to its high fibre, protein, fat (rich in unsaturated fatty acids) and mineral composition. The protein made up of pearl millet lacks gluten proteins making it an ideal alternative food for celiac and gluten-intolerant people (Asrani et al. 2023). Pearl millet has carbohydrates of 60-62%, protein of 9-11% and lipids of 5-

7%. Various cohort workers prepared pearl millet biscuits (Mehra and Singh, 2017). Textural properties such as chewiness, hardness, gumminess, breaking strength and cutting strength of biscuits increased with the replacement of wheat flour as observed by Kulkarni et al. (2021). Kulthe et al. (2017) reported that replacing wheat flour with pearl millet flour caused a decrease in the diameter and spread ratio of biscuits, while the thickness of the biscuits improved. The colour values of muffins were considerably decreased with a 50% replacement of wheat flour with pearl millet in muffins (GM et al.2024).

Biscuits are high-fat and high-sugar products (Moriano et al. 2018). Since fat is responsible for the flavour, texture, and aesthetic quality of the finished product, reducing and replacing fat is a significant challenge for researchers. A wide range of ingredients is being used as fat substitutes such as carbohydrates, proteins, fat and gels, which provide distinct attributes to the product. Also, it is crucial to consider how well these replacements work so that they can be compared to standard products. Sudha et al. (2007) reported that with the reduction of fat level, the biscuit dough becomes stiffer, more springer and more cohesive. Physical properties such as the spread of biscuits reduced and the breaking strength of biscuits increased. Arepally et al. (2020) reported that the texture of low-fat cookies increases in cookie's hardness and brittleness with the replacement of fat. Aggarwal et al. (2016) formulated low-calorie biscuits using polydextrose as a fat replacer, the prepared biscuit had a lower sensory score compared to a biscuit made from full fat.

Taking into account the difficulties in the preparation of biscuits from fat replacers, an underutilized dairy by-product called ghee residue is utilized to substitute bakery fat. The by-product obtained is usually considered waste with no apparent economic value, but it is rich in fat and protein. GR is a rich source of natural flavour compounds like carbonyls, lactones and free fatty acids and its concentration is 10,132 & 11 times higher than ghee, respectively (Varma and Raju, 2008). GR is a good source of phospholipids (lecithin, cephalin, sphingomyelin and cerebroside) and nitrogenous compounds.

On an industrial scale, ghee residue is utilized in the production of certain food products and as a flavour enhancer (Wani, 2022). Traditionally, ghee residue has been widely used by mixing it with milk or skimmed milk powder, khoa, sugar and flavours for the preparation of sweets like chocolate, burfi, peda, pinni, toffees etc. and in certain food preparations like soups, spreads, muffin, etc. (Dua et al. (2018); Agrawal et al (2017); Janghu et al (2014); Singh and Rani (2019); GM et al. 2024). Rajan et al. (2020) attempted to prepare a cake and muffin by replacing refined wheat flour with GR (10-40 %). The cake & muffins made from 60% refined wheat flour and 40% GR had the highest overall acceptability. The body & texture, flavour and taste improved compared to the control sample (without GR) sample. The product was nutritionally better, especially in calcium content. Sojan et al. (2019) developed cookies and biscuits by replacing the bakery fat with GR. It was noticed that up to 10% shortening can be replaced with GR and that reduced the cost of production by ~16.6%.

The present investigation aims to prepare a biscuit using pearl millet and refined wheat flour and to substitute bakery fat with GR. Considering the nutrients, flavours and phospholipids present in GR, an attempt has been made to replace it with bakery fat. In this study, we mainly studied the effect of GR on the physicochemical and sensory properties of the biscuit.

Materials and Methods

Materials

Commercial pearl millet flour (moisture~ 6.53%, fat~5.62%. protein~9.14% & ash~1.66%) and refined wheat flour (moisture~9.33%, fat~1.67%, protein~9.88% & ash~0.65%) were supplied by the B.D Super Store market (Karnal, Haryana) and the proximate was carried out according to the AACC method. Fresh ghee residue (TS~28.19%, Fat ~8.18%, Protein~12.18% & Ash~1.49%) was procured from the Model dairy plant of ICAR-NDRI, Karnal (Haryana). Bakery fat- (Marvopride, Bunge India Pvt. Ltd., Mumbai) ground sugar, skim milk powder, salt, baking powder, ammonium bicarbonate, sodium bicarbonate and ammonium iron citrate were purchased from the local market.

Biscuit preparation

Composite flour was made using pearl millet flour (PMF) and refined wheat flour (RWF) in a 1:1 ratio. Biscuits were prepared using the creaming method (Raju et al. 2007) with a slight modification. Fat was substituted with ghee residue at 10%, 15%, 20% & 25% levels. The sample without the ghee residue was used as a control. Fat and sugar were creamed (40% of flour) to a cream consistency in a Hobart planetary mixer (M/sHobart Corporation, Ohio, USA). The GR, salt (1%) and ammonium iron citrate (2 ppm) were dissolved in water and added at the final stage of creaming. The accurately calculated amount of dry ingredients like flour (100 %), SMP (4%), baking powder (1%), ammonium bicarbonate (0.6%) and sodium bicarbonate (0.4%) were sieved to provide aeration and remove larger particles. These dry ingredients were added to the above cream and mixing continued at low speed until the dough reached a smooth homogeneous mass. The dough was rolled into a thin sheet of 2–3 mm thickness and 4 mm diameter using a wooden rolling pin and then cut into the desired shape using a biscuit cutter mould. The cut pieces were baked at 175°C for 13±3 minutes in the oven (Hcs Enterprises, Haryana).

Proximate analysis

Moisture, fat, protein and ash were carried out according to the AACC (1999) method and acid insoluble ash as per ISI (1989).

The acidity of the extracted fat was performed as described in IS SP: 18 (Part V) by ISI (1982).

Colour and water activity

A Tristimulus spectrophotometer Hunter Lab model Colour Flex® (MiniScan XE plus, Hunter Associates Laboratory Inc. Reston, Virginia, U.S.A.) was used to measure the colour of the biscuit and the results were expressed in terms of the CIE-LAB system. Measurement was carried out according to the method mentioned by Agrahar-Murugkar et al. (2015). The Aqua lab water activity meter (Model Series 3 TE) supplied by M/s Decagon Devices, WA, USA was used to determine the water activity of biscuits. The instrument was calibrated with charcoal then the sample readings were taken in triplicates. Three random readings of colour per sample were recorded and averaged. Furthermore, the values of L*, a*& b* were used to calculate the browning index of a biscuit (Isleroglu et al. 2012).

$$\text{Browning index} = \frac{100 \times \left(\frac{a + 1.79L}{5.645L + a - 3.012b} - 0.31 \right)}{0.17}$$

Spread ratio

The biscuits were physically evaluated by measuring their thickness and diameter. The thickness of the biscuits was determined by piling six biscuits and then taking their average value. Similarly, the diameter of six biscuits was measured and then their average value was taken. The spread ratio was calculated by taking the ratio of diameter to thickness.

Hardness of biscuit

Sample biscuits were evaluated for hardness using Texture analyser TA-HD plus (Stable Microsystems, USA) fitted with a 50 kg load cell. The equipment was fitted with HDP/BS blade and the biscuit was kept on the heavy-duty platform. Blade cuts the biscuits and the maximum force required to cut the sample was recorded. The test conditions were pre-test speed- 2 mm/s, test speed- 3 mm/s, post-test speed- 10 mm/s and distance- 10mm. The hardness of the biscuits was obtained by taking the absolute peak force from the cutting strength curve (Tyagi et al. 2007).

Sensory evaluation

The sensory evaluation of biscuits was evaluated by an expert panel of 10 judges on a 9-point hedonic scale in which a score of 1 represented 'dislike extremely' and a score of 9 represented 'like extremely' (Agrahar-Murugkar & Jha, 2011). The samples for evaluation were appropriately coded before serving the samples to the judges for sensory assessment. Evaluated parameters are taste, texture, colour, flavour and overall acceptability.

Statistical analysis

The data obtained from experiments were recorded as mean ± Standard deviation and subjected to statistical analysis to arrive at valid and meaningful influences. Data was analysed using one way-ANOVA. The least significant differences were calculated by the Tukey (HSD) test and the significance at $p<0.05$ was determined. Correlation was carried out using a partial correlation coefficient. These analyses were performed using SPSS for Windows Version 26.0. Principal component analysis was carried out using the R package 'factoextra'.

Results and Discussion

Proximate composition of biscuits

Biscuits and cookies are shelf-stable, low moisture, moisture-convenient products. The proximate composition of pearl millet-based biscuits is presented in Table 1. The moisture percentage of the biscuit samples ranged from 0.55% to 1.52%, moisture content of all treatments varied significantly ($p<0.05$) except between control and 20% GR biscuits. According to Sanni et al. (2018) to keep biscuits for a longer period and to reduce microbial proliferation, the moisture content of biscuits should be minimal. GM et al. (2024) observed an increase in the moisture trend of muffins with the addition of ghee residue powder. Moisture content increased due to high moisture content in GR and also proteins present in the GR held water during the baking process. The fat content of the biscuits ranged from 18.66 to 22.35%. Fat content was reduced ($p<0.05$) as GR levels increased in biscuit formulation due to lower GR fat levels (8.18%) than shortening (min. 80% fat). Borawake and Bhosale (1996) reported that the incorporation of GR decreased the fat content of biscuits. However, the acidity of the extracted fat increased slightly due to the presence of higher free fatty acids in the GR, but there was no significant increase in acidity ($p>0.05$) of extracted fat between the control and the biscuit made with 10% GR. GR had little effect on protein content because there was no significant change in the protein percentage of all biscuits ($p>0.05$). Florence et al. (2014) reported almost similar protein values for pearl millet cookies. Ash content shows the amount of minerals present in the biscuits and also the purity of the flour used in the preparation of the biscuits. Ash gradually increased with increasing GR levels, but there was not much significant difference in the ash content ($p>0.05$). The acid insoluble values signify the impure compounds and siliceous material present in the biscuits. Ranjan et al. (2020) reported a similar trend of increasing calcium content with the addition of ghee residue in a cake muffin product. It is evident from Table 1 that there is no adequate trend with an increase in ghee residue levels, but the values are significantly different from each other excluding 10% and 20% GR biscuits.

Colour and water activity of biscuits

Consumer acceptability purely depends upon the appearance of the product; if the product appeals aesthetic to the consumer, he/she decides to purchase it. Similarly, water activity is critical, as it determines the product's shelf life and texture. Colour is an important factor that talks about the amount of heat dissipation on biscuits. Surface colour is usually measured in terms of L*, a* and b* which is adopted internationally by the Commission Internationale d'Eclairage. Table 2 shows the colour of the biscuit samples expressed in terms of tri-stimulus characteristics, L*, a*, and b* values. According to the results, the colour values of all biscuit samples were significantly different (p<0.05). In Table 2, as GR levels increased, the lightness levels increased significantly (p<0.05), while the values of redness (a* values) and yellowness (positive b* values) values were gradually decreased. Biscuits incorporated with 25% GR had the highest lightness value (p<0.05), whereas the control biscuit had the lowest (p<0.05). The lightness increases with an increase in the GR as a result dilution of components. Bala et al. (2019) found that the addition of whey protein isolates to quality protein maize based muffins resulted in an increased lightness value. Colour development depends upon the individual components in a product composition and baking conditions (Lazaridou et al. 2007). As GR contains a higher moisture content, it reduces the browning reaction during the baking process leading to an increase in the lightness value of the biscuit. Redness and yellowness values of 10% GR biscuits were higher (p<0.05) than those of 25% GR biscuits while the water activity was highest (p<0.05) for 25% GR biscuits and lowest (p<0.05) for 15% GR biscuits. We can correlate the values of colour and water activity, as water activity increases, the values of a* (r= -0.794, p<0.001) and b* (r= -0.882, p<0.001) values decreased, and lightness increased. In the literature, no reports are available on the effect of GR on bakery-related products.

Another parameter, the browning index, provides a brief idea of the extent of heat treatment and acrylamide formation (Isleroglu et al. 2012). The colour of baked products is an important criterion for their preliminary acceptability. Furthermore, the amount of browning determines the flavour of the final product (Mundt and Wedzicha, 2007). The Maillard reaction and caramelization are primarily responsible for colour formation. Coloured compounds, such as hydroxy methyl furfural and melanoidins, accumulate during baking and depend on the compounds present

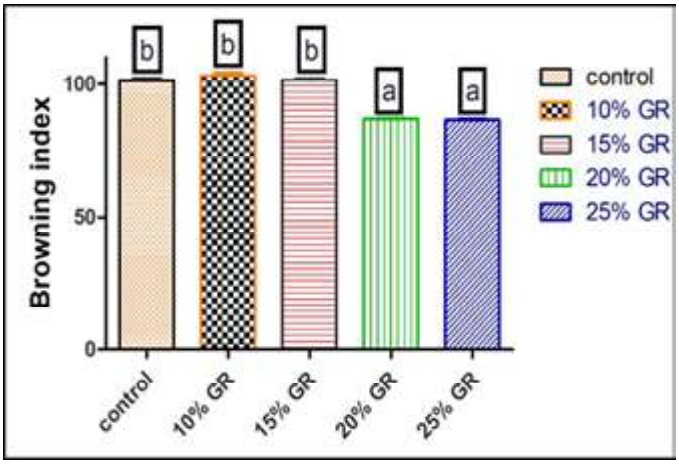


Fig 1. Browning index of biscuit samples

Note: control- Biscuit from Wheat: pearl-millet flour (50:50) with 100% shortening, 10% GR- 10 % fat replaced with ghee residue, 15% GR- 15 % fat replaced with GR, 20% GR- 20 % fat replaced with GR & 25% GR- 25 % fat replaced with GR

Table 1: Effect of different levels of GR on the proximate values of PM-based biscuit

Treatments	Moisture (%)	Fat (%)	Acidity of extracted fat (% oleic acid)	Protein (%)	Ash (%)	Acid insoluble ash (%)
Control	0.95±0.11^{b}	22.35±0.83^{b}	0.30±0.01^{a}	6.14±0.27	1.21±0.04	0.062±0.03^{b}
10 % GR	0.55±0.15^{a}	21.62±0.81^{b}	0.31±0.01^{a}	6.79±0.05	1.15±0.04	0.016±0.00^{a}
15% GR	0.66±0.04^{ab}	20.96±1.15^{ab}	0.33±0.02^{b}	6.86±0.89	1.17±0.05	0.026±0.00^{ab}
20% GR	1.01±0.10^{b}	19.21±0.79^{a}	0.34±0.00^{b}	6.44±0.80	1.19±0.04	0.033±0.00^{a}
25% GR	1.52±0.22^{c}	18.66±1.90^{a}	0.35±0.00^{b}	6.64±0.25	1.21±0.09	0.042±0.01^{c}

*Data are presented as Means ± S.D (n=3)

^{abc}Mean with different superscripts in different rows of a columns differ significantly (p<0.05)

Note: control- Biscuit from Wheat: pearl-millet flour (50:50) with 100% shortening, 10% GR- 10 % fat replaced with ghee residue, 15% GR- 15 % fat replaced with GR, 20% GR- 20 % fat replaced with GR & 25% GR- 25 % fat replaced with GR

in the biscuit dough, the temperature, and the water activity of the system (Purlis, 2010). Baking time, moisture, dough composition, sugar, and protein content were all other considerations. However, over-baking results in the formation of toxic compounds like acrylamide and 5-hydroxy methyl furfural and its derivatives, which negatively impact consumer health (Mariotti Celis et al. 2017). A maximum value of 103.10 browning index was obtained for 10% GR and a minimum value of 86.48 was obtained for 25% GR (Fig.1). The browning index was reduced with the substitution of GR (p<0.05), which could be due to an increase in moisture content of the dough or a slight change in the composition of dough. Among the mean values of the biscuits control, 10% GR and 15% GR did not differ, whilst a higher level of ghee residue substitution, viz. 20% and 25% differ significantly (p<0.05) from a former biscuit (Figure 1). Leiva-Valenzuela et al. (2018) reported browning reduces with an increase in moisture content, as it affects the Maillard reaction.

Physical and textural properties of biscuits

The spread ratio is one of the most important quality parameters for biscuits as it defines characteristics related to texture, chewiness, and overall mouth feel (Bose and Shams-ud-din, 2010). It is evident from Figure 2 (a) that there is an increasing trend of spread ratio with ghee residue levels. The spread ratio ranged from 5.49 to 6.19 and all samples differed from each other (p<0.05) in their mean values except for 10% GR and 15% GR biscuits. As in Figure 2(a), the control had a spread ratio of 5.49, while the spread ratio increased to a maximum of 6.19 after the substitution of GR (25% GR). According to Florence et al. (2014), biscuits made from pearl millet flour have a low spread ratio. The spread ratio increased with an increase in GR substitution due to an increase in moisture content in the dough and a slight reduction in the fat content of the biscuits, as is evident from the fat content in Table 1.

The hardness of the biscuits was measured in terms of cutting strength. The effect of ghee residue levels on cutting strength is

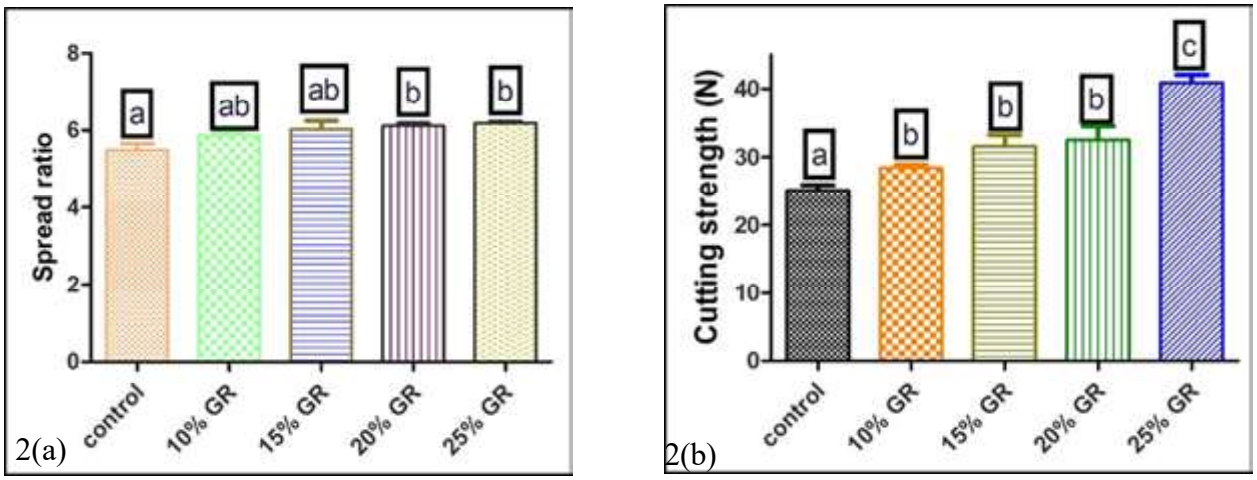


Fig 2. Effect of ghee residue substitution on (a) Spread ratio (b) Cutting strength
Note: control- Biscuit from Wheat: pearl-millet flour (50:50) with 100% shortening, 10% GR- 10 % fat replaced with ghee residue, 15% GR- 15 % fat replaced with GR, 20% GR- 20 % fat replaced with GR & 25% GR- 25 % fat replaced with GR

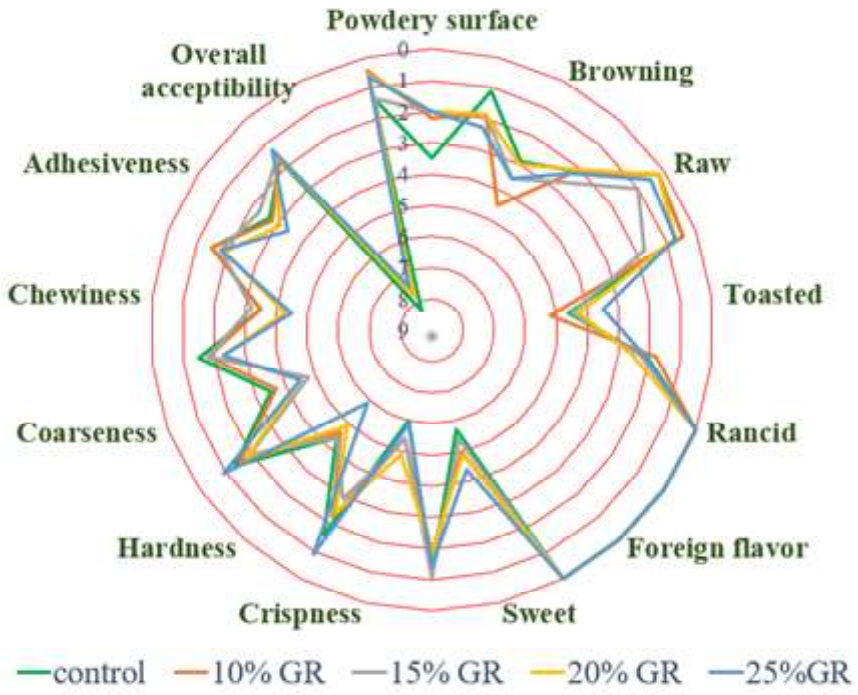
Table 2 Effect on colour and water activity of biscuits upon GR substitution

Treatments	L*	a*	b*	a_w
Control	49.51±0.42^{ab}	11.36±0.04^{b}	27.71±0.16^{c}	0.183±0.01^{b}
10 % GR	48.93±0.23^{a}	11.43±0.13^{b}	28.33±0.23^{d}	0.168±0.02^{a}
15% GR	49.82±0.24^{b}	11.11±0.05^{b}	27.49±0.27^{c}	0.164±0.01^{ab}
20% GR	50.81±0.14^{c}	09.90±0.06^{a}	26.40±0.13^{b}	0.171±0.00^{a}
25% GR	52.82±0.05^{d}	09.75±0.25^{a}	25.42±0.16^{a}	0.195±0.01^{c}

*Data are presented as Means ± S.D (n=3)
^{abc} Mean with different superscripts in different rows of columns differ significantly (p<0.05)

Fig 3. Sensory attributes of pearl millet-based biscuits

Note: control- Biscuit from Wheat: pearl-millet flour (50:50) with 100% shortening, 10% GR- 10 % fat replaced with ghee residue, 15% GR- 15 % fat replaced with GR, 20% GR- 20 % fat replaced with GR & 25% GR- 25 % fat replaced with GR



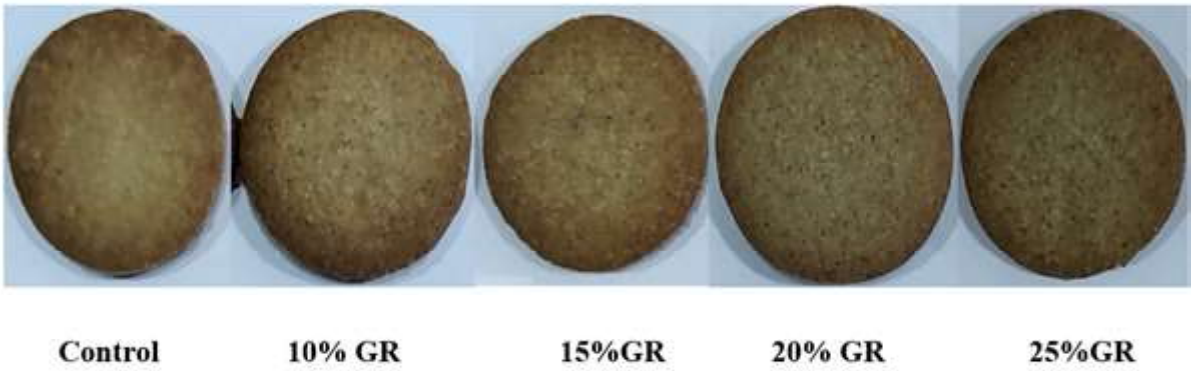


Fig 4. Images of biscuits prepared from different levels of ghee residue and control

Note: control- Biscuit from Wheat: pearl-millet flour (50:50) with 100% shortening, 10% GR- 10 % fat replaced with ghee residue, 15% GR- 15 % fat replaced with GR, 20% GR- 20 % fat replaced with GR & 25% GR- 25 % fat replaced with GR

shown in Figure 2 (b). The control has a minimum cutting strength of 25.10 N whereas the maximum cutting strength is obtained with 25% GR substitution (40.89 N). The mean cutting strength values of the control and GR substituted biscuits differed significantly (p<0.05). However, the cutting strength of biscuits containing 10, 15 and 20% GR did not differ significantly from each other. The substitution of GR significantly increased the cutting strength of the biscuits (p<0.05) and this could be attributed to the reduction in the fat content of biscuits that resulted in increased hardness. A study by Chugh et al. (2015) found that the use of fat replacers increased the hardness and brittleness of cookies with fat replacement. Chugh et al. (2013) reported similar findings in which reducing fat levels increased the hardness of a composite biscuit. Gallagher et al. (2005) observed a higher hardness when sodium caseinate was used at 10 & 15%. They surmised due to the high water holding capacity and gelling capacity of sodium caseinate. In our experiment, the harder biscuits may be due to the protein and ash content of GR.

Sensory analysis of GR substituted biscuits

Descriptive sensory analysis is a standard tool that provides detailed information on the nature and intensity of sensory attributes as perceived by humans while djudging food (Omoba et al. 2015). The sensory characteristics of biscuits treated at

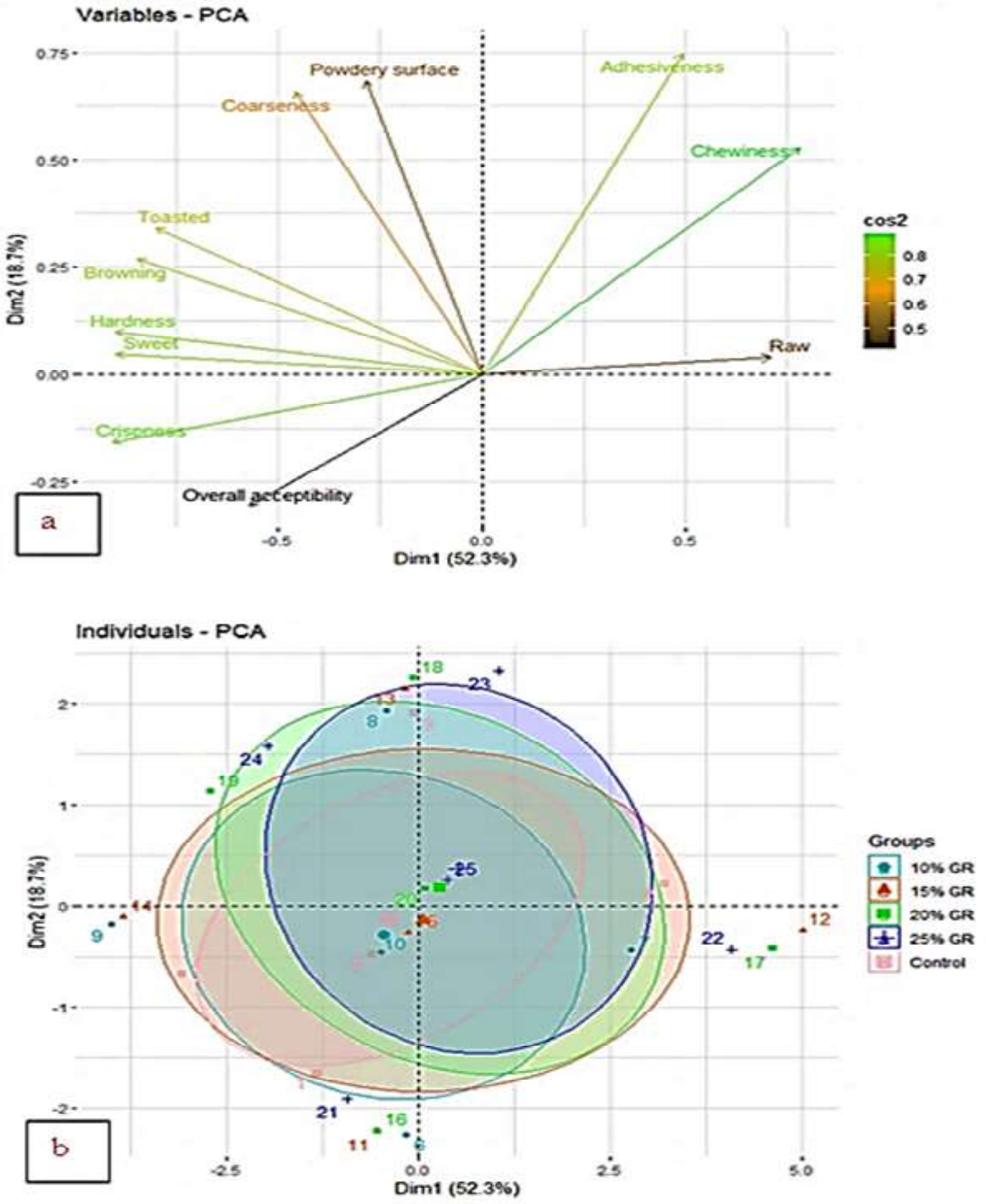


Fig. 5 Principal component analysis (a) Linear projection of loading plot of variables (b) PCA grouping of treatments with linear projection of sensory characteristics

various levels of GR are depicted in Figure 3. No variations in the overall acceptability of biscuits were observed. However, the control had a higher acceptability than the ghee-residue substituted biscuits. Among the GR substituted biscuits, 20% of the GR biscuits were more acceptable. It was also observed that with the substitution of GR for a pearl millet biscuit, the powdery surface was decreased. Furthermore, the biscuits showed no signs of rancid and foreign flavour development with the substitution

of GR. Additionally, the sweetness of a product decreased, but there was no significant difference ($p<0.05$). Crispness, coarseness, adhesiveness and chewiness were found to be higher with no significant difference ($p>0.05$). The hardness of a product was increased due to fat replacement with GR which could be significantly correlated with a reduction in the tenderness of a product, leading to an increase in the hardness. Product images are shown in Figure 4.

PCA is used to characterize the effect of GR levels on pearl millet-based biscuits. All sensory scores were converted to z-score (standardized) to ensure equal influence of all sensory attributes, and subjected to multivariate analysis. On the basis of the sensory score of the panellists, PCA was applied. PCA of all attributes resulted in three principal components with Eigenvalues greater than 1 (Kaiser-Meyer-Olkin criterion) (Massart et al. 1988; Borgognone et al. 2001) explaining 85.14% of total data variation. PCA showed that the PC1, PC2 and PC3 explained 52.3%, 18.7% and 14.16% variability, respectively. Varimax rotation was applied to these retained PCs to bring them into closer alignment with the original variables (Lawless and Heymann, 2013). The Varimax rotated factor loadings, which represent correlations between PC and the original attribute measurements. The biplot (product attribute) and PCA loading plot are shown in Figure 5. Factor loadings with an absolute value greater than 0.6 represent a strong influence. PC1 was found to be positively correlated to coarseness, browning appearance, toasted and sweet flavour. PC2 has the most positive loadings for crispness, hardness and overall acceptability, and negative with raw flavour. Kayitesi et al. (2010) stated that a third principal component is needed when the first two principal components fail to differentiate. The PC3 is largely positively correlated with adhesiveness, chewiness and powdery surface appearance. Omoba et al. (2015) obtained PC1 distinguished factors for pearl millet-based biscuits like colour and visual attributes, dry and crisp texture.

Conclusions

Replacement of bakery fat with ghee residue reduced the fat level in the biscuit without affecting the other nutritional parameters. Lightness (L*) increased with fat substitution, while redness and yellowness decreased with increasing ghee residue level. However, the browning index of biscuits decreased with the addition of ghee residue which is a positive sign to reduce the toxic compounds while baking. The spread ratio decreased while the hardness of the biscuit increased with the fat replacement. The 20% GR biscuit had the highest overall acceptability among the fat-substituted biscuit components. Further, PCA reveals that adhesiveness, chewiness and raw flavour are positively correlated.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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RESEARCH ARTICLE

Exploring the dynamic dielectric response of yogurt throughout fermentation across frequencies ranging from 10 to 3000 MHz

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Abstract: Our study delves into the intricate dielectric properties of yogurt, navigating its transformation during fermentation at 40°C and post-fermentation modulation across temperatures from 40°C to 10°C. Employing a sophisticated setup comprising a coaxial probe (Speag DAK-12, Schmidt & Partner Engineering AG, Switzerland) and a vector network analyzer (VNA) (Agilent Technologies, E5071C), operating seamlessly within the frequency spectrum of 10-3000 MHz, we meticulously examined yogurt's behavior. Our findings unveil a compelling narrative: as yogurt undergoes fermentation, its pH gradually decreases from 6.5 to 4.3, inducing a remarkable escalation in the loss factor across all frequencies. This intriguing correlation between pH and loss factor underscores the intricate dynamics of yogurt's fermentation process. The rich dataset gleaned from our investigation not only sheds light on the kinetics of yogurt gel formation but also holds promise for optimizing post-fermentation dielectric heat treatments, enhancing yogurt's shelf life and ensuring its quality during storage.

Keywords: Yogurt, VNA, Open ended coaxial probe, Fermentation, dielectric heat treatment

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Introduction

Yogurt, which is produced by the fermentation of pasteurized milk with bacterial cultures consistency of a mixture of Streptococcus subsp. thermophilus and Lactobacillus delbrueckii subsp. bulgaris, is one of the common dairy products and is consumed all over the world due to its high nutritional value and organoleptic properties (Tamine & Robinson, 2007; Shah, 2014; Aleman, 2023). Yogurt is also considered as a vehicle to carry the probiotic bacteria, which keeps health promoting properties, such as antimicrobial and antidiarrheal properties, protection against gastrointestinal upsets, improvement in lactose metabolism, lower blood cholesterol (Shah, 2006; Gill, 2023).

Yogurt is one of the perishable food materials, therefore, in order to have a longer shelf life, it is protected from spoilage during its preparation, storage, transportation and distribution. Since acidity of yogurt is one of the main indices for consumers acceptability, so just after producing the yogurt at desired acidity (pH ~ of 4.5), it is to be cooled to refrigerated temperature of less than 10 °C in order to control post acidification (Shah, 2006). Cooling slows down the activities of starter culture and contaminants, if any, such as yeasts and molds. Generally, the shelf life is 8-10 days when stored at temperature less than 10 °C (Entrup, 2005). Maintaining a low temperature while transporting and distributing especially during hot climate in poor countries is very difficult and it results into shelf life less than 8-10 days. Shelf life and quality not only depends upon refrigerated temperature, but also on milk quality, composition, homogenization, heat treatment, starter culture, production equipment, process techniques, packaging, additives, standard of hygiene used, storage conditions and health of dairy personnel (Nelson et al. 2006). In fact, there are three main indicators that limit the shelf life during storage period, which are occurrence of post-acidification; synthesis and oxidation; and growth of contaminants, such as yeasts and molds (Entrup, 2005; Yeboah, 2023). Out of many methods (Ramesh, 2007) for the food safety control and preservation, food items prepared by chemical methods approved by the respective regulatory bodies, are generally not being respected by the consumers due to risk perception of chemicals in food (Spillman et al. 2011). To some extent, this view of consumers appears to be correct in view of

some relevant findings (Julhin, 1981; Eigenman & Haenggeli, 2004; McCann et al. 2007; Maier et al. 2010) showing that added chemicals in food items may induce long-term health risk. On the other hand, heat treatment is one of the old, important and physical technique and it is reported that post-fermentation heat treatment (PFHT) in temperature region 60-65 °C has been found (Chandan & O'Rell, 2013) to increase the shelf life of yogurt to 8-12 weeks at 12 °C by destroying the live nature of yogurt, which is due to presence of lactic acid bacteria (LAB). Such destruction of LAB is against the properties of yogurt that are due to its live nature. Also, according to code of FAO/WHO (2013) yogurt must contain 10^6 cfu/g of micro-organisms, but such amount has not been mentioned in the standards of FDA (2015). Heat treatment, however, is allowed by both FAO/WHO and FDA. But such yogurt is to be labelled as either 'heat treated fermented milk' or 'heat treated after culturing'. Recent findings (Siefarth et al. 2014[a]; 2014[b]) made the comparison of conventional heat treatment (CHT) and dielectric heat treatment (DHT) based on radio-frequency showing that yeasts and molds didn't survive on applying these techniques individually even at 58 °C, while LAB were found partially surviving on application of DHT to 58 and 65 °C, whereas these were inactivated by CHT at the same temperatures. This shows that DHT is better than CHT as undesirable micro-organisms in yogurt did not survive, while desirable ones partially survived. These findings motivated the authors to undertake the present work. Now-a-days, food industry is the major user of dielectric heat treatment for various purposes, such as baking, cooking, thawing and tempering, drawing, pasteurization, sterilization and blanching (Ahmed & Ramaswamy, 2004; Awuah et al. 2014). In the present work, co-axial probe Dielectric assessment kit (DAK) connected to vector network analyzer (VNA) has been used, which is based upon reflection method. The primary aim of present work was to acquire the reliable data of dielectric properties of plain set yogurt in support of the development of radio-frequency as well as micro-wave applicators that are expected to be useful to provide post-fermentation dielectric heat treatment (PFDHT) in order to extend its shelf life. The secondary target, which is pre-product of primary aim was to monitor in real time the fermentation process of milk to produce to set yogurt in view of potential benefits of dielectric study (Pethrick & Hayward, 2002) in understanding the microscopic changes occurring in material under investigation in real time. The dielectric study of fermentation process seems to be helpful in understanding the mechanism and kinetic for the formation of yogurt gel, which however is a challenging subject matter despite the availability of number of techniques, such as small amplitude oscillating rheometry, diffusing wave-spectroscopy, ultrasonic spectroscopy, transmission and scanning microscopy and confocal laser scanning microscopy (Mezzenga et al. 2005).

In the present study the Dielectric Heat treatment (DHT) process is used, in which the rise in temperature of the dielectric material by applied electromagnetic field depends upon the complex permittivity ε^* of that material (Metaxas and Meredith, 1983) which is given by

$$\varepsilon^* = \varepsilon' - j\varepsilon'' \quad (1)$$

where $j = (-1)^{1/2}$ and ε', the real component is called dielectric constant and ε'', the imaginary component is called loss factor. ε' measures the ability of the material to store electromagnetic energy and measure dissipation of electric energy into heat.

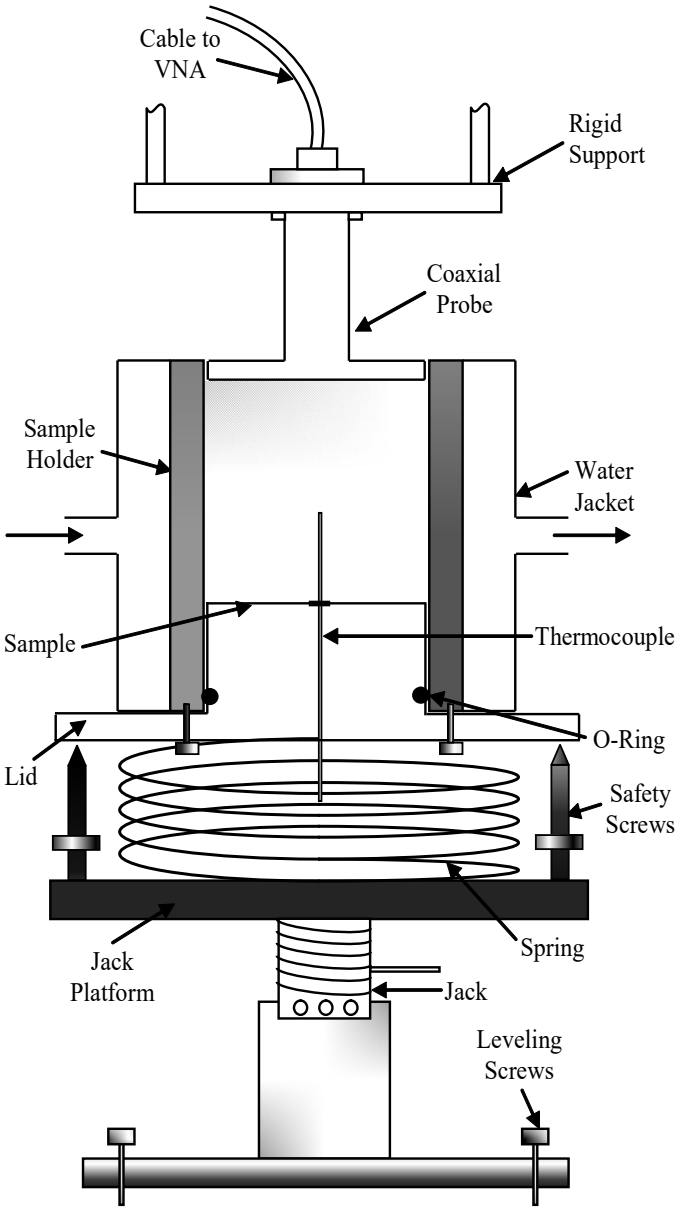

Fig. 1 Experimental setup

Further the penetration depth (d_p) of electromagnetic wave is measured using the values of and . It is defined as the depth where incident wave power density reduced to 1/e (~37%) of its value at the surface of the sample and is determined by the following equation (Von Hippel, 1954)

$$d_p(in\ cm) = \frac{c}{2\pi f\sqrt{2\varepsilon'[\sqrt{1+(tan\delta)^2}-1]}} \quad (2)$$

Where is frequency of electromagnetic wave in Hz, is speed of light in free space ($3x10^{10}$ cm/s) and . Measured mean values of and are used to determine. Smaller the values of and higher value of is obtained. During dielectric heating, plays a vital role in selection of the proper thickness of food material for uniform heating (Tang et al. 2002; Wang et al. 2003; Wang et al. 2008; Hu et al. 2024).

The objectives of the present study is to access the (a) pH dependent dielectric properties in the frequency region 10-3000 MHz of acidified milk during fermentation at incubation temperature of 40 °C to produce plain set yogurt at pH ~4.3, (b) temperature dependent dielectric properties so formed yogurt in the temperature region 40-10 °C and frequency region 10-3000 MHz, (c) penetration depth of electromagnetic field so formed yogurt at industrial scientific and medical (ISM) frequencies of 13, 27, 40, 915 and 2450 MHz.

Materials and methods

In this study, we investigate the dielectric properties of Verka Standard Milk, a widely consumed milk variety in Punjab, India. Renowned for its quality and nutritional content, Verka products hold significant prominence in the region. Verka Standard Milk, specifically, is esteemed for its balanced composition, boasting 8.5% solids-not-fat, 4.5% fat, and 3 g/100 ml of protein, rendering it an ideal choice for maintaining health and palatability. Given the increasing demands for advanced analytical insights into dairy products, particularly regarding their dielectric behavior, our research aims to address this need. Verka Standard Milk samples were procured from local markets in Sangrur, Punjab, and subjected to dielectric property analysis within the frequency range of 10-3000 MHz across a spectrum of temperatures ranging from 10 ^{0}C to 40 ^{0}C. By systematically exploring these parameters, we seek to elucidate the intricate interplay between dielectric properties and the composition and temperature variations of Verka Standard Milk, offering valuable insights into its quality and suitability for various applications.

Dielectric properties measurement apparatus consists of an open-ended coaxial probe (Speag DAK-12, Schmidt & Partner Engineering AG, Switzerland), which works in the broad frequency region of 10-3000 MHz and can be used for liquid, solid as well as semi-solid materials; VNA (Agilent Technologies, E5071C) Agilent. (2005) and a computer. A phase and amplitude stable cable is used to connect the probe to VNA. The calibrated face of the probe is placed in contact with the flat surface of compressed ground sample of rapeseed and the complex reflection coefficient () of electromagnetic field are recorded by VNA. The computer that controls the VNA precisely converts the measured into and with the help of software (DAK, 2014) based on the algorithm developed by Ellison and Moreau (2008).

The arrangement of probe, sample holder, spring and jack is shown in Figure 1. Cylindrical sample holder (50 mm inner diameter with 100 mm height) made of steel is used to hold the yogurt sample. The sample holder containing sample is put under the hydraulic press and pressed in such a way that at top side of sample holder, a depression of ~4 mm is created by inserting a removable brass disc of thickness ~4 mm with diameter just less than 50 mm so as probe can be mounted in this depression. The face of this disc in contact with sample was kept as smooth and plane as possible in order to create minimum aberrations in the surface of sample. Heat is provided to the sample by circulating the hot water at required temperature through jacket fitted with sample holder and temperature of sample is recorded with thermocouple (DTM 3000-Spezial, LKM, Electronics GmbH) that passes through the center of the lid. Sample holder is placed on platform of spring and jack system. To avoid the slipping, ends of spring are fixed in grooves made in lid and jack platform. Jack provides the necessary compressive force to the spring while compressed spring helps in minimizing the air gap between plane of probe, which is rigidly fixed in horizontal direction and face of sample the plane of which can tilt upto certain small angle. In other words, horizontal as well as vertical components of force of compressed spring keep the planes of probe and sample in contact with each other as well as parallel to each other. To avoid the tilting of sample holder beyond a small angle due to any accidental impact, jack platform is provided with three rigidly fixed safety screws (two shown in Figure 1), which can be adjusted in length so that a small gap always remains between the tips of screws and lid of sample holder during measurements so as whole weight of sample holder rests on the spring.

The milk sample was taken in the sample holder. It was heated to 40 °C by circulating the hot water and a starter culture of lactic acid bacteria 0.035 g (1.52×10^{10} cfu/g) was added into it. The initial pH of sample was determined by a standard portable pH meter and calibrated pH probe and was found to be 6.5. After that, the sample was left undisturbed and the constant temperature of (40±1) °C was maintained for next 5 h. The dielectric properties were measured from 10 to 3000 MHz at an interval of 0.5 h and each time the pH was also noted till pH of ~4.3 was obtained. The sample was then cooled from temperature 40 °C to 10 °C by circulating cold water and dielectric properties were again measured at an interval of 10 °C.

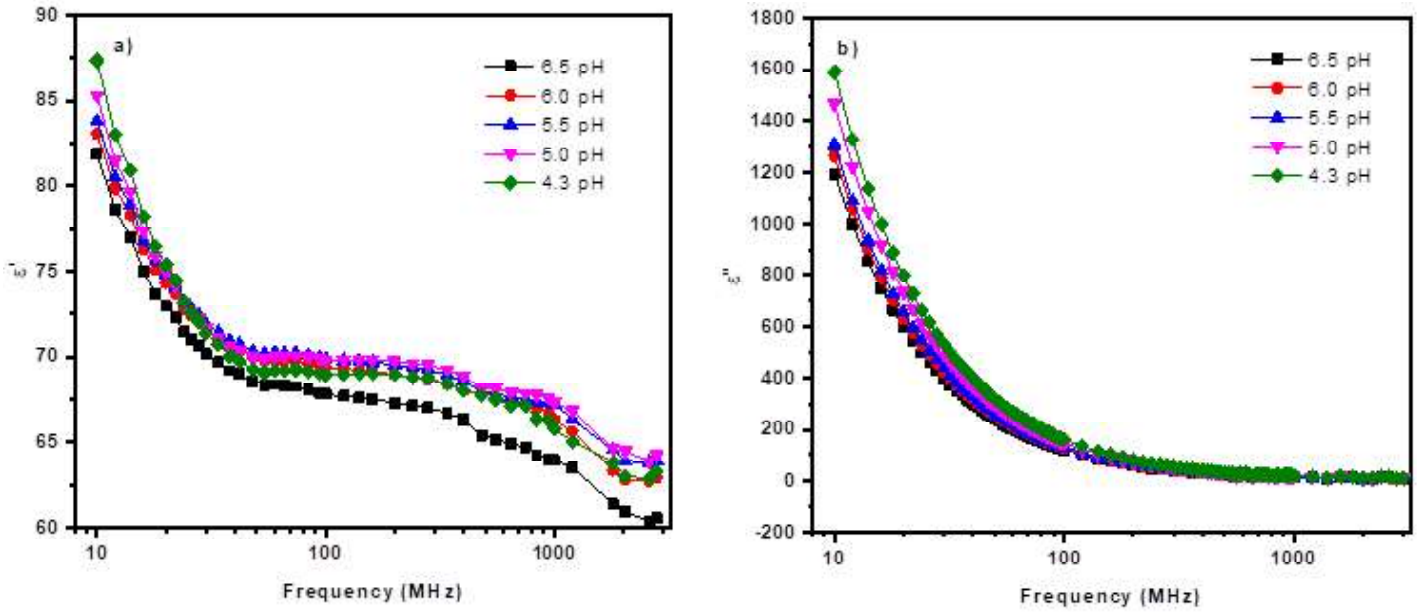

Fig. 2 Variation of a) dielectric constant, and b) dielectric loss factor with frequency at different pH of 4.3, 5, 5.5, 6, and 6.5

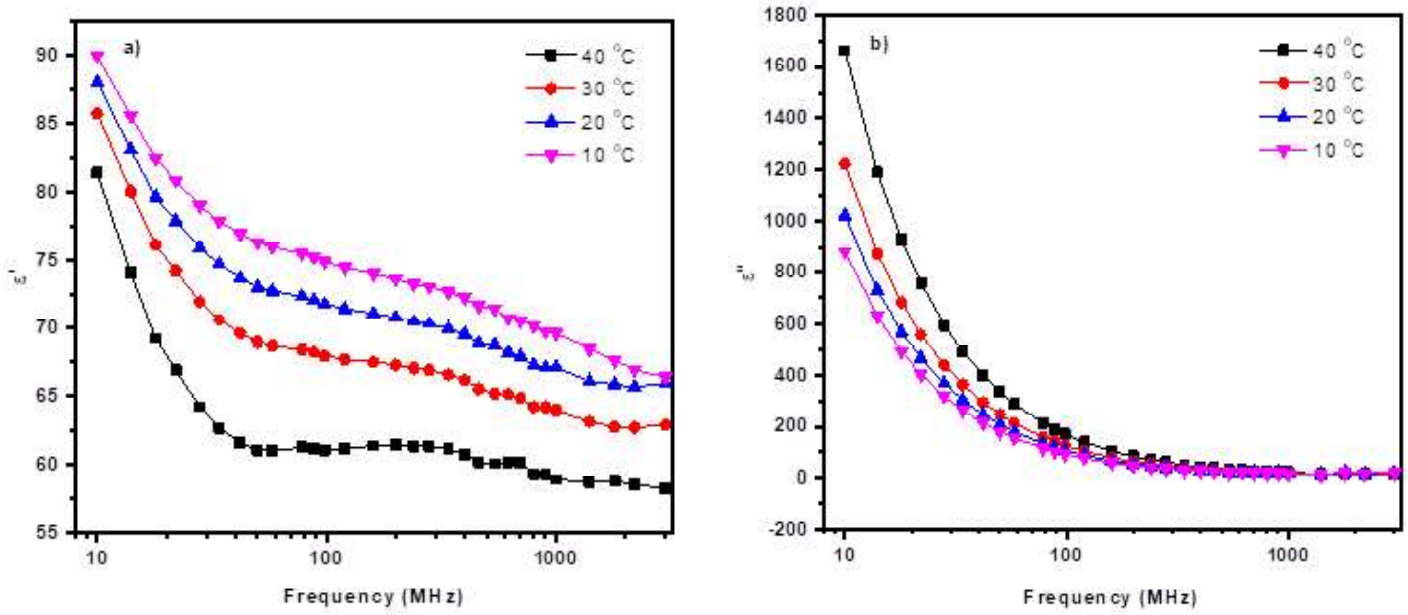

Fig. 3 Variation of a) dielectric constant, and b) dielectric loss factor with frequency at different temperatures of 10 °C, 20 °C, 30 °C, and 40 °C

Results and Discussion

Variation of dielectric constant and loss factor with pH

The obtained values for the dielectric constant (ε×) and loss factor of the milk sample during fermentation at different pH by varying frequencies from 10 MHz to 3 GHz has been shown in Fig. 2a and 2b, respectively. Figures show that both dielectric constant and loss factor decrease with increase in frequency. With increasing pH content, dielectric constant increased with a maximum of around 87 at 4.3 pH and 10 MHz. At each frequency, behavior of increasing dielectric constant was observed with rise in pH, except for pH values of 4.3 and 5. The values of dielectric constant have been found to be decreased at pH 4.3 and 5 as compared to other pH values for the frequency 20 MHz onwards.

Table 1: Calculated penetration depths (in cm) of incident electromagnetic field onto yogurt at five ISM frequencies and at temperatures 10 °C, 20 °C, 30 °C, and 40 °C

Temperature (°C)	Penetration Depth (cm) Frequency (MHz)				
	13	27	40	915	2450
40	7.439	5.297	4.437	1.707	1.028
30	8.793	6.330	5.365	2.070	0.966
20	9.733	7.060	6.015	2.237	0.917
10	10.584	7.744	6.648	2.317	0.851

Dielectric loss factor observed to be increased with increasing pH content as shown in Fig. 2b for the frequency below 600 MHz. There is no influence of pH at higher frequencies of 600-3000 MHz as the loss factor has almost the same value at each pH as well as at each higher frequency. It shows the negative correlation of pH with loss factor during fermentation. With decreasing pH, concentrations of lactic acid increases, but in addition to that, calcium phosphate from the casein micelles would start to solubilize. The acidification of milk causes major physiochemical modifications to both casein micelle and serum. The slow acidification causes a greater rearrangement of casein micelles leading to the formation of a homogenous gel in the entire milk volume. These variations can be explained on the basis of milk composition, which is a mixture of colloidal dispersion and solution consisting of different components like fat, protein, lactose, minerals, vitamins, and water. The milk can be fermented only when lactic acid bacteria convert lactose into lactic acid making milk more acidic through the consumption of orotic and hippuric acids present in yogurt starter culture (Valenzuela et al. 2024). During this process, other contents fat and protein are also disintegrated into several acids declining the pH value of milk (Guo et al. 2018). So, the overall pH value decreased with time increasing the conductivity of yogurt.

Variation of dielectric constant and loss factor with temperature

The yogurt prepared from fermentation of milk is needed to be stored at low temperatures for its safety, shelf life in food industry. In the present work, milk has been fermented at a constant temperature of 40 °C. Therefore, to study the behavior of as prepared yogurt, dielectric properties have been investigated during its cooling at temperatures of 40 °C to 10 °C. The variations of dielectric constant and loss factor have been presented in Fig. 3a and 3b. The values for both dielectric constant and loss factor were observed to be decreasing with increasing frequencies at each temperature. But loss factor has no influence of temperature above 600 MHz due to alike values (Fig. 3b). During cooling, dielectric constant was found to be increased with decreasing temperature at each frequency with a maximum of ~90 at 10 MHz and 10 °C (Fig. 3a). Whereas, loss factor decreases with fall in temperatures having minimum of ~20 after 800 MHz. The maximum value of loss factor was ~1700 at 10 MHz and fermented temperature of 40 °C. The Guo et al. (2018) determine the coefficients of dielectric loss factor with pH and titratable acidity decreased with increasing frequency and present study also show similar trends.

Penetration depth of electromagnetic field in prepared yogurt at ISM frequencies

Penetration depths of incident electromagnetic field in yogurt calculated using Eq. 2 at ISM frequencies of 13, 27, 40, 915 and 2450 MHz in RF and MW region at four temperatures given in Table 1. The values of penetration depth, decreases with rise in frequency and temperature. The results suggest that for dielectric heating of yogurt, thick layered samples can be used in RF system due to larger penetration depth, while thin layered samples are required for MW treatments to overcome the lack of penetration and achieve uniform heating. The results of Dobozi, R. (2023) strengthens our findings on the penetration depth of electromagnetic field.

Conclusions

pH dependent dielectric properties of acidified milk during fermentation at incubation temperature of 40 °C to produce plain set yogurt have been measured and presented by varying frequency from 10 to 3000 MHz. The temperature dependent dielectric properties of so formed yogurt have also been measured with decreasing temperature for storage of yogurt. The results showed that as pH changed from 6.5 to 4.3 during formation of yogurt, the loss factor increased at all frequency showing negative correlation between pH and loss factor during fermentation process. The changes in dielectric properties with reducing pH appear to be useful in monitoring of fermentation process. The measured dielectric properties of formed yogurt by decreasing temperature can be used to design RF/MW applicators for providing the post-fermentation treatment to set yogurt to prolong its shelf life.

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RESEARCH ARTICLE

Impact of microwave treatment on paneer: A study of image analysis and quality

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Abstract: The image processing technique was employed to analyze microwave-treated paneer samples. These samples were prepared through the standard heat-coagulation process of milk to produce paneer. A microprocessor-controlled semi-automatic pressing technique was utilized for paneer pressing, and its textural parameters were assessed using image-analysis methods. Microwave heating was introduced to extend the shelf-life of the paneer. Subsequently, paneer samples were placed in a 50 mm diameter dish and scanned using a Canon scan Mark II 9000F scanner to analyze colour parameters. The structure visible in the images was evaluated both before and after microwave treatment. It was observed that longer durations of microwave heating affected the browning index, while shorter exposures did not significantly enhance shelf life. The microbial quality shelf-life of microwave-heated paneer samples was extended by up to 15 additional days compared to control samples.

Keywords: Paneer, Semi-automatic Pressing, Microwave treatment, Image analysis, Shelf-life

Introduction

Paneer is one of the most nutrient rich and delicious milk products consumed worldwide. It is highly perishable because of its composition and unit operations involved in its preparation. Paneer is used for the preparation of variety of tasty, healthy and nutritious dishes and snacks in India. Preparation method of chhana or paneer involves heat-acid coagulation of cow or buffalo or standardized milk at about 85 to 90 C temperature then followed by pressing of the coagulum for a specified duration (5 to 15 minutes) in a mechanical or hydraulic or an automated press (Chitranayak et al. 2017a; Chitranayak et al. 2021). Ammu et al. (2020) had reviewed and reported about the improvement in overall paneer quality by application of mechanization method in paneer and chhana production. The effect of milk composition and milking season on quality characteristics of chhana was reported by Chakraborty et al. (2021). The effect of heating technique on the solid amount present in chhana recovered from cow and buffalo milks was studied and reported by Choudhary et al. (1998). Khan SU and Pal MA (2011) had reviewed on the various methods of paneer production and Kulshreshtha et al. (1987) and Arvind et al. (2019) had studied on the quality of paneer related to pressing conditions.

Thermal treatments of food and dairy products, such as heating, drying, sterilization, pasteurization etc. can be chosen on the

Nomenclature

2-D	two-dimensional
C_p	specific heat
d	penetration depth
E	applied microwave field strength (V/cm)
Exp	exponential
f	frequency of the applied microwaves (Hertz)
GHz	Giga Hertz
h	surface heat transfer coefficient (W/m^2K)
K	thermal conductivity (W/m K)
K’	constant, for expressing the heating in desired temperature and time
MW	microwaves
P_D	power dissipation (W/m^3)
SNF	solid not fat
tan δ	loss tangent
TPC	total plate count
Y & M	yeast and mold
ε’	relative dielectric constant
ϵ”	dielectric loss factor of the material under observation
ρ	density (kg/m^3)
α	attenuation factor

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basis of level of temperature required and the intended purpose (Aref et al. 1972). Heat treatment of milk is essential to destroy the pathogenic as well as spoilage microorganisms. It also denaturates whey proteins, reduces solubility of colloidal calcium phosphate, thus co-precipitating them along with the casein upon acidification of milk. These constituents increase the yield of curd, which are otherwise lost in whey (Rose and Tessier 1959; Fox and Morrissey 1977; Brule et al. 1978; Walstra and Jenness 1983). Heat treatment at 90 °C for 10–15 min was necessary to achieve desired yield (Muller et al. 1967). However, the application of thermal treatment produces some changes which may be desired, such as the softening of the texture, protein coagulation and aromatic components formation in addition to inactivation of pathogens present in the products. The heating technique by the application of microwaves could be successful in the products having high and intermediate moisture contents, since the water molecules present in the moisture are polar in nature. Therefore, the dairy and food products with higher moisture content, fat and sugar have great affinity to the applied high frequency microwaves to heat the sample instantaneously in a uniform way. The application of microwave treatment method has various field of dairy and food processing such as drying, pasteurization, cooking and increasing the shelf life of the food materials for their longer preservation (Chandrasekaran et.al. 2013). In comparison to the methods of conventional heating, microwave treatment have many advantages such as, high volume handling capacity, uniform and instant distribution of heat energy throughout the food particles which would yield products with higher qualities in terms of texture, taste, flavour, nutrition, and the overall increased production (Venkatesh, and Raghavan, 2004; Ahmed and Ramaswamy, 2007).Microwave heating is useful in controlling microbial growth in food and dairy products and also a fast technique for heating cooked food items.Moreover, the microwave heating technique changes the taste, nutrition, colour of dairy products much less than the other heating methods during cooking foods or reheating process of the pre-cooked food products. The prospects of microwave processing in dairy industry need to be investigated extensively. When employed this technology to Indian dairy products, due concern is to be given to the shelf-stability of products as well as quality changes.

Paneer is highly prone to contamination by different microorganisms as it contains high moisture content, in the range of 45 to 65 percent. The growth of microorganisms such as, yeasts and molds, and pathogens such as, *Salmonella sp*., *Staphylococcus aureus*, *E-coli* and *Listeria monocytogenes*, which spoil paneer and deteriorate its physiochemical attributes, taste, colour and flavor.*Listeria monocytogenes*, a food pathogen, development in paneer sample by PCR method was conducted by Ashwani et al. (2012).A research work on Escherichia coli O157:H7 and *Listeria monocytogenes* detection in different kinds of milk products was reported by Singh et al.(2009). Rani et al. (2014) worked on the preparation method of ready-to-serve low cholesterol masala paneer, its storage condition and the microbial quality. Kaur et al. (2013) worked and reported on the analysis of microbial quality, of food items kept in household at refrigerated condition. The highest quality of paneer remains intact for about one-day at ambient temperature and for about six to seven days under refrigerated temperature in the range of 4 to 6 °C and the spoilage of paneer is mainly due to the bacterial action, which can be controlled by applying good manufacturing practices and different heat treatment methods (Chitranayak et al. 2017b). Many researchers have been regularly working to extend the shelf life of paneer, including the application of microwaves.

Image processing is the technique which uses many complicated mathematical operations to process different captured images of food product or any other sample by using the processing of signal. In this technique digital photography or an optical scanner can be used for capturing or importing the images of the sample under test or sample to be analyzed. Further, the images captured from the optical scanner or by a digital camera have to be analyzed for later processing and finally the result will be produced based the final analysis of the images produced. For normal printouts, the analog image processing may be used, but for higher analysis of images digital technique and method has to be applied, which is done with the help of software and computers. For conducting the image analysis of the prepared paneer samples, the digital images of control and microwave heat energy treated paneer samples were captured as reported by Russ (1975). Giardina and Dougherty (1988) also worked and reported on the morphological methods in image and signal processing.

In the present study, microwave heating, which is being applied for heating of foods including paneer at every household in urban and semi-urban society, was applied with an intention to enhance microbial quality and shelf-life of paneer. Further, image analysis of the microwave treated and control samples of paneer were conducted. Therefore, the present work is conducted to know the effect of microwave treatment on paneer quality.

Materials and methods

Procurement of milk

Cow milk was procured from cattle yard of SRS of ICAR-NDRI, Bengaluru**.**

Preparation of paneer

The self-explanatory process flow chart for paneer preparation is given in Fig.1. For the continuous production of paneer, the performance of an impact type device was reported by Das S and Das H (2009).

Microwave treatment

The microwave heating technique was applied over paneer sample for the enhancement of its shelf life and microbial quality. Paneer samples were exposed to microwave for 40 seconds (M_{40}) and 60 seconds (M_{60}). Untreated paneer samples were kept as control (C).

Physico-chemical quality analysis

Physico-chemical analysis of microwave-treated paneer involves studying the changes in its physical and chemical properties after microwave treatment.

Moisture content

The moisture content of the paneer was determined by gravimetric method (AOAC, 2005). The moisture content of the paneer should not be > 70% (Chitranayak et al. 2017a). Microwaving can affect the moisture content which has significant impact on the paneer's consistency, shelf life, and texture. Increased evaporation can lead to drier paneer, while insufficient evaporation might result in soggy texture.

Density & porosity estimation

For measuring porosity of paneer, 1.5cm*1.5cm*1.5cm (V_0=3.375cm^3) paneer cubes were taken. Its initial weight was recorded (M_0). Paneer cubes were soaked in distilled water for overnight and kept in the refrigerator. Thereafter wiped the surface of the sample and taken the final weight (M_1).

Porosity of paneer cube was calculated by using formula.

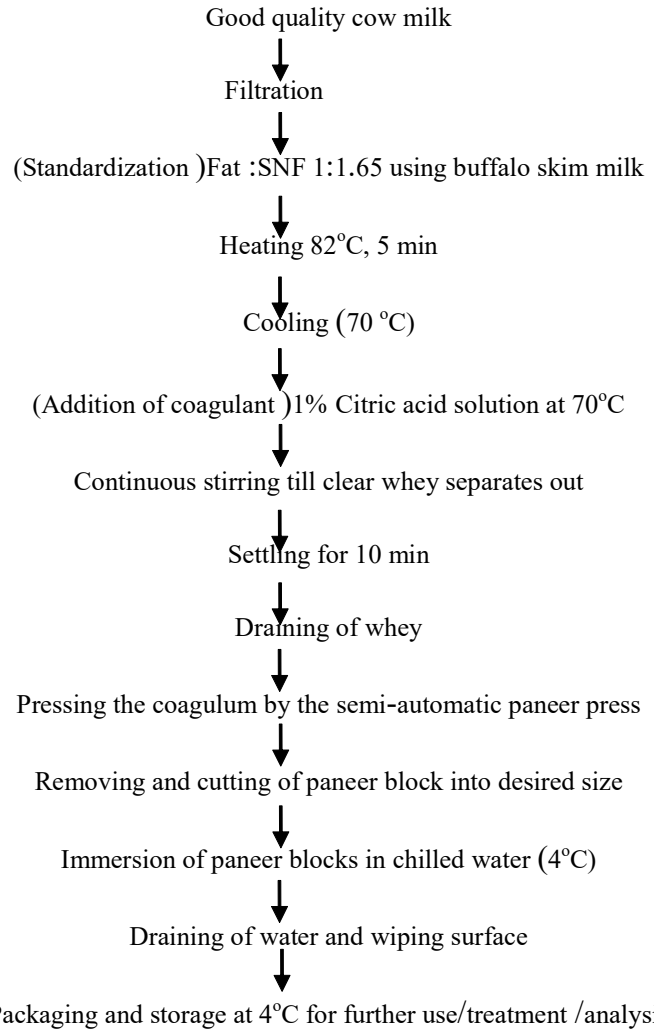

Fig. 1 Flow diagram for manufacture of paneer by semi-automatic paneer press

$$\text{Porosity}=\frac{(M1-Mo)}{V0}\times density\ of\ water$$

Instrumental texture profile analysis (TPA) of paneer

Microwave treatment may cause the paneer to become either softer or harder, which could affect its culinary applications and consumer acceptance. TPA tests were performed using texture analyzers of stable micro system equipped with 5kg load cell. The analyzer is linked to a computer that recorded the via a software program exponent 32. Experiments were carried out by compression test that generated plot of force (g) vs. time (s), from which texture values were obtained. A cylindrical probe (P/75) was used to compress 2cm*2cm*2cm paneer samples. The speed of the probe was fixed at 5mm/s during the test, compression & relaxation of the samples will take place. During the testing, the samples were held manually against the base plate.

Image acquisition

For the image analysis of the paneer samples prepared the sample was placed in the 50 mm diameter petri dish over a Canon scan Mark II 9000F scanner to get the image of the paneer. The set of images of paneer samples scanned were saved in a different folder for the analysis of its fractal dimension, pixel intensity, colour, skeletal analysis and particle analysis. Image analysis is actually the determination of structure of paneer samples before and after the microwave heating treatment. There are two parameters of the paneer samples analyzed using Image Analysis and they are, Colour Parameter and Textural Parameter.

Image analysis

Each sample had net quantity of 250 g, out of which 6 samples of area 2.5 cm^2 were cleanly cut without any protrudes in all four sides of paneer. Out of the 6 pieces taken in every sample, 3 were microwave heated at 40 seconds and the other 3 were microwave heated at 60 seconds. Then the sample was placed in the 50 mm diameter petri dish over a Canon scan Mark II 9000F scanner to get the image of the paneer for image analysis. The structure of the image was analyzed before and after the microwave treatment for comparing both the control and the microwave treated pieces. Image analysis and the analysis of the colour parameters of the paneer samples prepared using the automated press were done. Color parameters of the paneer samples were analyzed using the Adobe Photoshop software; where,

'L' is the lightness ranging from 0 to 100 (the minimum value of 0 is for black and the maximum value of 100 is for white).'a' is the chromatic component whose value ranging from -120 to +120 (the minimum value of -120 is for the colour green and the maximum value of +120 is for the colour red); 'b' (Chromatic component) ranging from -120 to +120 (the minimum value of -120 is for the colour blue and the maximum value of +120 is for the colour yellow).

The "L, a and b" values obtained from the scanned images were used for the evaluation of L*; a*; b*; and further the values of hue; chroma; yellowness index; whiteness index; browning index were determined as per the equations and formula given in the Table 1. Hue defines the pure colour in terms of green, red and blue. This is used to determine a certain colour. Chroma is the quality that distinguishes a strong colour from a weak colour. Yellowness Index (YI) is the number evaluated on the basis of data obtained from spectrophotometer which describes kind of changes in colour of the product from white or clear towards yellow. The test of yellowness index is used for evaluating the colour variations in a product or test sample produced by actual or stimulated exposure at outdoor. Whiteness Index (WI) gives the measurement that correlates the visual ratings of whiteness for certain white and near white surfaces. Browning Index (BI) of the sample is used to find the browning appearance or effect on

Table 1 Image parameters and corresponding Formulae

Image parameters	Equations or Formula
Lightness	$L^*=\frac{L}{255}\times 100$
Chromatic component	$a^*=\frac{(240\times a)}{255}$ -120
Chromatic component	$b^*=\frac{(240\times b)}{255}$ -120
Hue	$\tan^{-1}\frac{b*}{a*}$
Chroma	$\sqrt[2]{a*^2+b*^2}$
Change in colour	$\sqrt{(ax-ao)^2+(bx-bo)^2+(cx-co)^2}$
Browning index	$\frac{(m-0.31)\times 100}{0.172}$
	$m=\frac{(a+175L}{5.645L+a-3.012b}$
Yellowness index	$\frac{b^*}{L^*}\times 142.86$
Whiteness index	$100-(\sqrt{(100-L*)^2+a*^2+b*^2})$

the sample surface, which indicates the browning index. Image parameters and corresponding formulae were shown in Table 1.

Results and Discussion

Physico-chemical analysis of microwave treated and control paneer samples

Moisture content, density and porosity of microwave treated and control paneer samples were analyzed. Moisture content of microwave treated paneer samples was observed to be increased with its storage period (from 0th day to 12th day). Whereas moisture content of control paneer samples showed an initial increase and thereafter a decreasing trend with storage period (from 0th day to 20th day). There was no significant difference in density of microwave treated paneer samples and some random deviation was observed in the density values of control samples with storage. Kumar et al. (2014) mentioned that the moisture content in paneer varies between 50.72 to 56.99 % prepared from cow and buffalo milk with fat content ranging from 3.5 to 6%. The initial moisture content of the paneer and the rate at which moisture evaporates are critical in the microwave heating process. The behavior of water in the paneer is dependent on its phase (liquid water vs. solid ice) and the amount of free water present. Further, the variations in pressure and time have a significant impact on the moisture levels in paneer. This suggests that both factors must be carefully controlled to achieve desired results. The study observed that varying the duration of microwave application did not significantly affect the moisture content, structural integrity, or texture of the paneer. This could imply that once a certain threshold of heating is reached, additional microwave exposure might not lead to substantial changes. According to Khan and Pal (2011), paneer made from buffalo milk typically contains between 51–54% moisture. This benchmark is useful for understanding the typical moisture range in paneer. In summary, while microwave heating can influence the moisture content and characteristics of paneer, the duration and specific pressure conditions need to be optimized. The typical moisture content range for paneer from buffalo milk is well-established, but practical outcomes depend on controlling microwave parameters effectively.

Porosity values of microwave treated samples were less compared to its control sample. There was no trend observed in the values of porosity with storage period for both microwave treated and control samples.

Texture profile analysis of microwave treated and control paneer samples

Texture profile analysis of microwave treated and control paneer samples were done. Hardness, Adhessiveness, Springiness, Resilience, Cohessiveness, Gumminess and Chewiness values were obtained. Hardness values of both microwave treated and control samples were showed a decreasing trend with storage period. Microwave treated sample was harder than control samples. Springiness and chewiness values of both microwave treated and control samples exhibited an increasing trend with storage period. The increase in chewiness was also reported by Dongare et al. (2019), Singh et al. (2014) and Shashikumar and Puranik (2012)

Image analysis of microwave treated and control paneer samples

The samples of paneer cubes prepared were treated with microwaves and then their digital images were analyzedDigital Images of control paneer sample, digital images of 40 Second-Microwave heat energy treated paneer sample and the digital images of 60 Second-Microwave heat energy treated paneer sample has been depicted in Fig.2. The values of hue of the sample paneer defined the pure colour in terms of green, red and blue (Table 3). Chroma values distinguished between the strong colour zones of the paneer sample from the weak colour zone. The results and the data obtained from the spectrophotometer explain the yellowness index, change in colour of the product under test from white or clear towards yellow. The change found in the value of whiteness index actually gives the measurement that correlates visual ratings of whiteness for the near white surface of the paneer and the certain white surface of the paneer sample. The WI of control and microwave treated paneer samples were at par with the findings of Barnwal et al. (2023). The ability of food products for the absorption of microwaves and convert the energy of microwaves into heat depends on their dielectric

Table 2 Physico-chemical analysis of microwave treated and control paneer samples

	Treated			Control		
Days	Moisture **(%)** wet basis	Density **(**g/cc)	Porosity (%)	Moisture**(%)** wet basis**(**	Density **(**g/cc**)**	Porosity (%)
0th day	51.10	1.2053	7.722	47.2059	1.1426	19.93
4th day	54.27	1.121	3.7	48.6847	1.4202	10.21
8th day	55.72	1.2096	4.1013	51.1723	-	-
12th day	56.7253	1.2073	4.6873	54.2753	-	-
16th day	-	-	-	44.1482	0.5637	5.2951
20th day	-	-	-	42.3762	0.9848	9.7259

property. The heating by microwave takes place because of the dipolar rotation and ionic polarization mechanism (Li et al. 2019).

Pant et al. (1993) analyzed the texture profile attributes of paneer prepared from tofu and milk after deep-fat frying and before frying by conventional heating method. The observation made for the microwave treated paneer samples that the variation in the value of the browning index is large for higher duration of microwave exposure, which finds the effect of more browning of the paneer sample. This may happen due the application of microwave heating of the sample for 40 and 60 seconds, which indicated the rise in the value of browning index from -0.56 to 36.85 after the microwave treatment of 40 seconds and it further increased to 104.61 for 60 seconds of microwave application as given in the table below. Similar pattern was observed for the yellowness index of the paneer sample. Browning and yellowness both for the paneer sample increased with the exposure of microwave

Fig.2 Digital Images of control and microwave treated paneer samples

(a) Control sample, PC1

(b) Digital Images of 40 Second-Microwave heat energy, operated at 2450 MHz frequency, treated paneer sample-PM1

(c) Digital Images of 60 Second-Microwave heat energy treated paneer sample, PM1, Source operated at 2450 MHz frequency

Table 3 Texture profile analysis of microwave treated and control paneer samples

Sample	Parameters	Days of interval		
		0^{th} day	8^{th} day	20^{th} day
Control	Hardness	82.3480	19.4613	42.2288
	Adhessiveness	-2.7665	-0.5194	-1.3670
	Springiness	0.6764	0.8441	0.9071
	Resilience	0.2200	0.2877	0.2872
	Cohessiveness	0.3654	0.6274	0.6438
	Gumminess	30.0899	12.2100	27.1893
	Chewiness	20.3528	10.3064	24.6634
Treated	Hardness	103.556	-	56.6989
	Adhessiveness	-0.9285	-	-1.4996
	Springiness	0.7527	-	0.9267
	Resilience	0.1781	-	0.2689
	Cohessiveness	0.3147	-	0.5455
	Gumminess	32.589	-	30.9315
	Chewiness	24.5297	-	28.6642

Means in column with the same letters do not differ significantly ($p<0.05$)

Control: Untreated paneer sample, M40: Microwave treated paneer sample for 40 second, M60: Microwave treated paneer sample for 60 second

Table 4 Effect of microwave treatment on CIE L*, a*, b* and Δ E

Treatment	CIE L*	CIE a*	CIE b*	Δ E
CONTROL	84.50±0.68[a]	2.53±0.11[a]	16.72±0.82[a]	48.87±0.50[a]
M40	84.12±1.56[a]	1.96±0.14[b]	17.38±1.00[a]	48.27±1.62[a]
M60	84.50±0.44[a]	2.58±0.15[a]	16.53±0.63[a]	34.95±0.7[b]

Table 5 Effect of microwave treatment on hue, chroma, BI, YI and WI

Treatment	Hue	Chroma	BI	YI	WI
CONTROL	81.38±0.43[a]	16.91±0.81[a]	23.51±1.03[a]	28.25±1.18[a]	77.05±0.26[a]
M40	83.57±0.46[b]	17.49±1.00[a]	24.11±1.83[a]	29.54±2.04[a]	76.36±1.61[a]
M60	81.15±0.27[a]	16.73±0.64[a]	23.29±0.89[a]	27.94±0.92[a]	77.18±0.18[a]

Means in column with the same letters do not differ significantly ($p<0.05$)

energy. Apart from these three important colour, indices, the yeast and mold count of paneer samples prepared was also undertaken for control and microwave treated samples. It was found that the microbial growth was much less in the microwave treated paneer samples in comparison to control samples after 24, 48, 72 and 96 hours. However, the detailed study on microbial growth and total plate count was conducted and discussed in another related research work. The results given in the tables' showed that the exposure of microwaves over the paneer samples for 40 seconds found to be optimum. Statistical analysis was carried out for the image data obtained using multivariate analysis with LSD post doc test to compare means. It was found that the microwave treatment of a domestic microwave appliances used in home appliances operated at 2450 MHz frequency on paneer had significant ($p<0.05$) effect on CIE a*, hue and Δ E (Table 2).

Conclusion

On the basis of the experimental data of image analysis and microbial analysis obtained for the microwave exposure over the paneer samples, it was observed that the up-keeping quality of microwave treated paneer was improved. On the basis of the values obtained for image parameters for paneer samples based on standard equation, the statistical analysis was carried using multivariate analysis with LSD post doc test to compare means. It was found that the microwave treatment of paneer had significant effect on CIE a*, hue and Δ E. The samples with 60 seconds exposure of domestic microwave appliances, normally used in home appliances operate at 2450 MHz frequency have shown higher amount of changes in colour parameters, as shown in the tables than 40 second and control samples of paneer. Therefore, it can be concluded that the duration of 40 second exposure of microwaves energy over the paneer samples could be optimum for its shelf life improvement, without compromising much of its colour, taste and appearance. Moreover, it can be stated that microwave technology is a successful technology for the processing of food without compromising its quality.

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RESEARCH ARTICLE

Effect of incorporation of dietary fibers on reduced calorie *kulfi* containing whey protein concentrate

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Abstract: To improve the dietary value of *kulfi*, 50% of the sugar was replaced by stevia (at 0.05%), 3.0% whey protein concentrate (WPC) was added along with 0.5 and 1.0% dietary fiber pectin and wheat bran (WB). As the level of pectin and WB addition increased the specific gravity increased and freezing point decreased. At 1.0% pectin and WB addition the melting rate decreased and at 1.0% pectin and 0.5 and 1.0% WB addition there was a significant increase in hardness. Based on sensory analysis, *kulfi with* 0.5% added pectin was judged to be at par with the control.

Key words: *Kulfi*, Pectin, Wheat bran, Dietary fibers, Stevia, Whey protein concentrate

Introduction

Today, foods are not only intended to satisfy hunger and provide necessary nutrients for humans but also to prevent nutrition-related diseases and improve physical and mental well-being of the consumer. In this regard, functional foods play an outstanding role. Consumers more and more believe that foods contribute directly to their health. So, the current trend is functional foods development, to enhance the health attributes of widely consumed foods by fortifying with functional food ingredients. Functional foods are the foods which have some biological functionality besides the basic nutritional value or they may be regarded as conventional food products with health-promoting ingredients or components that go beyond their traditional nutritive value. Those health-promoting ingredients are called as functional ingredients. Some examples of these active ingredients are dietary fiber, phytosterols, bio-active peptides, prebiotics, conjugated linoleic acid, omega-3-fatty acids, etc. (Giri and Kanawjia, 2013a, 2013b, 2014; Giri et al. 2014a).

Among different functional ingredients regular consumption of dietary fiber provides several health benefits. It possesses great potential for modulating the action of gut in both digestion and absorption of food. It improves gut motility, increases bowel passage and also plays a role in the reduction or prevention of diseases of the colon such as constipation, cancer and ulcers. Intake of dietary fiber reduces risk of coronary heart disease, stroke, hypertension and diabetes (Partula et al. 2020). Beside this, it reduces weight and improve immune function. The recommended daily dietary fiber intake is 28 g/day for adult women and 36 g/day for adult men (El-Salhy et al. 2017). Unfortunately, most persons consume less than half of the recommended levels of dietary fiber daily.

Pectin is a plant fiber obtained from the rind and peel of citrus fruits such as lemons, grapefruits, oranges and tangerines (Wang et al. 2015). Structurally, pectin is classified as a water soluble, complex polysaccharide, rich in the sugar – galactose and it is suggested to have health benefits to humans. It has the potential to lower serum cholesterol, particularly low-density lipoprotein (LDL) cholesterol, improve insulin resistance, and relief diarrhea. Pectin acts as de-toxicant, as regulator and protectant of the gastrointestinal tract, as immune system stimulant and as anti-ulcer and anti-nephrotic agent (Sandei, 2018). Pectin, as the other dietary fiber components, helps to prevent a surge in blood glucose levels by promoting satiety, and possibly by reducing the rate of glucose uptake. Pectin is used in food as a gelling agent, particularly in jams and jellies. It reduces syneresis in jams and marmalades and increases the gel strength. It can also be used to stabilize acidic protein drinks, such as drinking yogurt, to improve the mouth-feel and the pulp stability in juice based drinks including as a fat substitute in baked goods. In general, levels of pectin used as a food additive are between 0.5 and 1.0%. It is also used as a source of dietary fiber.

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Wheat bran (WB) which is also considered as dietary fiber is the outer layer of wheat consists of thin-walled long rectangular cells. WB is packed with nutrition, and offers many dietary benefits. A cup (58 g) of WB contains 99% of the US recommended daily allowance (RDA) of fiber, 9.0g of protein, and 34% of the RDA for iron. WB is also high in magnesium, manganese, niacin, phosphorus, zinc and vitamin B_6. lt helps to maintain normal bowel function and relieves occasional constipation, such as caused by changes in dietary habits or travel (McRorie, 2019). WB can be added to muffins, pancakes, biscuits, waffles, or even in cookies. A small amount of WB can be added to smoothies, especially when it is finely powdered.

Kulfi is a traditional Indian frozen dairy product which is liked by people of all ages from all over India. It has a composition almost similar to that of ice cream. *Kulfi* may be prepared from cow or buffalo milk or a combination thereof or from cream, and / or other milk products, with or without the addition of glucose, cane sugar, dextrose and, eggs, fruits, preserved fruits, fruit juices, nuts chocolate, edible flavors and permitted food colors. It may contain permitted stabilizers and emulsifiers, not exceeding 0.5% by weight. The mixture shall be suitably heated before freezing. The product shall contain not less than 10% milk fat, 3.5% protein and 36% total solids except that when any of the content of milk fat shall not be less than 8.0% by weight. Starch may be added to a maximum extent of 5.0% under a declaration on the label (Aneja et al. 2002). It differs from ice cream in that, it is not aerated. In traditional method, it is frozen in ice-salt mixture. In industrial production, standardized milk is concentrated to about half of the original volume and sugar is added. The mix is cooled and frozen at -20°C for 6 h. Typical *kulfi* formulation constitutes 9.0% milk fat, 17% milk solids-not-fat, 13% sugar and 1-2% nuts (optional).

Milk and dairy products are poor in food fibers and it is recommended to eat them along with fiber. The nutritive value of combined products is very high and they are tasty. To improve the dietary value of *kulfi* in our early studies in *kulfi* 0, 50, 60 and 70% sugar was replaced with 0, 0.05, 0.06 and 0.07% refined stevia extract powder (Rao and Giri, 2009), respectively. *Kulfi* prepared by replacing half the sugar content with stevia was adjudged at par with the control in sensory characteristics whereas more than 50% sugar replacement resulted in bitterness, lack of brownish appearance and presence of icy texture. In 50% sugar replaced with 0.05% stevia-added *kulfi*, whey protein concentrate (WPC) at 0, 2.0, 3.0 and 4.0% levels were separately incorporated. The 3.0% WPC-added *kulfi* was adjudged as best by a panel of judges. Above 3.0% WPC addition, the product was very soft and possessed undesirable whey flavor (Giri et al. 2013; Giri et al. 2014b).

To further enrich the stevia sweetened WPC incorporated *kulfi*, in the present study it was incorporated with different levels (0.5 and 1.0%) of dietary fibers (pectin and WB) separately and its effect on physical and sensory characteristics were studied.

Materials and Methods

Ingredients

Fresh cow milk was taken from Students' Experimental Dairy Plant, Dairy Science College, Bangalore, for preparation of *kulfi* in this investigation. Fresh cream (40% fat and 6.0% solids-not-fat) which was obtained after separating the fresh whole milk and 'Sagar' brand skim milk powder were used for standardization of milk (5.0% fat and 8.5 % solids-not-fat). Good quality cane sugar was purchased from the local market. Refined stevia extract powder (containing 91.1% stevioside) was obtained from Kuber Botanicals, Hubli. 'Aloch' brand Sodium alginate, used as stabilizer, was purchased from local market. WPC (70% protein), used in the present study, was obtained from Mahaan Group, New Delhi. Pectin as a dietary fiber used in the present study, was obtained from Ganesh Chemicals, Bangalore. 'Elite' brand WB was obtained from the local market and used for this experiment.

Production of dietary fiber added WPC incorporated stevia sweetened *kulfi*

Standardized milk (5.0% fat, 8.5% SNF) was condensed to half of its original volume in an open pan. Then at 65 °C, 0.3% sodium alginate, 6.5% Sugar, 0.05% stevia (Giri et al. 2014b), 3.0% WPC (Giri et al. 2013) and different levels of pectin (0.5 and 1.0%) and WB (0.5 and 1.0%) were added for different batches and mixed thoroughly (Table 1). The mix after cooling to room temperature was filled in moulds and hardened at -20 °C for 8 h. The products thus prepared were subjected to analysis for different physical and sensory attributes to maintain compare with control *kulfi* (6.5% sugar, 0.05% stevia, 3.0% WPC and no dietary fiber).

Analytical methods

Fat content in milk was determined by Gerber method and in cream and *kulfi* by Rose–Gottlieb method (IS: Part XI 1981). The milk solids-not-fat content in milk and protein content in *kulfi* were determined according to the method outlined in IS:10083 (1982) and AOAC (1980), respectively. Methods as described in IS: Part XI (1981) were followed to estimate contents of ash and moisture (gravimetric method) in *kulfi*. Carbohydrate content of *kulfi* was estimated by subtracting moisture, fat, protein and minerals from 100.

Specific gravity of *kulfi* mix was estimated at 30 °C by using a standard specific gravity bottle of 50 ml capacity, taking distilled water as the standard liquid. The melting rate of the *kulfi* was observed by drawing 50 g of the sample on a wire net placed on a funnel over a beaker immediately after removal from the hardening chamber. The time taken by the sample for complete

melt down and dripping into the beaker at room temperature (30°C) was noted. The melting rate was expressed as ml/15 min. Penetration value was determined using penetrometer (AIMIL, Associated Instrument Manufactures Pvt. Ltd., Bangalore) to assess hardness of *kulfi*. Penetration value was determined as soon as *kulfi* was drawn from the moulds after hardening. The distance in millimeters travelled by the cone in 5 s into the sample at room temperature was noted. For each sample, readings were recorded at 3 different spots and the mean value was noted. The freezing point of the *kulfi* mix was determined by using cryoscope (Cryostar I, Advanced Milk Instruments Manufacturer, USA).

Sensory analysis

Samples were judged by a panel of ten judges with a 9-point hedonic-scale score card (9 for liking extremely and 1 for disliking extremely). The judges were from the Faculty of Dairy Technology Department, Dairy Science College, Hebbal, Bangalore. The judges have not been trained for the product evaluation, but they are dairy professionals having sufficient knowledge about the sensory evaluation methods and the product characteristics. Scoring system for the characteristics was provided with standard descriptive phrases to help the judges to arrive at a decision. Judges were supplied with 4 to 5 coded samples of 30 g each as per IS: 6273 (1971). The analysis was performed in Sensory Laboratory. The panelists were allowed to use water and bland crackers for palate cleansing between the samples. The samples were evaluated always 2 h before or after the meals (Makhal et al. 2011; 2014; 2013b).

Statistical analysis

All experiments were done in triplicate. The significant difference among the samples was determined by one way analysis of variance (ANOVA) using IBM SPSS Statistics 20 software package. The data are presented as Means±Standard Error. When significant (5.0% level) differences were observed, individual means were compared using Tukey's Post Hoc multiple comparison test (Sau et al. 2014).

Results and Discussion

Effect on composition

Due to addition of pectin or WB, net weight of *kulfi* increased, which decreased the concentrations of fat, protein, ash and moisture content in the product (Table 2) and increased the carbohydrate percentage.

Physical properties

Specific gravity

The specific gravity of control *kulfi* mix recorded was 1.095, as against 1.104 and 1.112, for 0.5 and 1.0%pectin addition, respectively (Fig. 1). The specific gravity of control sample was significantly (p<0.05) lower than both the pectin added *kulfi* mix samples. Between two pectin added *kulfi* mix, specific gravity significantly (p<0.05) higher at 1.0% of pectin addition, as against 0.5% level. Increased specific gravity may be due to high water solubility of pectin.

The specific gravity of 0.5 and 1.0% WB added *kulfi* mix recorded were 1.099 and 1.104, respectively. The specific gravity of control sample was significantly (p<0.05) lower than that of both WB added *kulfi* mix samples. There was significant (p<0.05) difference between 0.5 and 1.0% WB added *kulfi* mix on specific gravity.

Table 1: Levels of different ingredients added in 100 g concentrated milk for preparation of *kulfi*

Ingredients	Control	0.5% pectin added kulfi	0.5% WB added kulfi	1.0% pectin added kulfi	1.0% WB added kulfi
Sugar (%)	6.5	6.5	6.5	6.5	6.5
Stevia (%)	0.05	0.05	0.05	0.05	0.05
WPC (%)	3	3	3	3	3
Pectin (%)	0	0.5	0	1	0
WB (%)	0	0	0.5	0	1

Table 2: Effect of different levels of dietary fiber (pectin or WB) on composition of reduced calorie *kulfi* containing WPC

Levels of dietary fiber	Constituents (%) Fat	Protein	Carbohydrate	Ash	Moisture
Control	10.4	8.7	16.8	1.0	63.0
0.5% pectin/ WB	10.3	8.6	17.2	0.99	62.7
1.0% pectin/ WB	10.3	8.6	17.6	0.99	62.4

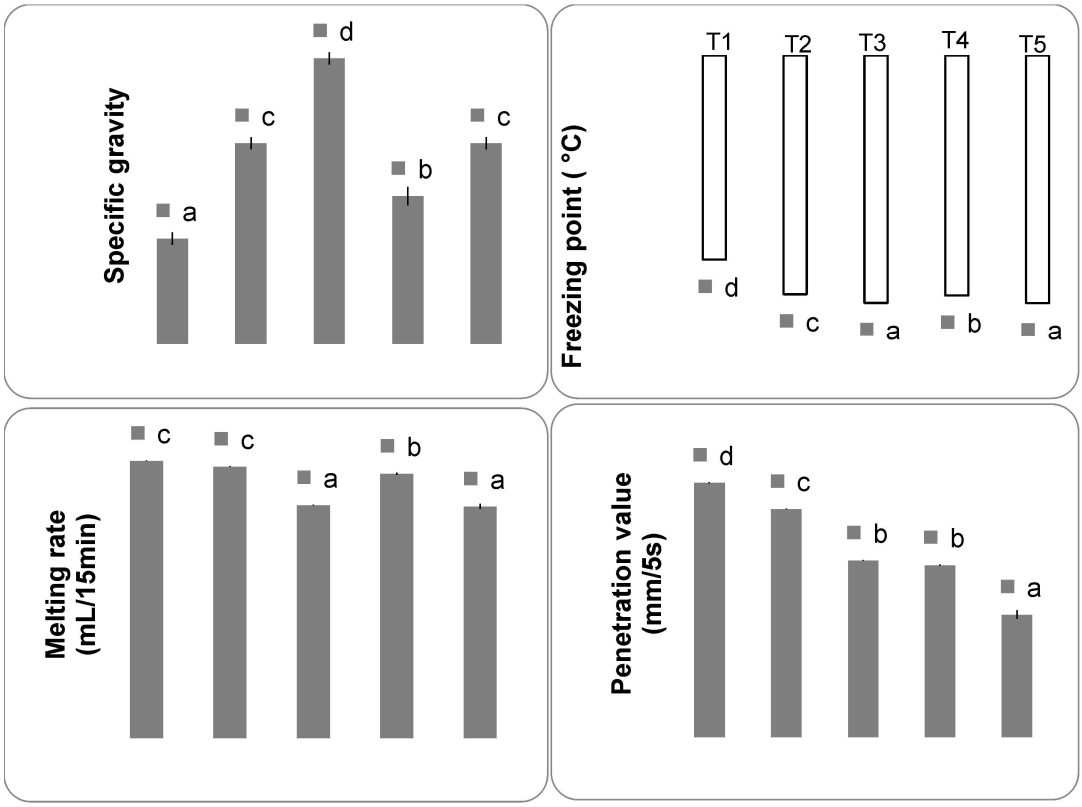

Fig. 1 Effect of different levels of fiber addition on specific gravity, freezing point, melting rate and penetration value of WPC incorporated sugar reduced, stevia sweetened *kulfi* mix/ *kulfi*; T1, T2, T3, T4, T5 imply control, 0.5% pectin, 1.0% pectin, 0.5% WB, 1.0% WB added *kulfi*, respectively; *n*=3; Different small alphabets indicate significantly different (p<0.05); Vertical bars indicate errors of means.

Addition of WB increased specific gravity due to higher water binding property of WB.

Freezing point

The freezing point of control *kulfi* mix recorded was -2.3620°C, as against -2.7628°C and -2.8628°C for 0.5 and 1.0% pectin addition, respectively. The freezing point of control sample was significantly (p<0.05) higher than both pectin added samples. Between two pectin added *kulfi* mix, freezing point was significantly (p<0.05) lower at 1.0% of pectin addition, as against 0.5% level. Pectin was soluble in *kulfi* mix. Due to increase of soluble particle in the solution freezing point decreased.

The freezing point of 0.5% and 1.0% WB added *kulfi* mix were -2.7768°C and -2.8648°C, respectively. The freezing point of control sample was significantly (p<0.05) higher than both WB added samples. There was significant (p<0.05) difference between 0.5 and 1.0% WB added *kulfi* mix on freezing point. When the WB was added since it binds water, the soluble constituents in the free water is concentrated, so freezing point decreased.

Melting rate

The melting rate (ml/15 min) of control *kulfi* recorded was 12.582, as against 12.322 and 10.582 for 0.5 and 1.0% pectin addition, respectively. There was no significant (p<0.05) difference in melting rate between control and 0.5% pectin added *kulfi*, but there was significant (p<0.05) difference of melting rate between control and 1.0% pectin added *kulfi*. Between two pectin added *kulfi,* melting rate significantly (p<0.05) lower at 1.0% of pectin addition as against 0.5% level.

The melting rate (ml/15 min) of 0.5% and 1.0% WB added *kulfi* were 12.00 and 10.52, respectively. There was significant (p<0.05) difference of melting rate between control and 0.5% WB added *kulfi* and control and 1.0% WB added *kulfi*. Melting rate of 0.5% WB added *kulfi* recorded significantly (p<0.05) higher than that of 1.0% addition.

Dietary fiber added *kulfi* samples with WB recorded lower melting rate compare to pectin addition. This could be attributed to the fact that WB with minute quantities of starch, possessed higher water binding property (Sui et al. 2018).

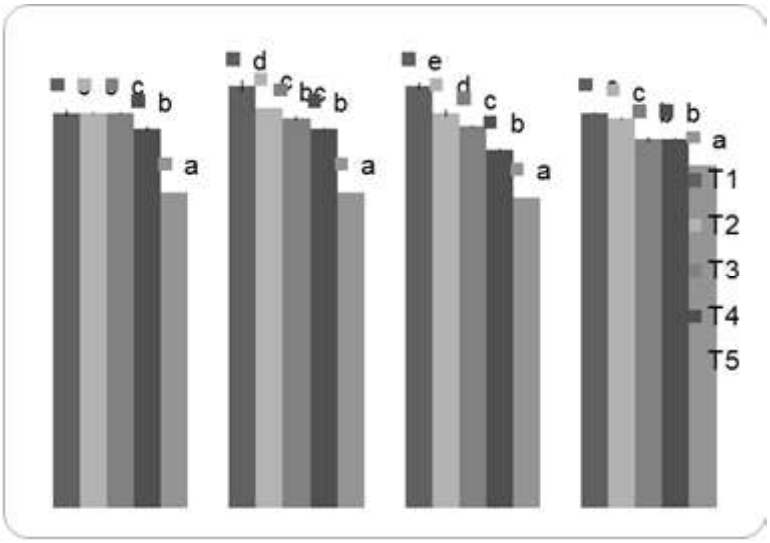

Fig. 2 Effect of different levels of fiber addition on sensory characteristics of WPC incorporated, sugar reduced, stevia sweetened *kulfi;* T1, T2, T3, T4, T5 imply control, 0.5% pectin, 1% pectin, 0.5% WB, 1.0% WB added *kulfi,* respectively;*n*=3;Different small alphabets indicate significantly different (p<0.05); Vertical bars indicate errors of means.

Pectin was highly soluble. However, it did not allow the mix viscosity to increase to the extent as that of WB added sample. Among the pectin added samples, as the level of fiber increased, the melting rate also decreased. This could be because of higher viscosity resulting from higher levels of pectin incorporation. The increased viscosity results in a tight, compact body which resisted melting.

At 0.5% level of fiber incorporation there was no significant (p<0.05) difference in melting rate of control and pectin added *kulfi* samples. This could be attributed to the limited water binding capacity of the pectin at lower level (0.5%). As the level of pectin incorporation increased (l.0%), greater quantities of water being bound by fiber resulted in significantly (p<0.05) lower melting rate of pectin added *kulfi* samples as compared to control *kulfi.*

Penetration value

The penetration value (mm/5 s) of control *kulfi* recorded was 30.96, as against 30.45 and 29.45 for 0.5 and 1.0% pectin addition, respectively. There was significant (p<0.05) difference in penetration value among control, 0.5% and 1.0% pectin added *kulfi.*

The penetration value of 0.5 and 1.0% WB added *kulfi* was 29.36 and 28.40 for 0.5 and 1.0% WB addition, respectively. The penetration value of all WB added samples were significantly (p<0.05) lower as compare to control. There was significant (p<0.05) difference between 0.5 and 1.0% WB added *kulfi* on penetration value.

Pectin and WB being water soluble, reduce the portion of free water, there by increased the viscosity of the product (Sui et al. 2018). This was responsible for lower penetration values of pectin and WB added samples, at all levels. As the level of dietary fiber in the *kulfi* increased, the penetration value of the sample decreased on account of greater amounts of water being bound by the increased fiber content and as a result harder, compact body of *kulfi*.

Sensory characteristics

Color and Appearance

Control and all pectin added samples awarded same score 7.5 on color and appearance but for 0.5 and 1.0% WB added *kulfi* awarded 7.2 and 6, respectively (Fig. 2). The color and appearance score of control sample was significantly (p<0.05) higher than that of all WB added *kulfi* samples. There was significant (p<0.05) difference between 0.5 and 1.0%WB added *kulfi* on color and appearance score. Pectin had no negative effect on the color and appearance of the treated *kulfi. So*, pectin treated *kulfi* did not differ significantly (p<0.05) from the control *kulfi*. However, WB treated samples differ significantly (p<0.05) from the control. Judges opined that WB had dark specks of bran, which were visible on the product body.

Body and Texture

The body and texture score of control *kulfi* awarded was 8, as against 7.6 and 7.4 for 0.5 and 1.0% pectin added *kulfi,* respectively. There was significant (p<0.05) difference on body and texture score of pectin added samples with control sample. However, there was no significant (p<0.05) difference between 0.5 and 1.0% pectin added *kulfi.*

For 0.5% and 1.0% WB added *kulfi* 7.2 and 6 were awarded, respectively on body and texture score. There was significant (p<0.05) difference in all WB added *kulfi* when compared with control *kulfi* as well as between 0.5 and 1.0% WB added *kulfi* on body and texture score.

Flavor

The flavor score of control *kulfi* was awarded 8, as against 7.5 and 7.2 for 0.5 and 1.0% pectin added *kulfi* respectively. The

flavor score of control *kulfi* was significantly (p<0.05) higher than that of all pectin added *kulfi*. Probably higher level of pectin addition may have negative influence on the flavor.

For 0.5 and 1.0% WB added *kulfi* 6.8 and 5.9 were awarded, respectively on flavor score. The flavor score of control *kulfi* was significantly (p<0.05) higher than that of all wheat bran added *kulfi*. There was significant (p<0.05) difference of flavor score between 0.5 and 1.0% WB added *kulfi* with control *kulfi.* When levels of wheat bran addition increased the flavor score decreased significantly (p<0.05) due to its powdery flavor.

Overall acceptability

The overall acceptability score of control *kulfi* awarded was 7.5, as against 7.4 and 7.0 for 0.5 and 1.0% pectin added *kulfi,* respectively. There were no significant (p<0.05) difference between control and 0.5% pectin added *kulfi* on overall acceptability score, but there was significant (p<0.05) difference between control and 1% pectin added *kulfi* on overall acceptability score. Between 0.5 and 1.0% pectin added *kulfi* there was significant (p<0.05) difference on overall acceptability score. Probably higher level of pectin addition may have negative influence on the flavor.

For 0.5 and 1.0% WB added *kulfi* 7 and 6.5 were awarded, respectively on overall acceptability score. There was significant (p<0.05) difference between 0.5 and 1.0% WB added *kulfi* with control *kulfi* on overall acceptability score. Between 0.5 and 1.0% WB added *kulfi* there were significant (p<0.05) difference on overall acceptability scores. As the levels of addition of WB increased the score of overall acceptability decreased significantly (p<0.05) because WB treated *kulfi* possessed chalky flavor and chewy body at higher level. The judges adjudged the 0.5% pectin added *kulfi* on par with the control *kulfi*

Conclusion

Due to water soluble characteristics of pectin and water binding property of wheat bran the physical properties of *kulfi* or *kulfi* mix changed significantly (p<0.05) at the higher level of pectin and WB addition. At the higher levels of WB addition lower sensory scores were obtained due to visible dark specks of WB, and a chalky or powdery flavor and chewy body. Even at the higher levels of pectin lower flavor scores were evident. So, it is concluded that a fiber fortified, WPC incorporated, stevia sweetened *kulfi* with good sensory properties could be made by incorporating 0.5% pectin.

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RESEARCH ARTICLE

Process optimization and characterization of spice oleoresins infused ice cream for enhancing its functional attributes

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Abstract: Different spice oleoresins, such as black pepper (0.15, 0.20, and 0.25% w/w), ginger (0.10, 0.15, and 0.20% w/w), and turmeric (0.02, 0.05, and 0.07% w/w), were added at specific concentrations to make the spice-flavored functional ice cream. The effects of concentration of oleoresin on the physico-chemical, biochemical, microbiological, and organoleptic attributes of the spice-flavored ice cream was evaluated. Addition of 0.20% black pepper oleoresin obtained the highest overall acceptability score of 8.5, having an overrun of 33.64%, melting rate of 0.85g/min, total solids of 43.86%, fat content of 12.33% and total phenol content of 393.72 mg GAE/ml and ranked first. This was followed by ginger flavoured ice cream with the concentration of 0.15% with overall acceptability score of 7.7, overrun of 30.53%, melting rate of 0.87 g/min, total solids of 43.82%, fat content of 12.53% and total phenol content of 384.57 mg GAE/ml. Among the three spice oleoresins added, turmeric oleoresin at the concentration of 0.05% had the lowest overall acceptability score of 7.3, overrun of 29.63%, melting rate of 0.87g/min, total solids of 48.93%, fat content of 12.66% and total phenol content of 378.47 mg GAE/ml. The addition of oleoresin enhanced the total phenol content in the ice cream which could provide therapeutic benefits to consumers.

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Introduction

Ice cream is a frozen dessert made from pasteurized mixture of milk, milk solids, sugar or corn syrup, flavourings, stabilizers and emulsifiers, with or without eggs. Ice cream is the most popular dairy product, loved by people of all ages (Goff and Hartel, 2013). Due to the low storage temperature, ability for stabilizing ingredients, and widespread consumer appeal, frozen dairy desserts act as excellent carriers for nutrients fortification. Therefore, ice cream can be successfully employed to provide consumers with special added ingredients and therapeutic benefits in addition to the basic nutrition. In order to attract consumers who are health-conscious, new ice cream flavours with functional properties are being developed.

Spice components give food its distinctive flavour, aroma, piquancy, and colour. These possess functional properties like anti-oxidative, anti-inflammatory, anti-diabetic, anti-hypertensive, and antibacterial activities (Srinivasan, 2005). Spices contains flavonoids, phenolic compounds etc. which have an intrinsic ability to protect products by inhibiting microbial growth, oxidation and enzymatic reactions in food (Torre et al. 2017). Flavors of spices are due to these phenol compounds. Eugenol, apiol, sufranol, vanillin, piperine, beta caryophyllene, alfa pinene, carvacol, thymol, sabinene, cinnamaldehyde, and gingerol are a few significant chemical constituents for the flavouring potential of spices (Torre et al. 2017). Spices can also be used as a flavouring to ice creams (El-Sayed & Youssef, 2019). Pinto et al. (2004 and 2006) developed ginger ice cream adding ginger juice in ratios of 3, 4, 5% and ginger pieces at 4, 6, 8% levels of ice cream mix and compared with vanilla as the control. The optimum concentration of curcumin to add to ice cream as a natural colouring agent, according to Manoharan et al. (2012), was 0.5% by evaluating the sensory properties of ice cream. David (2014) found that ice cream incorporated with 4% ginger juice was the most acceptable in terms of sensory and microbiological analysis. Gabbi et al. (2018) has manufactured ice cream with ginger juice, paste, candy and powder. According to Chamchan et al. (2017) ice cream with 15% ginger extract or lemon grass extract infused with 90% xylitol

was found to be the most acceptable in terms of microbiological, chemical and physical properties. Vedashree et al. (2020) prepared ice cream with freeze-dried ginger extracts at different concentrations, and analyzed its physico-chemical and microbial qualities. The effect of cinnamon and black pepper powders addition on the sensory and physico-chemical characteristics of ice cream was investigated by Aumpa et al. (2022). Various spice powders such as fenugreek, coriander, black pepper and cinnamon were added to ice cream by Dhanavath et al. (2022) at various, concentrations, viz. 1%, 1.5%, and 2%. The spice powders with a 1.5% incorporation level showed a higher level of acceptance. In order to investigate the effect on the chemical and physical characteristics of ice cream, MacÝt et al. (2017) used spice essential oils (coconut, lemon bark, clove, and cinnamon) in two different concentrations (0.2% and 0.4%).

Although researchers have tried to create ice creams with spice flavours using spice powders and other related forms, there are no reports on ice creams with spice oleoresin infusions. No research has been done on the effect of spice oleoresin addition on the physico-chemical, bio-chemical, and sensory attributes of ice cream. Oleoresins serve as an alternative to synthetic additives and contribute to flavour, aroma, colour and therapeutic properties like anti-inflammatory, antioxidant, antimicrobial, and anticancer properties, besides additional health benefits (Habashy et al. 2018). The use of oleoresins is preferred for the food industry because it has a specific flavor and aroma. Oleoresins offer better heat stability and flavour compared to spice powders and essential oils (Shahidi and Hossain, 2018).

The objective of the current study was to develop functional foods by incorporating spice oleoresins such as black pepper, ginger and turmeric into ice cream. The study was carried out to investigate the physico-chemical and bio-chemical properties of spice oleoresin-flavored ice cream as well as the optimization of spice oleoresin concentration for the production of spice-flavored ice cream.

Materials and methods

Selection of ingredients

Both dairy and non-dairy products were used for the preparation of ice cream. All the dairy and non-dairy products (Fresh cream, Toned milk, Skim milk powder, Sugar, Stabilizer-emulsifier blend (premitex), Spice oleoresins (Naturalich: Ozone Naturals), Natural colour (Symega food ingredients Pvt. Ltd.)) were procured from the local market at Kozhikode, Kerala, India.

Formulation of ice cream

The percentage composition of each ingredient used in the preparation of ice cream mix was calculated using Algebraic method as described by De (1980). The composition of each ingredient is estimated as toned milk – 31.10, cream - 46.65, sugar – 15.55, skim milk powder – 6.22 and stabilizer-emulsifier – 0.46 (%, w/w). The ice cream was made by the following procedures provided by De (1980), with some minor modifications made to fit the conditions in the laboratory.

Optimization of ice cream mix and ice cream

The ice cream mix was prepared by mixing all liquid ingredients and dry ingredients separately. All liquid ingredients such as toned milk and cream were mixed and blended thoroughly in a stainless-steel vessel. The dry ingredients such as sugar and skim milk powder were added to the liquid mix. After proper stirring, the ice cream mix was heated. When the temperature attained 50°C, the stabilizer and emulsifier was added. The mixture was further heated to pasteurization temperature (80°C). The temperature was maintained at 80°C and held for 5-8 mins. After pasteurization, the ice cream mix was cooled immediately to 3-4°C by placing the stainless-steel vessel in the chilled water for 20mins. At this stage, the required concentration of spice oleoresin was added to obtain the spice flavour and aroma. The different spice oleoresins used for the preparation of ice cream was black pepper (0.15, 0.20 and 0.25% w/w), ginger (0.10, 0.15 and 0.20% w/w) and turmeric (0.02, 0.05 and 0.07% w/w). The concentration of oleoresin in the ice cream mix was decided based on the preliminary trials. The mix was homogenized using an electric blender (iBELL hand mixer IBL HM 390L, 200 W) for 5 min. Additionally, the mix was kept for aging at 5 °C for 4 hours to enhance the whipping ability, texture, and overrun. The aged mix was frozen in an ice cream maker (kitchenif automatic digital ice cream maker,1.5L) for 20 minutes at a speed of 55 rpm. After freezing mix to semi-solid consistency, it was drawn directly into the ice cream cup and covered. The containers were then transferred to the freezer for hardening at a temperature of -18ºC or below for 12 h. After the ice cream was hardened, it was subjected to the physical, biochemical, microbial and sensory analysis.

Analysis of physical characteristics of ice cream mix and ice cream

The physical properties studied were acidity, pH, melting rate, overrun and colour. The method outlined in IS: 1166 - 1960 was used to determine the titratable acidity of the ice cream samples. The results were expressed as a percentage of lactic acid equivalent. The specific gravity of ice cream was measured using a pycnometer (Standard Specific Gravity Bottle) as per the AOAC procedure (AOAC 925.22 1925). The method described by Rajor and Gupta (1982) was used to assess the melting rate of ice cream. A long stem glass funnel was used to hold 30 g of ice cream that had been spread on a wire mesh measuring 2 sq. inch. Over a 100ml measuring cylinder, the funnel with wire mesh carrying the ice cream slice was placed. The weight of the melted ice cream for the first 10 min was recorded and further measured at every 5 min until the ice cream melted completely. The melting rate was measured as:

$$\text{Melting rate (g/min)} = \frac{\text{weight of melted ice cream (g)}}{\text{time (min)}} \quad (1)$$

The overrun of ice cream was determined according to the method described by De (1980). Known volume of ice cream mix was taken in a pre-weighed 200 ml glass beaker before ageing. Similarly, after freezing the mix in the ice cream maker, the partially frozen ice cream was immediately drawn and filled into the same beaker, and the initial and final mass was recorded. The overrun was determined by using the formula:

$$\text{Overrun, \%} = \frac{\text{weight of unit volume of mix} - \text{weight of unit volume of ice cream}}{\text{weight of unit volume of ice cream}} \times 100 \quad (2)$$

The pH of ice cream was determined using a digital pH meter (Eutech Instrument pH tutor) according to the method described by Dhanavath et al. (2022).

Colour of sample was determined by the method outlined by Kwon et al, (2019) using colour meter (Color Flex EZ, Hunter Lab). Spice flavoured ice cream was filled to half the volume of the sample cup and it was placed on the instrument. Digitally, the sample's colour was represented as L*, a*, and b* by the International Commission of Illumination. The L* measures brightness from 0 (black) to 100 (white). The values of a* and b*, which correspond to the two chromatic components of green to red and blue to yellow, respectively, range from -120 to 120. Triplicate readings for each sample were taken.

Determination of physico-chemical and biochemical properties

Ice cream's total solid content was calculated using the method outlined in Indian Standard Procedure IS: 1479-1961 (PARTII). Fat content was estimated by Gerber method as outlined in AOAC 2000.18, 2002. The protein content was estimated using the Kjeldhal method, as mentioned in AOAC 930.29, 2005. The ash content of ice cream was calculated by dry ashing method as outlined in AOAC method (AOAC 930, 2005). The sucrose content in ice cream was determined by the difference between the total reducing sugar and reducing sugar. The total reducing sugar and nonreducing sugar was determined by anthrone method and Nelson-Somogyi method, respectively as described by Sadasivam and Manickam (2008).

With some minor modifications, the Folin-Ciocalteu method reported by Perera and Perera (2021) was used to estimate the total phenol concentration. 0.1ml of sample was taken in an ambered test tube and dissolved in 7ml of distilled water and mixed thoroughly. 0.5ml of Folin-Ciocalteu reagent was added (1:1 v/v water) and mixed well, then incubated the test tubes for 8 min at room temperature, followed by the addition of 1.5ml of 2% sodium carbonate and 0.9ml of distilled water. The sample was stored at room temperature for 2 hours in the dark. At 765 nm, absorbance was measured using a blank. The calibration curve was prepared using gallic acid, also referred to as phenolic acid from spices, as a standard. The calibration curve was used to calculate the sample's total phenolic content, and the results were represented as milligram of gallic acid equivalent/g of sample (mg/GAE/ml).

Microbiological analysis

The total plate count of ice cream was analyzed by the Indian Standard procedure described in IS: 1166 – 1986 with slight modification. 10g ice cream was sampled in a bottle that has been sterilized, and it was tempered in a water bath at 40°C. 1ml of ice cream sample was transferred to 9ml of sterile water and then subsequent serial dilutions were made up to 10^{-8} for each sample. 1ml from 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilution was plated in sterile petri dish using nutrient agar media. The plates were further incubated for 24 h at 37°C (IG216IC: IGENE LABSERVE, Delhi).

Sensory evaluation of ice cream

The sensory evaluation of ice cream was performed using 9-point hedonic scale. The ice cream cups were served immediately after withdrawing from the freezer (after hardening) and promptly offered to the panelists. The serving sequences were totally randomized, and the samples were coded with three-digit random numbers. Sensory evaluation for spice flavoured ice cream was conducted by 10 trained panel members (Isleten and Karagul-Yuceer, 2006) and the attributes evaluated were colour, flavour, taste, texture and overall acceptability.

Statistical Analysis

A single factorial completely randomized block design (CRD) was followed to determine the effect of addition of each oleoresin in the spice flavoured ice cream. Experiments were conducted by adding each oleoresin (black pepper, ginger and turmeric) at three different levels (black pepper - 0.15, 0.20 and 0.25% (w/w), ginger - 0.10, 0.15, 0.20% (w/w) and turmeric - 0.02, 0.05, 0.07% (w/w)). Three replicates of each treatment were taken for the evaluation of its physical, biochemical, microbiological and organoleptic qualities. The quality parameters of spice flavoured ice cream developed for different concentrations of oleoresins were analyzed using R Studio (R version 4.2.1, R Core team 2022) statistical software.

Results and Discussion

Effect of addition of spice oleoresin on the qualities of ice cream

Experiments on effect of addition of spice oleoresin for production of spice flavoured ice cream were done for varying concentrations *viz.*, Black pepper (0.15, 0.20,0.25%), Ginger (0.10, 0.15, 0.20%) and Turmeric (0.02, 0.05, 0.07%). The spice flavoured ice cream

was evaluated for its physical, biochemical, microbial and organoleptic characteristics.

Effect of addition of oleoresin on the physical qualities of ice cream mix

The ice cream mix was analyzed for acidity and specific gravity. The acidity of ice cream mix remains constant for all the concentrations of oleoresins added and the value corresponded to 0.18% (Table 1). The specific gravity of ice cream mix varied from 1.090 to 1.099 (Table 1) as the concentration and type of oleoresin was varied. The ANOVA indicated that specific gravity was significantly influenced ($p \leq 0.001$) by the addition of oleoresin. Oleoresins are thick, resinous substance with a flavour that is five to twenty times stronger than that of the corresponding spice. Because of resinous nature it is dense and vicious. Sp gravity of pepper oleoresin ranges from 0.86 to 0.88, turmeric oleoresin from 0.916 to 0.936, and that of ginger oleoresin from 0.862 to 0.878. The increase in sp. Gravity ice cream may be attributed to the increased specific gravity of oleoresins. But however, in black pepper and turmeric oleoresins, it was found that an increase in oleoresin there is a decrease in specific gravity.

Effect of addition of oleoresin on the physical qualities of ice cream

Variation in overrun of ice cream: As the oleoresin concentration increased, the overrun value increased from 33.52 to 33.69%, 29.37 to 30.70% and 29.37 to 29.70% for black pepper, ginger and turmeric oleoresin infused ice cream, respectively. Low overrun values are due to the non-inclusion of air during freezing process as it was done in lab scale. The ANOVA indicated that the overrun significantly ($p \leq 0.001$) affected by the addition of oleoresin (Table 2). The yield and profit of ice cream production are directly affected by the overrun, which also affects the product's body, texture, and flavour (Pinto et al. 2004). In the findings of Yeon et al. (2017), ice creams with fermented pepper powder had a significantly lowered overrun than plain ice cream. This may be related to viscosity; if the mixture has a high viscosity, less air will get into the ice cream during production. Ice cream with ginger shreds in it reduced overrun, with higher addition levels having a significantly greater effect (Pinto et al. 2006).

Variation in pH of ice cream: For ice cream added with a black pepper oleoresin flavour, the pH ranged from 6.58 to 6.60.

Table 1: Physical properties of spice oleoresin flavoured ice cream mix

Oleoresin	Concentration of oleoresin (%)	Acidity (% as lactic acid)	Specific gravity
Black pepper	0.15	0.18[a]	1.099[a]
	0.20	0.18[a]	1.095[b]
	0.25	0.18[a]	1.093[c]
Ginger	0.1	0.18[a]	1.097[b]
	0.15	0.18[a]	1.098[b]
	0.2	0.18[a]	1.099[a]
Turmeric	0.02	0.18[a]	1.099[a]
	0.05	0.18[a]	1.097[b]
	0.07	0.18[a]	1.090[c]

Different letters indicate significant differences at $p \leq 0.001$

Table 2: Physical properties of spice oleoresin flavoured ice cream

Oleoresin	Concentration of oleoresin (%)	Over run %	pH	Acidity (% lactic acid)	Melting rate (g/min)	Colour value L*	a*	b*
Black pepper	0.15	33.52[c]	6.58[a]	0.18[a]	0.83[b]	87.61[a]	-4.10[c]	17.86[c]
	0.20	33.64[b]	6.60[a]	0.18[a]	0.85[b]	87.42[b]	-4.82[b]	18.10[b]
	0.25	33.69[a]	6.60[a]	0.18[a]	0.97[a]	85.94[c]	-6.29[a]	19.56[a]
Ginger	0.10	29.37[b]	6.41[b]	0.20[c]	0.84[b]	89.42[b]	-0.65[c]	21.48[b]
	0.15	30.53[a]	6.47[b]	0.19[b]	0.87[b]	89.67[a]	-0.87[b]	21.48[b]
	0.20	30.70[a]	6.54[a]	0.18[a]	0.91[a]	89.73[a]	-1.21[a]	22.95[a]
Turmeric	0.02	29.37[c]	6.42[b]	0.20[c]	0.85[b]	90.15[a]	-5.92[c]	36.59[c]
	0.05	29.63[b]	6.56[a]	0.18[a]	0.87[b]	88.99[b]	-6.64[b]	48.22[b]
	0.07	29.70[a]	6.58[a]	0.18[a]	0.99[a]	87.74[c]	-6.68[a]	56.41[a]
Control	Nil	-	6.52	0.20	0.75	-	-	-

Different letters indicate significant differences at $p \leq 0.001$

However, the variation was non-significant. The pH of ginger and turmeric flavoured ice cream varied from 6.41 to 6.54 and 6.42 to 6.58, respectively. The pH of ginger and turmeric flavoured ice cream was significantly influenced ($p \leq 0.01$) by the addition of oleoresin. The pH of the developed ice creams was compared to ice cream available in the market (as control) and the value was found to be 6.52. It is observed that the pH of all concentrations was closer to the market sample. In the study by Dhanavath et al. (2022), the pH of ice cream added with spice powders was found to be in the range of 6.58 to 6.68. As reported by Gabbi et al. (2018), the addition of ginger juice and powder resulted in a notable rise in acidity and fall in pH of the ice cream samples.

Variation in acidity of ice cream: The acidity ice cream from 0.18 to 0.20% for different concentrations of black pepper, ginger and turmeric oleoresin infused ice creams (Table 2). The addition of ginger oleoresin had a significant effect on the acidity of ice cream. Sagdic et al. (2012) reported that due to the acidic character of phenolic compounds, their addition into ice cream, increased the acidity. When processed amla which is rich in phenolic compounds (Gooseberry) was added, the acidity and pH of the ice cream significantly increased (Goraya and Bajwa, 2015). The acidity of oleoresin infused ice cream was compared to melted market sample (as control) and the value was found to be 0.20%. Dhanavath et al. (2022) reported that ice cream incorporated with various spices powders had an acidity of 0.28% and also stated that titratable acidity of the samples had significantly increased during storage period.

Variation in melting rate of ice cream: As the concentration of oleoresin increased, the melting rate of ice cream increased from 0.83 to 0.99 g/min. The addition of oleoresins considerably ($p \leq 0.01$) affected the melting rate. According to Aumpa et al. (2022), the ice cream melted at a rate ranging from 0.23 and 0.52 g/min. The melting rate increased as black pepper powder and cinnamon powder were added. This is due to the use of fibre powder in an ice cream matrix can promote thermal diffusion when the food is heated by the environment. The fat network plays an important role in influencing how quickly ice cream melts (Muse and Hartel, 2004). According to Gabbi et al. (2018), the added solids, including some starch from the ginger, might be the reason why the melting rate decreased as the amount of processed ginger in the ice cream was increased. In accordance with Pinto et al. (2006), the melting resistance of ice cream containing ginger shreds increased with the addition of more shreds.

Variation in colour value: Colour value of ice cream prepared for varying concentrations of oleoresin is presented in terms of L*, a* and b* values (Table 2). As the concentration of oleoresin increased from 0.15 to 0.25%, the L* value of for black pepper oleoresin infused ice cream decreased from 87.61 to 85.94. the a* value of colour, which indicates the greenness also decreased from -4.10 to -6.29. The b* value, which indicated the yellowness of ice cream was increased from 17.86 to 19.56.

Ice cream's L* value increased from 89.42 to 89.73 as the concentration of ginger oleoresin increased from 0.10 to 0.20%. The a* value reduced from -0.65 to -1.21, while its b* value increased from 21.48 to 22.95 for ginger flavoured ice cream. The L* value for ice cream reduced from 90.15 to 87.74 as the concentration of turmeric oleoresin increased from 0.02 to 0.07%, while the a* value declined from -5.92 to -6.68 and the b* value increased from 36.59 to 56.41. The addition of oleoresin considerably ($p \leq 0.001$) affected the ice cream's colour values L*, a*, and b*. Aumpa et al. (2022) reported that increase in black pepper powder and cinnamon powder resulted in a considerable reduction in the ice cream's L* value while an increase in its a* and b* values. The presence of many pigment molecules in the spices, including the yellow, red, and brown carotenoids and flavonoids, which are responsible for the product's altered colour qualities, caused the alterations in colour intensity. Sagdic et al. (2012) observed that adding phenolic material to ice cream significantly altered its colour qualities. Yeon et al. (2017) reported that L* decreased while a* and b* increased, resulting in a lowering colour value in ice cream containing fermented pepper powder. Pepper powder and cinnamon powder was added to create a lighter shade of brown that produced more redness and yellowness. The colour value L*, a* and b* varied significantly and when ice creams are supplemented with different additives (Akalýn et al. 2008; Hwang et al. 2009; Sagdic et al. 2012).

Effect of addition of spice oleoresin on physico-chemical and biochemical qualities of ice cream

Physico-chemical characteristics such as total solids, protein, ash, fat, and sucrose, as well as biochemical characteristics including total phenol content of the oleoresin infused ice creams, were investigated.

Variation in total solids of ice cream: Table 3 shows the effect of spice oleoresins on the total solids of ice cream. The total solids had a highly significant ($p \leq 0.001$) effect on the addition of oleoresin. Total solids of ice cream increased significantly with the addition of fig paste, although milk fat levels at various levels had a little impact (Murtaza et al. 2004). According to Gabbi et al. (2018), the total solids of the ice cream significantly decreased as the levels of the ginger juice and paste increased because of the low solid content of those ingredients. On the other hand, due to the high dry matter of candy and powder, total solids increased when they were added.

Variation in protein content of ice cream: The protein of ice cream varied from 5.53 to 5.71%, however the variation was non-significant. The protein content of spice oleoresin flavoured ice cream was compared to market sample (as control) and the value found to be 4.9%. It is observed that the protein content of pepper ice cream was higher than that of the control. Perera and Perera (2021) reported that the protein composition of the ice creams did not differ significantly. According to Gabbi et al. (2018), the

protein level of processed ginger-infused ice cream was significantly lower except that of ginger powder, which had a comparatively higher protein content than other forms of ginger.

Variation in ash content of ice cream: The spice flavoured ice cream's ash content ranged from 0.66 to 1.00%, although the variation was not statistically significant (Table 3). Perera and Perera (2021) found that adding spices in small quantities had no noticeable effect on the amount of ash in spice flavoured ice cream. According to Pagthinathan (2020), ice cream added with ginger paste had a higher percentage of ash content than ice cream with ginger juice added. According to Gabbi et al. (2018), the addition of ginger paste and powder increased the ice cream's ash level; however, the addition of ginger juice and candy decreased the ice cream's ash content.

Variation in fat content of ice cream: As the oleoresin concentration varied, the fat content of the ice cream increased from 12.26 to 12.80%, although the change was not statistically significant (Table 3). According to Akaln et al. (2008), partial coalescence occurred during the freezing process of the ice cream mixture, wherein clumps and clusters of the fat globules form and enclose air to form an inner fat structure or network. According to Perera & Perera (2021), the fat percentages of regular coconut ice cream and spicy coconut ice cream were 11.66% and 11.06%, respectively.

Variation in sucrose content of ice cream: The ice cream gets a delightful sweetness from the sucrose. The sucrose content of spice oleoresin infused ice cream ranged from 15.11 to 15.74 %. The sucrose content was influenced ($p \leq 0.001$) by the addition of oleoresin. The sucrose content of the oleoresin infused ice cream complied with ISI requirements.

Variation in total phenol content of ice cream: The effect of spice oleoresin on total phenol content (TPC) of ice cream is presented in Table 3. As the pepper oleoresin concentration increased from 0.15 to 0.25%, the TPC increased from 330.42 to 445.33 mg GAE/ml. The TPC was significantly ($p \leq 0.01$) influenced by the addition of oleoresin. The TPC of ice cream rises from 356.02 to 407.54 mg GAE/ml as the ginger oleoresin concentration rises from 0.10 to 0.20%. But the difference wasn't statistically significant. The TPC of turmeric oleoresin infused ice cream rises from 368.81 to 425.16 mg/GAE/ml as the oleoresin concentration rises from 0.02 to 0.07%. The difference wasn't considerable, though. Pepper contains piperine, ginger has gingerol, and turmeric contains curcuminoids as phenolic substances. Perera & Perera (2021) investigated the antioxidant activity of ice cream and the total phenolic content was analyzed. Due to the addition of spices, spice flavoured coconut ice cream had greater levels of total phenol. Aumpa et al. (2022) reported that the difference between black pepper powder and cinnamon powder showed a considerable impact on spice ice cream's TPC and antioxidant properties. The TPC ranged from 216.7 to 484.4 mg of TE/g of sample. Antioxidant activities, which are distinct variables that measure a sample's ability to suppress free radicals and their ability to prevent lipid components from degrading, can also be impacted by polyphenol chemicals. Additionally, adding spices to food increased antioxidant activities, indicating a positive correlation between the antioxidant properties and phenolic content.

Effect of addition of spice oleoresin on organoleptic characteristics of ice cream

The black pepper oleoresin flavoured ice cream was evaluated to study the organoleptic properties like colour, flavour, taste, texture and overall acceptability. The effect of addition of oleoresin on the organoleptic characteristics of ice cream is presented in Fig. 1. The results indicated that, as the concentration of black pepper oleoresin increased from 0.15 to 0.25%, the score of colour, flavour, taste, texture and overall acceptability of ice cream was found to increase initially and then decrease. The figure shows that the score of colour, flavour, taste, and texture for ice cream with black pepper oleoresin addition increased from 6.3 to 8, 6.3

Table 3: Physico-chemical and biochemical qualities spice oleoresin flavoured ice cream

Oleoresin	Concentration of oleoresin (%)	Total solids %	Protein %	Ash %	Fat %	Sucrose %	Total phenol content (mg GAE/ml)
Black pepper	0.15	43.79^{b}	5.56^{a}	1.00^{a}	12.26^{b}	15.74^{a}	330.42^{c}
	0.20	43.86^{b}	5.56^{a}	1.00^{a}	12.33^{ab}	15.55^{b}	393.72^{b}
	0.25	46.20^{c}	5.53^{a}	0.77^{b}	12.53^{a}	15.11^{c}	445.33^{a}
Ginger	0.10	43.64^{b}	5.71^{a}	0.66^{b}	12.40^{b}	15.65^{a}	356.02^{b}
	0.15	43.82^{b}	5.71^{a}	0.88^{a}	12.53^{a}	15.49^{b}	384.57^{ab}
	0.20	53.06^{a}	5.71^{a}	1.00^{a}	12.60^{a}	15.24^{c}	407.54^{a}
Turmeric	0.02	44.98^{c}	5.70^{a}	0.88^{a}	12.53^{b}	15.70^{a}	368.81^{b}
	0.05	48.93^{b}	5.56^{b}	0.77^{a}	12.66^{ab}	15.57^{b}	378.47^{ab}
	0.07	52.06^{a}	5.53^{b}	0.66^{a}	12.80^{a}	15.36^{c}	425.16^{a}

Different letters indicate significant differences at $p \leq 0.001$

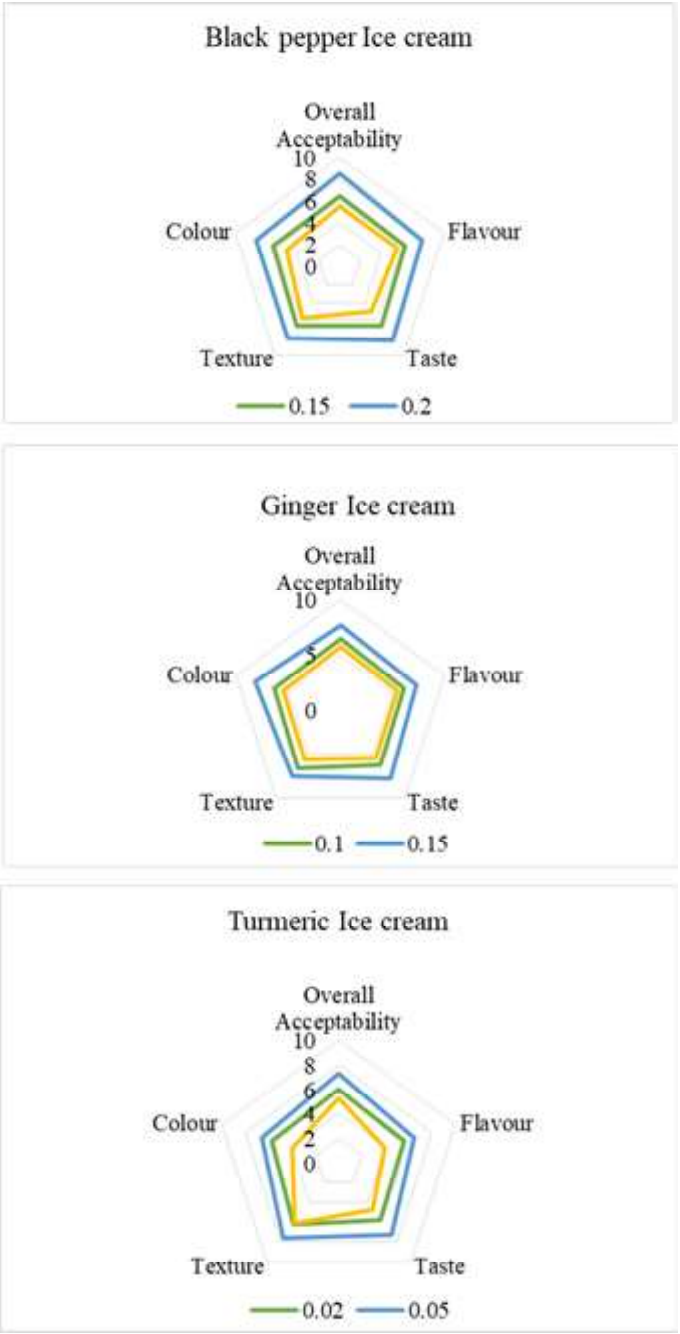

Fig. 1 Effect of addition of oleoresin on organoleptic properties of ice cream

to 7.9, 6.6 to 8.3, and 6.6 to 8 accordingly, when the concentration of oleoresin increased from 0.15 to 0.20%. Further increase in concentration of oleoresin to 0.25% decreased the score of colour, flavour, taste and texture to 5, 5.6, 5.5, 7 respectively. A key consideration in the organoleptic evaluation of black pepper oleoresin ice cream is the overall acceptability. For ice cream with black pepper oleoresin, as the amount of oleoresin increased from 0.15 to 0.20%, the overall acceptability increased from 6.5 to

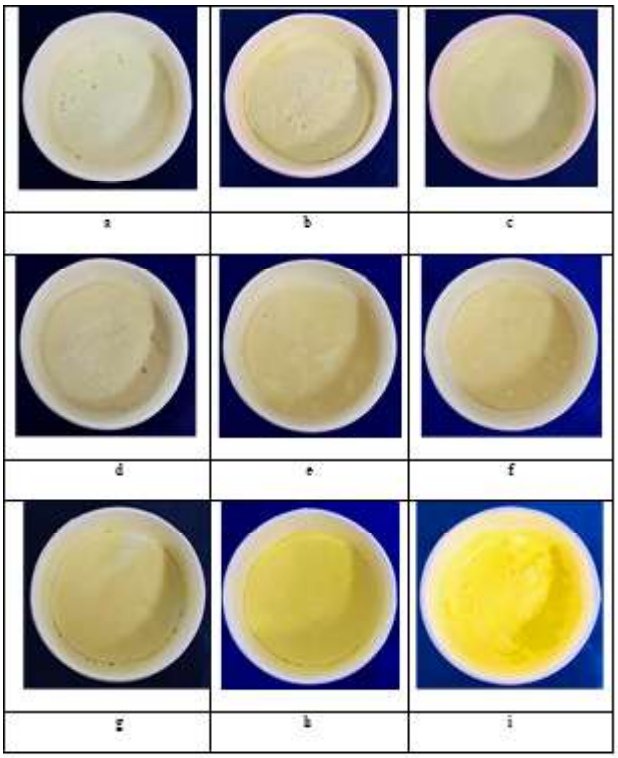

Fig. 2 Ice cream prepared with black pepper oleoresin at a concentration of a) 0.15%, b) 0.20%, c) 0.25%, ginger oleoresin at concentration of d) 0.10%, e) 0.15 % and f) 0.20%, turmeric oleoresin at a concentration of g) 0.02%, h) 0.05% and i) 0.07%

8.5. For the corresponding oleoresin concentration increased to 0.25%, overall acceptability score reduced to 5.5. The addition of oleoresin had significant effects on the overall acceptability ($p \leq 0.001$).

The ice cream with ginger oleoresin was evaluated for its organoleptic properties, including colour, flavour, texture, and overall acceptability. Fig. 1 depicts how the addition of oleoresin affected the organoleptic properties of ice cream. From the figure it was observed that for ice cream with ginger oleoresin addition, as the concentration of oleoresin increased from 0.10 to 0.15%, the score of colour, flavour, taste and texture showed an increase from 6.3 to 8.2, 6.1 to 7.3, 6.2 to 7.8, 6.6 to 7.5 respectively. Further increase in concentration of oleoresin to 0.20% decreased the score of colour, flavour, taste and texture to 5.5, 5.5, 5.5, 5. 6 respectively. The overall acceptability of developed ice cream increased initially and then decreased. For ice cream with ginger oleoresin, as the amount of oleoresin increased from 0.10 to 0.15%, the overall acceptability increased from 6.5 to 7.7. For the corresponding oleoresin concentration increased to 0.20%, overall acceptability score reduced to 5.7. The overall acceptability was significantly influenced ($p \leq 0.001$) by the addition of oleoresin.

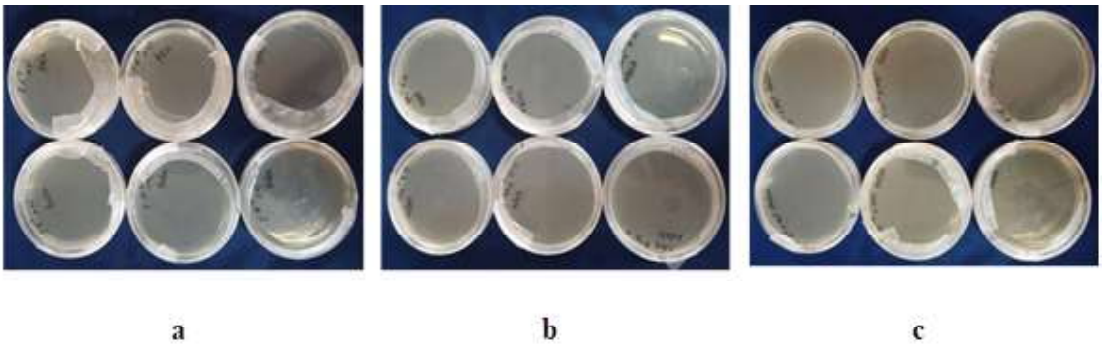

Fig. 3. Microbiological analysis of a) black pepper, b) ginger and turmeric oleoresin flavoured ice cream

The turmeric flavoured ice cream was evaluated to study the organoleptic properties like colour, flavour, taste, texture and overall acceptability. Fig. 1 shows the effect of oleoresin addition on the organoleptic properties of ice cream. The findings showed that the colour, flavour, taste, texture, and overall acceptability of ice cream increased first and then decreased as the concentration of turmeric oleoresin increased from 0.02 to 0.07%. As the concentration of oleoresin increased from 0.02 to 0.05%, the score of colour, flavour, taste and texture showed an increase trend from 5.7 to 6.6, 5.6 to 6.5, 5.8 to 7.3, 6.2 to 7.6 respectively. Further increase in concentration of oleoresin to 0.07% decreased the score of colour, flavour, taste and texture to 4, 4, 4.7, 6.1, respectively. For ice cream with turmeric oleoresin, as the amount of oleoresin increased from 0.02 to 0.05%, the overall acceptability increased from 6 to 7.3. For the corresponding oleoresin concentration increased to 0.07%, overall acceptability score reduced to 5.3. The overall acceptability was significantly influenced ($p \leq 0.001$) by the addition of oleoresin.

Optimization of Spice Flavoured Ice cream

The most acceptable spice oleoresin was chosen based on the overall acceptability of the ice cream as a whole. Ice cream with 0.20% black pepper oleoresin obtained maximum overall acceptability of 8.5. Ice cream with 0.15% ginger oleoresin obtained maximum overall acceptability of 7.7. The overall acceptability of the ice cream that was made with 0.05% turmeric oleoresin, was 7.3.

Microbiological Analysis

Ice cream with spice oleoresins were subjected to a microbiological analysis. The standard plate count was found to have a low microbial load after 24 hours. The increased level of spice oleoresin incorporation in the ice cream revealed a low microbial count. The antibacterial and antifungal activities spices could be the reason for the low microbial load in the spice flavoured ice cream. The plating results of 10^{-5} dilutions were shown in Fig. 3.

Conclusions

Different spice oleoresins, such as black pepper (0.15, 0.20, and 0.25% w/w), ginger (0.10, 0.15, and 0.20% w/w), and turmeric (0.02, 0.05, and 0.07% w/w), were added at variable quantities in order to make the spice-flavored functional ice cream. With a total solids content of 43.86%, a fat content of 12.33%, and a total phenol content of 393.72 mg GAE/ml, the addition of 0.20% black pepper oleoresin had the greatest overall acceptability score of 8.5 and was ranked top. Ginger flavoured ice cream with the concentration of 0.15% with overall acceptability score of 7.7, overrun of 30.53%, melting rate of 0.87 g/min, total solids of 43.82%, fat content of 12.53% and total phenol content of 384.57 mg GAE/ml. The turmeric oleoresin had the lowest overall acceptability score of 7.3, overrun of 29.63%, melting rate of 0.87g/min, total solids of 48.93%, fat content of 12.66%, and total phenol content of 378.47 mg GAE/ml among the three spice oleoresins infused ice creams. Taking into account the overall acceptability of the finished product, the most palatable spice oleoresin was selected.

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RESEARCH ARTICLE

Isolation, identification and investigation, of genetic diversity of *Lactobacillus* bacteria strains in traditional dairy products of Lorestan province (Iran)

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Abstract: Traditional dairy products contain a diverse set of bacteria that produce useful biological compounds called bacteriocins. Lorestan province is rich in natural flora due to its special ecosystem and traditional dairy products. In this regard, to isolate, identify and investigate the genetic diversity of *Lactobacillus* bacteria found in dairy products, 10 samples from each of the traditional yogurt, dough, and cheese products of Lorestan province were collected and evaluated. Primary bacteria were isolated using special culture medium (MRS) and various biochemical tests including gram test, catalase test and antibiogram test. In the next step, to confirm and identify the isolated bacteria, 16SrRNA gene amplification and sequencing were used. After identifying the bacteria, the genetic diversity between the isolated bacterial strains was evaluated using REP-PCR and BOX-PCR genetic markers. The antibiogram test showed that all isolated bacterial strains were resistant to vancomycin and kanamycin and sensitive to gentamicin. After the amplification of 1500 base pairs of 16SrRNA gene and its evaluation in databases, it was found that the strain isolated from the dairy products yogurt, dough and cheese were the most similar to *Lactobacillus casei*, *Limosilactobacillus fermentum* and *Lactobacillus helveticus* bacteria respectively. The grouping of the studied bacterial strains based on the similarity matrix of the combination of two markers showed that the studied bacteria formed 7 groups at a similarity coefficient of 45%, and the bacteria isolated from Noorabad local yogurt formed only one group. The results of the present study showed the high potential of traditional dairy products of Lorestan province in terms of having probiotic strains with high genetic diversity, which can be considered as suitable biological preservatives and used to produce of various dairy products commercially.

Keywords: Bacteriocin, dairy, genetic marker, *Lactobacillus*, Lorestan

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Introduction

In recent years, the reduction of food losses due to corruption in the stages of food production has become the focus a lot of important and practical research (Evivie et al. 2020). With the discovery of the positive effects of different species of bacteria on human health in the early 20th century, the first study steps were taken towards research in the field of probiotics. Today, probiotics are classified as live microorganisms that, when consumed in sufficient quantities, can provide many health benefits, and improve the immune system (Meyer-Torpa et al. 2021). The use of beneficial microorganisms in the food and pharmaceutical industries has a long history. Probiotics prevent the growth of spoilage bacteria and pathogens during food storage. Therefore, probiotics are an important way to preserve food and prevent the spread of pathogens through food. Probiotics also have very strong antioxidant properties that prevent food oxidation during food storage (Biolcati et al. 2022).

One of the most important things that must be considered to produce a probiotic product is the ability to transport and distribute, store and manage without losing its viability (Jarocki et al. 2020). Several physical and chemical factors, including oxidation, humidity changes, osmotic pressure, and temperature, affect the viability of probiotic microorganisms. In addition, the conditions of the human digestive system are not suitable for many probiotic species (Derrien and Hylckama Vlieg. 2015).

Lactic acid bacteria (LABs) are of high economic importance and play an important role in the fermentation process of traditional dairy products. Their metabolic properties help to develop the desirable characteristics of food products and allow the nutritional value of raw materials to be maintained and often increased (Linares et al. 2017). Many species of *Lactobacillus*

bacteria are in the group of probiotics. These bacteria are tolerant to many environmental changes during manufacturing and processing, storage and transportation, as well as digestive tract conditions. Therefore, they can be a suitable source for the development of probiotic products (Toole et al. 2017).

There are a wide range of laboratory techniques for the identification of lactic acid bacteria, and they can be evaluated based on morphological, biochemical, physiological, and genetic characteristics using different methods. Molecular genetic techniques are the main tools for the analysis of microorganisms and are expanding rapidly. One of the most widely used techniques for bacterial identification is the use of specific primers and amplification of all or part of the 16SrDNA gene sequence (Ferone et al. 2020).

Various DNA fingerprinting techniques exist to access the genetic diversity of different microorganisms, and the use of primers for specific genomic regions provide specific diversity patterns that are very efficient for isolating and grouping microorganisms. Repetitive exogenous palindromic sequence amplification (REP) and BOX are among the fingerprinting techniques developed for genetic analysis in prokaryotes (Borba et al. 2020). Analysis using primers of these regions with repetitive elements creates specific diversity patterns for each bacterium, which can be a suitable tool for distinguishing and evaluating genetic diversity between bacterial strains (Korvin et al. 2014).

LABs can inhibit food pathogens and increase the life of food (Fidana et al. 2022), therefore, adding these bacteria as food supplements is of great interest (Munekata et al. 2021). The natural flora of dairy products is complex and includes many strains of LABs. Since these dairy products containing these bacteria have better and unique flavors compared to products made from pasteurized milk (Biolcati et al. 2022). Due to the high importance of lactic acid bacteria in the food industry, identification, and evaluation of new strains of this group of bacteria can be a very important step in improving the production of dairy products. Based on this, the present study was carried out to isolate, identify and investigate the genetic diversity of lactic acid bacteria in traditional dairy products of western Iran, including different geographical areas of Lorestan province.

Materials and methods

Bacterial strains and growth conditions

A total of 30 acid lactic bacteria isolated from traditional yogurt, dough, and cheese. Samples were collected from different regions of Lorestan province with different geographical distances and weather conditions. After sampling, the samples were transferred to the laboratory in sterile containers on ice and kept in a refrigerator at 4°C for further processing. Isolates were inoculated in selective media MRS broth and incubated under anaerobic conditions (10% CO_2) at 37ÚC for 48 hours. The bacterial isolates examined, and their sources are listed in Table 1.

Morphological and biochemical characterization

For each strain, a liquid culture was grown from a single colony. All isolates were identified according to their morphological and biochemical characteristics. To determine the type of bacteria, gram test and observation under the microscope were used. For catalase test, a drop of 3% hydrogen peroxide was added to a fresh culture on a sterile glass slide and mixed well. Producing bubble or froth, indicated catalase-positive and no bubble or froth indicated catalase negative. After determining the type of bacteria, the antibiogram test was used to determine the pattern of sensitivity and resistance of bacterial strains. For the antibiogram test, 6 commercial antibiotics (tetracycline, vancomycin, amikacin, ampicillin, kanamycin, and gentamicin) were used by the disk diffusion method.

DNA isolation

Total DNA was extracted from a culture inoculated with a single colony. Bacteria cells were grown in 1.5 ml MRS broth for 24 h at 37° C temperature and genomic DNA extractions of the isolates were extracted using the Bacterial DNA Isolation Kit (Denazist, Iran) and the quality and quantity of DNA were measured to determine acceptable purity using spectrophotometry (Bio-Rad SmartSpec 3000 UV/Vis Spectrophotometer, USA) and electrophoresis on 1 % agarose gel, in 1X TAE buffer.

Oligonucleotide primers

Repetitive sequence based on polymerase chain reaction (REP-PCR) fingerprinting with BOX-PCR and REP-PCR was conducted to obtain the genomic fingerprinting of all bacterial isolates. The oligonucleotide primers used in present study are as listed in

Table 1: Bacteria isolated used in this study

Isolated code	Source	Isolated code	Source
B1	Yoghurt	B16	Dough
B2	Yoghurt	B17	Dough
BC	Yoghurt	B18	Dough
B4	Yoghurt	B19	Dough
B5	Yoghurt	B20	Dough
B6	Yoghurt	B21	Cheese
B7	Yoghurt	B22	Cheese
B8	Yoghurt	B23	Cheese
B9	Yoghurt	B24	Cheese
B10	Yoghurt	B25	Cheese
B11	Dough	B26	Cheese
B12	Dough	B27	Cheese
B13	Dough	B28	Cheese
B14	Dough	B29	Cheese
B15	Dough	B30	Cheese

Table 2.

16SrDNA Amplification and Sequencing

Fragments of the 16SrDNA genes were amplified using the universal primers (Table 2). PCR-mediated amplification was carried out in a gradient MyCycler™ thermal cycler system (Bio Rad, USA). The amplification conditions were performed in 25 µl volumes containing 50 ng of template DNA, 10X PCR reaction buffer containing 20 mM $MgCl_2$, 10 pmol each of the primers, 2.5 mM of the dNTPs mixture and 1 U of Sinaclon *Taq* DNA polymerase (Sinaclon, Iran).

The 16SrDNA-PCR consisted of an initial denaturation step at 95°C for 5 minutes, which was followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 4 minutes, and a final extension at 72°C for 10 minutes.

The PCR products were checked for correct amplification by electrophoresis on 1.5% agarose gel under UV illumination. PCR products were purified and sequenced with the same primers used in a PCR (Denazist, Iran). Identification and similarity search of the 16SrDNA sequences was carried out using the BLASTN program at the NCBI database (*http.www.ncbi.nlm.nih.gov*) for identification of bacterial isolates at species levels. Phylogenetic analysis was performed sequence alignment using ClustalW and phylogenetic trees were constructed using the neighbor-joining method of MEGA6 program.

BOX and REP-PCR conditions

PCR amplification was performed in 25 µl volumes containing 50 ng of template DNA, 10X PCR reaction buffer containing 20 mM $MgCl_2$, 10 pmol/µl each of the primers, 2.5 mM of the dNTPs mixture, and 1 U of Sinaclon *Taq* DNA polymerase (Sinaclon, Iran). PCR amplification was performed in a gradient MyCycler™ thermal cycler system (Bio Rad, USA).

The BOX-PCR consisted of an initial denaturation step at 95°C for 5 minutes, which was followed by 40 cycles of 94°C for 1 minute, 61°C for 1 minute, 72°C for 4 minutes, and final extension at 72°C for 10 minutes. For REP-PCR, the conditions were 95°C for 5 minutes, which was followed by 40 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and a final extension for 10 minutes at 72°C.

Amplified PCR products were evaluated by electrophoresis on 1.5 % agarose gel, in 1X TAE buffer and visualized under UV light by safe staining and photographed using Gel documentation system (Bio-Rad, USA). To determine the size of the amplified fragment, 100bp DNA ladder (Sinaclon, Iran), was used as size standard molecular marker.

Calculation of genetic diversity and cluster analysis

The presence (1) and absence (0) of REP-PCR and BOX-PCR products were recorded and assembled in a data matrix for each isolate. Analysis of the binary scores was performed using NTSYS-pc version 2.1 software (Rohlf. 2002). A similarity matrix 30 bacteria was calculated using of the simple matching coefficient. The clustering and draw of dendrogram was based on unweighted pair group method with arithmetic averages (UPGMA).

Results and Discussion

Gram, catalase and antibiogram test

Gram test results showed that most of the grown bacteria were Gram-positive bacteria (purple color) and bacilli and negative catalase. However, in some cases, Gram-positive cocci-shaped bacteria were also observed. After isolation of Gram-positive bacteria grown on MRS culture medium, antibiogram test and disc diffusion technique were used to group and more accurately identify the isolated bacteria. Based on the results, all the studied bacteria were resistant to vancomycin and kanamycin and sensitive to gentamicin. Based on resistance and sensitivity to three antibiotics, tetracycline, amikacin and ampicillin, the isolated bacteria had different patterns.

Bacteria isolated from yogurt showed resistance to tetracycline, amikacin and ampicillin antibiotics, while bacteria isolated from dough and cheese were sensitive to tetracycline antibiotics. Bacteria isolated from dough and cheese were generally sensitive to the antibiotic ampicillin, while bacteria isolated from yogurt were relatively resistant to the antibiotic ampicillin. In general, based on the level of sensitivity and resistance to the studied antibiotics, the isolated bacteria showed significant differences.

Table 2: Primers and conditions used in the PCR experiments

Name of primer	Sequence (5'-3')	Annealing Temperature
REP	5′-GTGGTGGTGGTGGTG-3′	56
BOX	5′-CTACGGCAAGGCGACGCT-3′	61
16SrDNA-F	5′-AGAGTTTGATCCTGGCTCAG-3′	60
16SrDNA-R	5′-AAGGTTACCTCACCGACTTC-3′	

Amplification and sequencing of 16SrDNA gene

The results of amplification of the 1500 bp fragment of 16SrDNA gene showed that the specific primers were able to amplify this gene in all the studied bacterial strains (Figure 1). After the successful amplification of 16SrDNA gene, the PCR product was sequenced. The target sequence was aligned against other genetic information of Lactobacillus family bacteria using BLAST tool in NCBI database. The results showed that the isolated bacteria belonged to different species of *Lactobacillus* genus.

To determine the phylogenetic relationships between the isolated bacterial strains, a multiple alignment was performed between the 16SrDNA gene sequences of three bacteria isolated from the dairy products of yogurt, dough, and cheese, and finally a phylogenetic tree was drawn. The results showed that the bacteria isolated from yogurt and cheese were placed in the same group with 87% similarity, and the bacteria isolated from dough formed a common group with these two bacteria at a further distance (Figure 2).

Phylogenetic tree and determination of bacterial strain

Based on the results of BLASTN and determining the similarity between the nucleotide sequences of the 16SrDNA gene, 22 bacterial strains from different species of *Lactobacillus* genus were selected based on the most genetic similarity with the isolated bacteria, and a phylogenetic tree was drawn based on the results of multiple alignment in MEGA software and ClustalW tool. The results showed that the bacterial strain isolated from yogurt (Lorestan-A) was 99% similar to *L.helveticus* bacteria and 95% similar to *Lactobacillus casei* bacteria. The bacterial strain isolated from the dough sample (Lorestan-B) was similar to *Limosilactobacillus fermentum* bacteria with 89% similarity, and the bacterial strain isolated from the cheese sample (Lorestan-C) belonged to *L.helveticus* species with 98% similarity (Figure 3).

Genetic diversity of the studied bacterial strains

The results of PCR product electrophoresis for two REP-PCR and BOX-PCR markers showed that the REP-PCR marker was polymorphic between bacterial strains with the amplification of 15 fragments (bands) and about 91% polymorphism. The BOX-PCR marker with 11 bands and 87% polymorphism showed differences between the studied bacterial strains (Figure 4).

The grouping of bacterial strains based on the combination of information from two markers showed that the studied bacteria formed 7 groups with a 45% similarity coefficient. Genotype number 3 (B3 isolated from yogurt sample) formed a group separately (Figure 5).

The results showed that the grouping was largely consistent with the type of dairy product. So that yogurt, dough and cheese samples were separately placed in similar groups. The grouping results obtained were not consistent with the geographical distances and the bacterial strains isolated from dairy products of similar geographical areas were placed in different groups (Figure 5). In order to check the accuracy of grouping, principal coordinate analysis (PCOA) and grouping based on two principal components (two-dimensional) and three principal components (three-dimensional) were performed. The obtained results confirmed the grouping and showed that the bacterial strains that were isolated from different dairy products were located at a

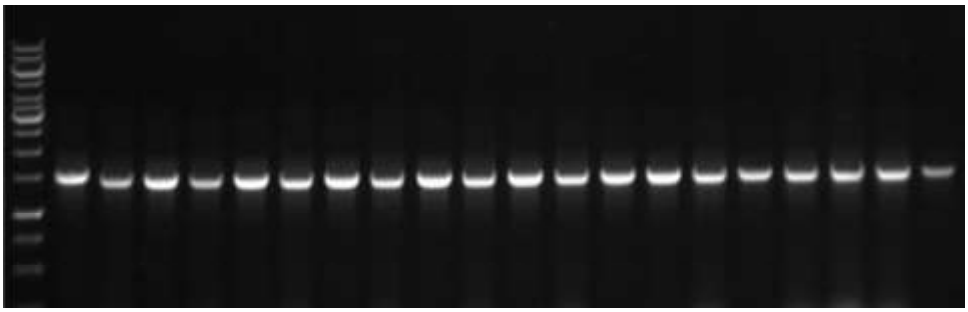

Fig. 1 Amplification of 1500bp of 16SrDNA gene using PCR technique (The numbers 1 to 20 indicate the number of the bacterial strain and M indicates the marker with a standard size of 1Kb)

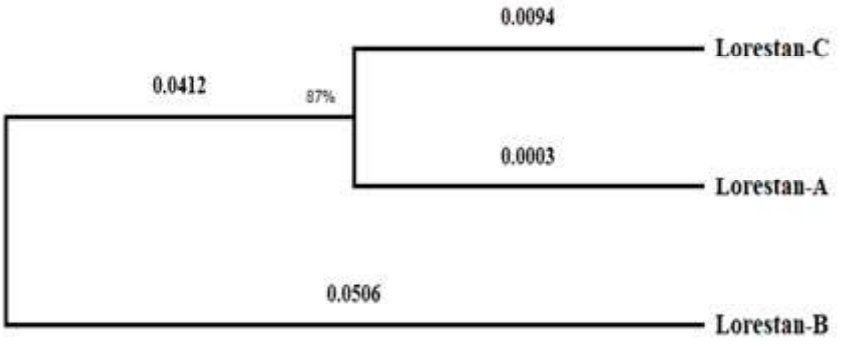

Fig. 2 Phylogenetic tree of selected bacterial strains based on the nucleotide sequence of 16SrDNA gene (Lorestan-A strain of bacteria isolated from yogurt, Lorestan-B strain of bacteria isolated from dough and Lorestan-C strain of bacteria isolated from cheese)

further distance from each other (Figure 6).

Identifying and collecting native strains of probiotic bacteria in traditional products, in addition to maintaining and organizing germplasm, can be used in the production of industrial products using useful and high-efficiency strains. Local dairy products and traditional fermented foods are rich in probiotics and have beneficial and health-promoting properties due to the presence of probiotic bacteria, including lactic acid bacteria (Callon et al. 2004). Probiotic microorganisms include a diverse group of bacteria and fungi that exist symbiotically with other organisms and play a very decisive role in creating the natural balance of ecosystems (Rojek et al. 2022).

Due to the rapid development of the probiotic industry, there is an urgent need to identify new probiotics. To obtain new lactic acid bacteria with high probiotic potential, it is very important to investigate and isolate bacterial species and strains in dairy products (Zhanget al. 2022).

The results of the present study showed that the use of morphological and biochemical techniques such as gram test and catalase test can be very effective in primary separation and reducing the number of examined samples. Lactic acid bacteria in traditional dairy products collected from Lorestan province were all gram-positive and catalase-negative, which was consistent with the results of previous studies. Borga et al. (2017) in their

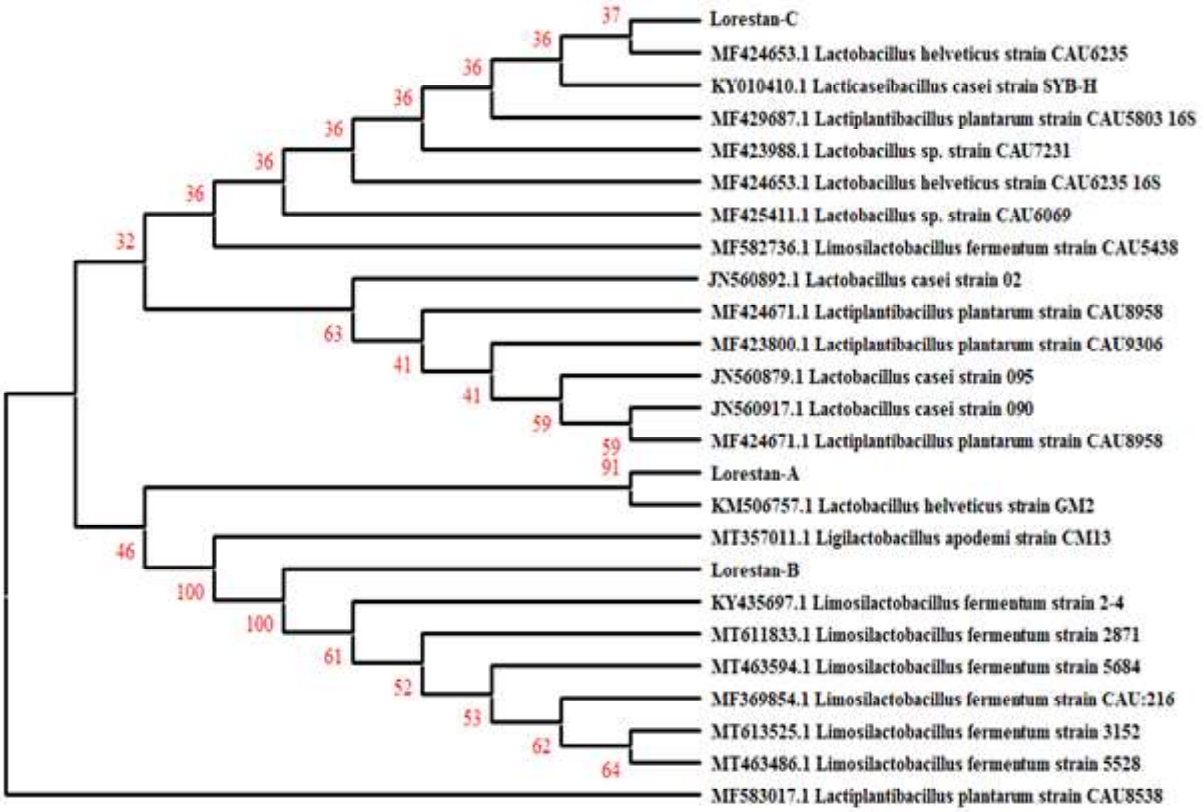

Fig. 3 Phylogenetic tree of selected bacterial strains with different species of *Lactobacillus* genus (Lorestan-A strain of bacteria isolated from yogurt, Lorestan-B strain of bacteria isolated from buttermilk and Lorestan-C strain of bacteria isolated from cheese)

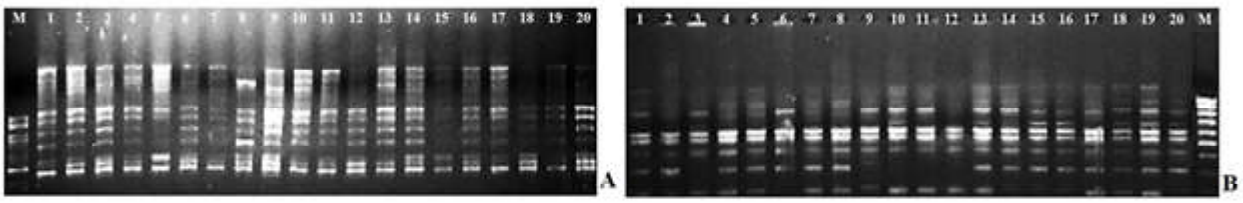

Fig. 4 The results of electrophoresis and amplification of fragments using the REP-PCR (A) and BOX-PCR (B) markers in bacterial strains isolated from the studied dairy products. (Number 1 to 20 include 20 bacteria strains and M represents a marker with a standard size of 100bp)

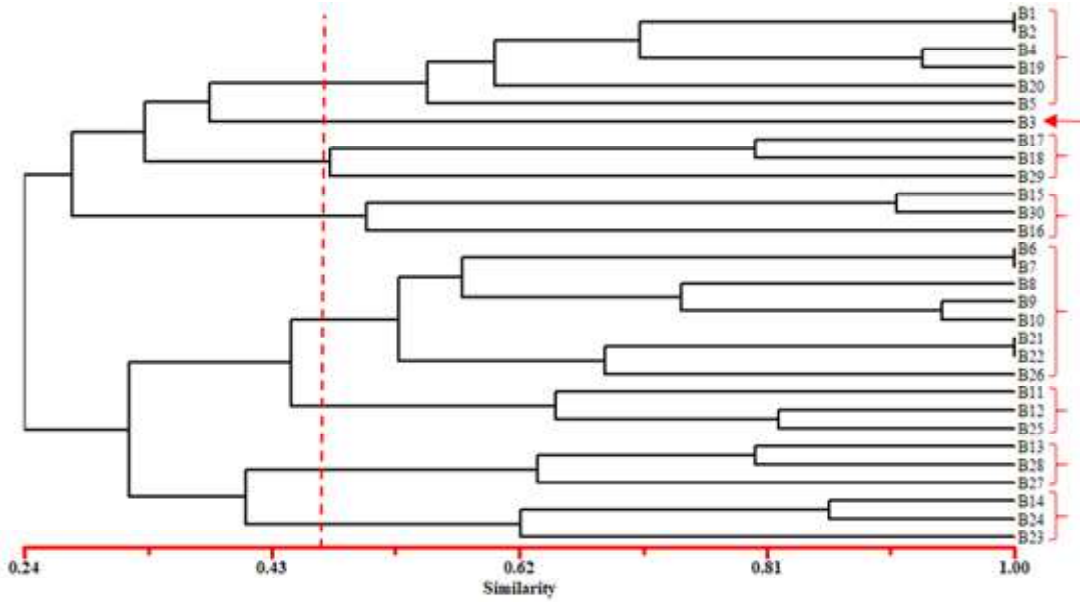

Fig. 5 Grouping of studied bacterial strains using the combination of REP-PCR and BOX-PCR information

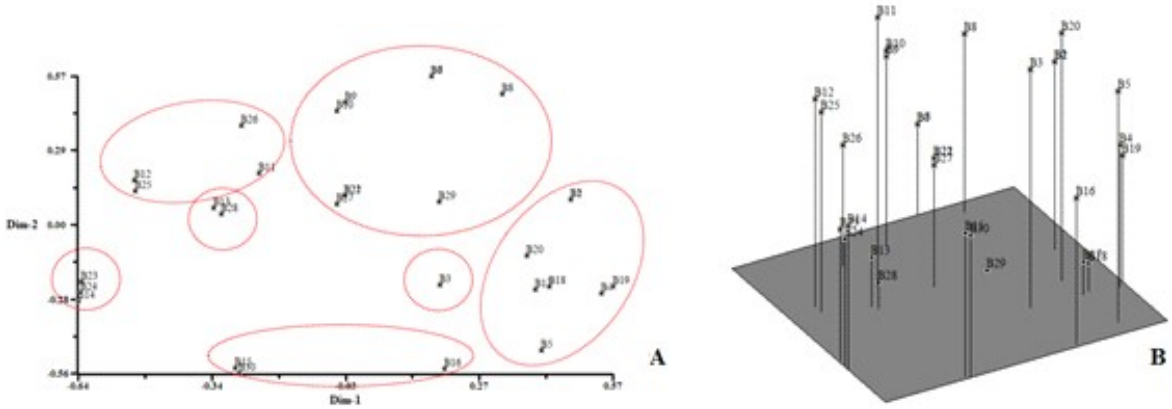

Fig. 6 Grouping of studied bacteria using two-dimensional (A) and three-dimensional (B) graphs drawn based on the PCOA test

research isolated 10 probiotic lactic acid bacteria including *L. acidophilus*, *L. brevis*, *Bifidobacterium,* and *Streptococcus thermophilus* from traditional yogurt samples. In their study, based on morphological, physiological, and biochemical tests, all strains was Gram-positive and catalase negative. In a research, Montazeri et al. (2020) were able to examine 60 strains from a total of 12 raw milk samples with morphological studies and gram test. A total of 36 catalase-negative and Gram-positive strains were found, including *L. gasseri* and *L. galottix*.

In the present study, the antibiogram technique and determining the pattern of sensitivity and resistance of the isolated bacteria showed a suitable grouping and the obtained pattern was like the results of previous studies. Although some observed differences can be attributed to the changes caused by the test method. In a similar study, Zhang et al. (2018) reported different sensitivity and resistance patterns of lactic acid bacteria in dairy products. In their study, 11 different antibiotics were used to group the isolated bacteria. In general, it was found that the use of antibiogram test can be a suitable tool to check the selection of different bacteria.

In another similar study conducted by Junior et al. (2015) on the isolation and identification of lactic acid bacteria, it was found that most of the studied bacterial strains are sensitive to chloramphenicol. They stated that the pattern of resistance and sensitivity depends on various characteristics of bacteria, including the type of bacteria and the presence of resistance and sensitivity genes in the bacterial cell.

The results of the present study showed that all the isolated bacteria were sensitive to the antibiotic gentamicin. Although these results do not confirm natural resistance to gentamicin in all bacteria present in traditional crops, this antibiotic can be used for other similar studies. All isolated strains showed resistance to vancomycin and kanamycin, and about 60% of the samples were resistant to amikacin antibiotic. Tulumoglu et al. (2013) found that 90% of *Lactobacillus* strains tested were resistant to gentamicin. These results indicate the weak inhibition

of bacteria in traditional products by aminoglycoside antibiotics.

Lactic acid bacteria (LABs) are mainly sensitive to clinical antibiotics such as penicillin, tetracycline, erythromycin, and chloramphenicol, and they have relatively high resistance to streptomycin and ciprofloxacin antibiotics. However, the pattern of resistance and sensitivity of these bacteria is very different at the level of species and strains, and species of the same genus may have different patterns. The antibiotic resistance gene in *Lactobacillus* bacteria is located on the chromosome and is highly conserved (Dzidic et al. 2008).

The results of molecular analysis of the isolated bacterial strains showed that the bacteria isolated from yogurt are similar to *L. helveticus* and *L. casei* bacteria. The bacterial strain isolated from the local dough sample belonged to *L.fermentum* and the bacterial strain isolated from Lorestan traditional cheese also belonged to *L.helveticus*. The results showed that according to the type of dairy product, the bacterial strain in them is also different.

The natural flora of traditional cheeses is complex and includes several strains of lactic acid bacteria, which are very important in cheese processing and creating its smell and taste. Since these cheeses have better and unique flavors compared to pasteurized and industrially pasteurized cheeses, there is more interest in studying the functional and structural diversity of lactic acid bacteria in traditional cheeses. Therefore, traditional cheeses are considered as the best sources of lactic acid bacteria strains useful for the food industry. The favorable properties of these bacteria are very important for use as starter cultures in dairy products (Coelho et al. 2022).

The results of grouping the studied bacterial strains based on REP-PCR and BOX-PCR markers showed that the studied bacteria have a high genetic diversity. In addition, significant genetic diversity was also observed among similar dairy products. The results generally indicated the appropriate efficiency of DNA-based genetic markers in the grouping of lactic acid bacteria of traditional dairy products.

Based on the results of the study by Gevers et al. (2011), the REP-PCR marker is an efficient and effective tool for the isolation of lactobacilli in traditional dairy products. In their study, they showed that this group of markers with high multiplication power of polymorphic fragments can be very efficient in grouping species and strains of lactic acid bacteria.

Physiological and biochemical tests have limitations and similar physiological characteristics are seen among many of isolated bacteria. In addition, the results of these methods are not always accurate. These results are only based on cultivation methods and phenotypic characteristics, and for this reason, it is recommended to use genetic methods along with phenotypic tests to identify the strain. For this purpose, in the present study, REP-PCR and BOX-PCR molecular markers as well as 16SrDNA gene sequencing were used to group and more accurately identify the isolated bacteria. The results of these techniques were able to identify the type of bacteria studied based on the type of species while grouping the isolated bacteria.

Isolation of new *Lactobacillus* bacteria strains and determination of their characteristics allows the introduction of innovative and competitive probiotic products with increasing applications. During isolation, there will be several limitations in the identification and confirmation of bacteria. Considering that a large number of strains isolated from a source are usually subjected to discriminant analysis, the selection of appropriate techniques will usually be influenced by factors such as the duration and cost of the analysis. Nowadays, methods based on DNA molecular markers such as amplification of repetitive sequences (REP-PCR) are different in terms of potential and required time (Jarocki et al. 2020).

Probiotics isolated from the traditional microbiome can be successfully stored in the genetic bank for use in the pharmaceutical and food industries, especially as culture starters in the future. However, these isolated lactic acid bacteria should be further investigated and their other potential probiotic properties, including anti-cancer properties, intestinal disorders, and anti-allergic properties should be evaluated. Also, due to the increasing use of probiotics, nutritional supplements, and therapeutic agents, there should be detailed information about their risks and benefits (Ahmadnejad and Dolatabadi. 2020).

Conclusion

Examining and evaluating the characteristics of native bacteria strains of each region can be the source of new lactic acid bacteria. This work, while preserving microbial and genetic resources, provides useful information for scientific and commercial applications, especially in the field of dairy and traditional industries. It is necessary to establish a regular structure for collecting, preserving and using lactic acid bacteria in traditional dairy products.

The high efficiency of REP-PCR and BOX-PR markers in identifying the genetic diversity of lactic acid bacteria in dairy products was confirmed. These products are known to be rich in different bacteria with significant probiotic potential. The results of the present study showed the high potential of traditional dairy products of Lorestan province in terms of having probiotic strains with high genetic diversity, which can be considered as suitable biological preservatives and used to produce various types of dairy products commercially. These bacteria can be added to different foods and benefit from their effects.

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RESEARCH ARTICLE

Characterization of *COX-2* gene using RNA based technique in endometrial epithelial cells of buffalo (*Bubalus bubalis*)

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Abstract: The present study has been reported towards molecular characterization of *COX-2* gene (*PTGS2* gene) in buffalo endometrium. Non-pregnant buffalo uteri were collected from the local abattoir immediately after slaughter and total RNA was extracted. First strand cDNA was synthesized from approximately 2 µg total RNA using iScript cDNA synthesis kit. Following cDNA synthesis, PCR amplification of cDNA was carried out using *COX-2* gene specific primers. To determine the optimum conditions, different concentrations of $MgCl_2$, template DNA, Taq DNA polymerase, primers, dNTPs as well as different cycling programmes were analysed. Reproducible amplification pattern was obtained using 2 µl cDNA template (50 ng/µl), 2.5 µl 10X Taq Buffer, 0.5 µl dNTP (50 mM), 2.5 µl $MgCl_2$ (25 mM), 0.5 µl each primer (10 pM/µl), 0.1 µl Phusion DNA polymerase (5 U/µl) and nuclease-free water. The purified PCR amplified product (449 bp) was sequenced using *COX-2* gene specific primers. Nucleotide sequence analysis of PCR amplified product exhibited 100 percent identity with the reference sequence of *Bubalus bubalis COX-2* gene using Clustal Omega Multiple Sequence Alignment Tool. Based on above findings, it is concluded that the optimized PCR conditions and the buffalo *COX-2* gene specific primers can be useful in pursuing further gene expression studies to explore the function of *COX-2* gene in buffalo.

Keywords: Buffalo; *COX-2* gene; DNA; Molecular characterization; PCR

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Introduction

Early embryonic mortality is one of the major causes of reproductive failure resulting in reduced pregnancy rate, slower genetic improvement and substantial economic loss to the farmers (Mondal and Prakash, 2002; Tripathi et al. 2018; Tripathi et al. 2021; Mondal et al. 2021). During early pregnancy, the embryo depends on uterine environmental support for survival and growth as it undergoes continual modifications to cope with the needs of embryo. Majority of the losses occur due to failure of signalling exchange at embryo-uterine interface. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from the uterus induces luteolysis and reduce the progesterone secretions from the corpus luteum (CL). CL produces progesterone which is needed to stimulate and maintain the endometrial function that is permissive for early embryonic development, implantation, placentation and maintenance of pregnancy (Mondal et al. 2013). Prostaglandins (PGs) are produced by endometrium that plays an important role in ovulation, luteolysis, implantation, maternal recognition of pregnancy and parturition (Dubois et al. 1998; Mondal et al. 2009; Nandi et al. 2012; Lacroix-Pépin et al. 2011). Prostaglandins consist of a diverse family of autocoids derived from cyclooxygenase metabolism of arachidonic acid to PGG_2 and then into PGH_2 which leads to generation of five principal bioactive prostaglandin metabolites; PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TXA_2 (Thromboxane A_2) (Breyer and Breyer 2000). The production of endometrial PGs is mainly governed by the rate limiting enzymes i.e., cyclooxygenases (COX-1 and COX-2). The COX-1 and COX-2 are also known as prostaglandin endoperoxide H synthase-1 and 2 (PGHS-1 and PGHS-2), respectively. These enzymes are responsible for the conversion of arachidonic acid into PGH_2, the common precursor of the various forms of PGs including PGE_2 and $PGF_{2\alpha}$. The downstream enzymes, PGE synthase (PGES) and PGF synthase (PGFS) catalyze the conversion of PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively. PGE_2 is luteotrophic in nature, whereas $PGF_{2\alpha}$ acts as the luteolytic agent in ruminant and oxytocin is responsible for its episodic release. In ruminants, oxytocin (OT), progesterone (P4), and estradiol (E2) regulate the uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that causes luteolysis (Mondal et al. 2015a; Mondal et al. 2015b; Mondal et al. 2017).

Two isoforms of cyclooxygenase have been identified viz., COX-1 and COX-2, that are encoded by two different genes known as *COX-1* and *COX-2* gene, respectively (Salamonsen and Findlay, 1990; Smith et al. 1996; 2000). The enzyme COX-1 is a constitutively expressed in a range of cell types, conversely COX-2 enzyme is inducible which plays an important function in various pathological and physiological circumstances in animal tissues (Smith et al. 1996; Funk, 2001). The expression and regulation of both the isoforms are tissue and species specific (Arosh et al. 2002). COX-3 has also been identified as a splice variant of COX-1, and it is present mainly in brain and spinal cord (Mondal et al. 2015b). The role of COX-3 is still not known. However, Blitek et al. (2006) reported a possible role in pain sensitivity based on the mechanism of action of acetaminophen (paracetamol), evoked as a selective inhibitor of COX-3. Depending on pathological and physiological circumstances, their expression varies within a specific tissue and species (Kniss, 1999; Funk, 2001; Arosh et al. 2002). Usually, COX-2 is regulated by growth factors, different cytokines and greatly elevated in both acute and chronic inflammation (Simon, 1999). Since, information on characterization and expression profile of *COX-2* gene in buffalo (*Bubalus bubalis*) is lacking. Therefore, in the present study, characterization of *COX-2* gene in the uterine endometrium of buffalo was undertaken.

Materials and Methods

Sample collection

Buffalo uteri were collected from the local abattoir immediately after slaughter and transported to the laboratory on ice. The stages of estrous cycle were determined based on colour, vasculature, size and consistency of CL. Accordingly, uteri were classified into three stages: stage I (days 3 to 5), stage II (days 6 to 15) and stage III (days 16 to 21) of estrous cycle (Ghosh and Mondal, 2006). Uteri were opened longitudinally and carefully cut out from the lamina propria of the intercaruncular endometrium. The endometrium was scrapped out using a sterile surgical blade.

Isolation of total RNA from buffalo endometrial tissue

Total RNA was isolated from the buffalo endometrial tissue samples (n= 6) using Trizol (Ambion, Life Technologies, USA) followed by RNeasy Mini Kit (Qiagen, USA). The quality and integrity of the purified RNA was checked through agarose gel electrophoresis, and quantity was measured using nanodrop spectrophotometer (Eppendorf, Germany) at 260 and 280 nm wavelengths. The purified RNA was preserved at -80°C until use.

Synthesis of cDNA using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The first strand cDNA was synthesized from purified RNA using iScript™ cDNA synthesis kit (Bio-Rad, USA). From each endometrial sample, 2 µl of total RNA was reverse transcribed using 4 µl 5× iScript reaction mixture and 1 µl of iScript Reverse Transcriptase to make a final volume of 20 µl with nuclease free water in a sterile 0.2 ml PCR tube on ice. This reaction mixture was incubated at 25°C for 5 min, later at 46°C for 20 min, after which the reaction was terminated (95°C for 1 min). The cDNA was stored at -20°C.

Designing of primers

Primers for *COX-2* (*PTGS*2) gene (Table 1) and *β-actin* gene (Table 2) for *Bubalus bubalis* were designed based on the available sequence in NCBI with accession number (NC_037549.1) and (NC_037568.1) respectively using Primer3 software. PCR amplification of cDNA was carried out using *β-actin* primers to check the quality of the cDNA. Primer sequences used for PCR amplification of coding sequence of *COX-2* gene is given in the Table 1.

Preparation of PCR product

The working solutions (100 µM) of both forward and reverse primers were prepared from stock solutions by adding Milli-Q water (autoclaved). PCR master mix was prepared by adding the reagents in the following order (Table 3) into a sterile 0.2 ml PCR tube. PCR reactions were performed in the thermal cycler

Table 1: Primers used for *COX-2* gene

Primer	Sequence (5′-3′)	Melting temp. (°C)	Length
COX-2 FP	TCAAGATCACATTTGATTGAGA	62.2	449 bp
COX-2 RP	TGTATCCTCCCACAGTCAAAGA	63.4	

Table 2: Primers used for *β-actin* gene

Primer	Sequence (5'-3' sequence)	Melting temp. (°C)	Length
β-actin FP	GACGACATGGAGAAGATCTGGCA	69.7	341 bp
β-actin RP	GAAGGATCTTATGAGGTAGTCTG	61.5	

(Eppendorf, Germany) under following general cycle conditions: Initial denaturation at 98ºC for 3 min, followed by 30 cycles of denaturation at 98ºC for 45 sec, annealing at 59ºC for 30 sec, and extension at 72ºC for 90 sec and final extension at 72ºC for 10 min. The PCR amplified products were stored at -20ºC.

Checking of PCR product by agarose gel electrophoresis

After amplification, the PCR product was checked on 1% agarose gel to verify the amplification of the target region. A volume of 5 µl PCR product was mixed with 2 µl of 6X gel-loading dye and loaded slowly in separate wells along with 100 bp DNA ladder in the first well of the gel.

Gel extraction of amplified product

The PCR amplified product was resolved from 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The electrophoresis was performed in 1X TBE buffer at 75 volts for 60 minutes till complete separation. The PCR amplified product (449 bp) was visualized on UV transilluminator and photographed with a gel documentation system (Bio-Rad, USA). PCR product was purified using gel extraction kit (Qiagen, USA) as per manufacturer's instruction.

Sequencing and analysis of PCR product

The purified PCR product was sent for sequencing (Eurofins, Bangaluru) with *COX-2* gene specific primers. The sequence data were analysed using Clustal Omega Multiple Sequence Alignment Tool and NCBI database. The analysis was done against the reference sequence of *Bubalus bubalis COX-2* gene. The obtained nucleotide sequence was also compared with different species by Nucleotide Basic Local Alignment Search Tool (nBLAST) of NCBI database. Clustal Omega Multiple Sequence Alignment Tool and nBLAST was used to calculate the appropriate match for the selected sequences to identify the similarities and differences between sequences.

Results and Discussion

Total RNA was extracted from the buffalo uterine endometrial tissue by Trizol and RNeasy Mini Kit method. The quantitative and qualitative estimation of isolated RNA was checked by Nanodrop spectrophotometer at 260 and 280 nm wavelengths and agarose gel electrophoresis, respectively. RNA concentrations ranged from 510 to 575 ng/µl with an average of 547 ng/µl, and optical density (OD at 260/280 nm) values ranged from 1.91 to 1.99 with an average of 1.93, which indicated that the isolated RNA was of good quality. The quality of isolated RNA was further checked by running on 1% agarose gel electrophoresis. Two distinct 28S and 18S ribosome of RNA bands were observed on agarose gel (Figure 1), which revealed that the RNA was of good quality. The cDNA was synthesized from the reverse transcription of 1000 ng of isolated RNA by using iScript™ cDNA synthesis kit (Bio-Rad, USA) and stored immediately at -20°C until its use.

Table 3: Composition of reaction mixture for PCR (Thermo Scientific, USA)

Sl. No.	Components	Volumes
1	cDNA Template (50 ng/µl)	2.0 µl
2	10X Buffer	2.5µl
3	50 mM dNTP mix	0.5 µl
4	25 mM $MgCl_2$	2.5 µl
5	Forward Primer (10 pmol/µl)	0.5 µl
6	Reverse Primer (10 pmol/µl)	0.5 µl
7	Phusion DNA Polymerase (5 U/µl)	0.1 µl
8	Milli-Q water (autoclaved)	16.4 µl
	Total Volume	25 µl

The quality of the cDNA was analysed by polymerase chain reaction (PCR) using *β actin* gene, NCBI Accession Number (NC_037568.1). A 341 bp amplification was observed for the *β actin* gene (Figure 2) indicated that the cDNA was in good quality. The *COX-2* gene sequence information of *Bubalus bubalis* was retrieved from NCBI database. Gene specific forward and reverse primers for *COX-2* gene coding sequence were constructed using the Primer3 software.

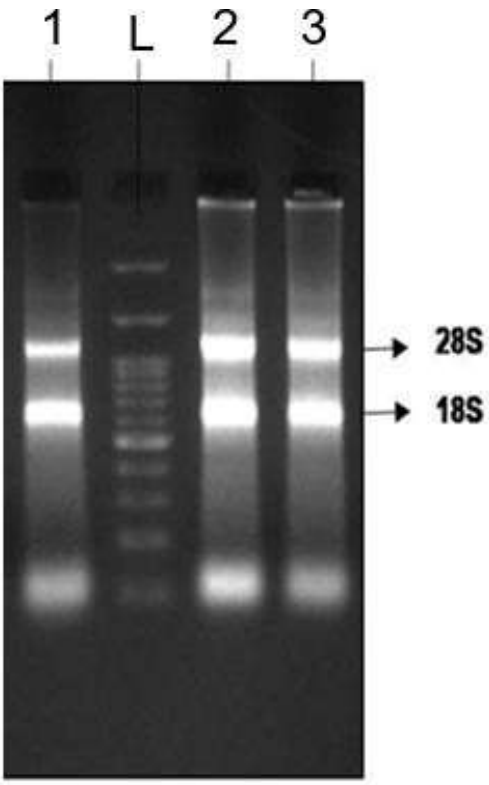

Fig. 1 Agarose gel electrophoresis exhibited amplification at 28S and 18S of RNA

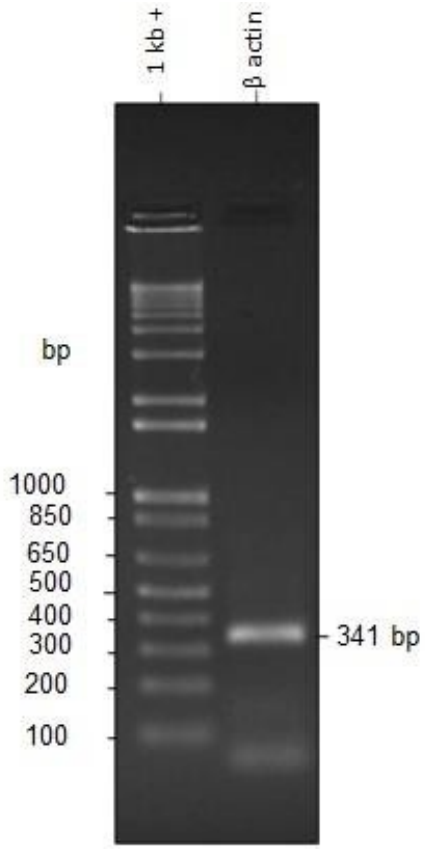

Fig. 2 Agarose gel electrophoresis of PCR product for β-*actin* gene (Lane 1: 1kb DNA ladder; Lane 2: β-*actin* gene

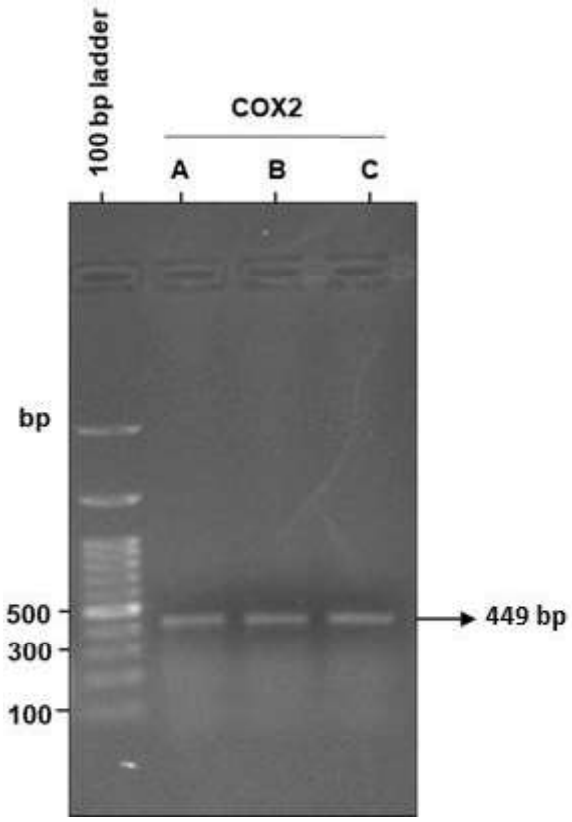

Fig. 3 Agarose gel electrophoresis of PCR product for *COX-2 gene* (Lane 1: 100 bp DNA ladder; Lane 2, 3 and 4: *COX-2* gene)

The PCR was performed to amplify the *COX-2* gene. The PCR product showed amplification of 449 bp (Figure 3) on agarose gel after electrophoresis. The amplified PCR product of 449 bp was cut, weighed and purified using QIAquick Gel Extraction kit (Qiagen, USA) and subsequently examined by electrophoresis in agarose gel stained with ethidium bromide.

Sequence analysis of PCR amplified product

The purified PCR product of 449 bp was sent for DNA sequencing (Eurofins, Bangalore). Custom DNA sequencing was done by Sanger sequencing. After sequencing of 449 bp PCR product, a length of 360 bp was matching with the *Bubalus bubalis COX-2* sequence along with primer binding. Clustal Omega Multiple Sequence Alignment Tool was used to study the appropriate match for the selected sequences to ascertain the similarities and difference between target and reference sequences. The sequence analysis using Nucleotide Basic Local Alignment Tool (nBLAST) revealed that there was 100 percent homology of each with nucleotide sequence (Figure 4 and Figure 5) with the *Bubalus bubalis COX-2* (*PTGS*2) gene (Accession Number: NC_037549.1). Multiple sequence alignment of nucleotide sequence obtained for 360 bp sequence of PCR product showed 100% homology with the *Bubalus bubalis COX- 2* gene (Figure 5). All these results clearly indicated that the PCR amplified product was from the *Bubalus bubalis COX-2* gene.

These sequence analysis showed 100, 98.32, 96.64 and 91.60 percent identical with cattle, goat, sheep and pig, respectively. Asselin et al. (1997) demonstrated that cDNAs of *COX-2* gene were cloned and sequenced in endometrial epithelial cells of *Bubalus bubalis*. The *COX*-2 gene was found 84, 86, and 87 percent of homology in relation to rat, guinea pig and human, respectively. Liu et al. (2001) reported cloning and characterization of the full-length bovine *COX-2* or *PGHS-2* cDNA of 1.8 kb size. Comparative analysis revealed that the amino acid sequence of the bovine protein was very similar to that of other mammalian homologs, being 89, 87, 87, 89, 90, 86 and 86 percent identical to human, rat, mouse, horse, rabbit, guinea pig and mink *PGHS-2* gene. Tsai et al. (1996) cloned a PCR product of 553 bp into a pCRTM II vector and subsequently sequenced the cloned product. The sequence was compared and found to be 87.5 and 86.2 percent identical with human and mouse PGHS-2, respectively. Sakaram et al. (2010) amplified the full length cDNA of DRA gene in Murrah buffalo and analysed to identify the

Fig. 4 Sequence of PCR amplified product (449 bp). Blue colour indicates *COX-2* specific primers used for sequencing, Purple colour indicates 360 bp sequence obtained from 449 bp PCR product

TCAAGATCACATTTGATTGAGAGTCCGCCAACTTATAATGTGCACTACAGCTATAAAAGCTGG
GAAGCCTTTTCTAACCTGTCTTATTATACCAGAGCTCTTCCTCCGGTGCCTGATGACTGCCCA
ACACCCATGGGTGTGAAAGGGAGGAAAGAGCTTCCTGATTCAAAAGAAGTTGTAAAAAAAGTA
CTTCTAAGAAGAAAGTTCATTCCTGATCCCCAGGGCACAAATCTGATGTTTGCATTCTTTGCC
CAGCACTTCACCCATCAATTTTTCAAGACAGATTTTGAACGAGGACCAGCTTTCACTAAGGGA
AAGAACCATGGGGTGGACTTAAGTCACATTTATGGTGAATCTTTAGAGAGACAGCATAAGCTG
CGCCTTTTCAAGGATGGAAAAATGAAATATCAGATGATTAATGGAGAGATGTATCCTCCCACA
GTCAAAGA

Fig. 5 Multiple alignment of PCR amplicon (449 bp) for *Bubalus bubalis COX-2* gene sequence

Download GenBank Graphics

PREDICTED: Bubalus bubalis prostaglandin-endoperoxide synthase 2 (PTGS2), mRNA

Sequence ID: XM_025285468.1 Length: 3387 Number of Matches: 1

Range 1: 348 to 707 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
665 bits(360)	0.0	360/360(100%)	0/360(0%)	Plus/Plus

```
Query  1    TAATGTGCACTACAGCTATAAAAGCTGGGAAGCCTTTTCTAACCTGTCTTATTATACCAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  348  TAATGTGCACTACAGCTATAAAAGCTGGGAAGCCTTTTCTAACCTGTCTTATTATACCAG  407

Query  61   AGCTCTTCCTCCGGTGCCTGATGACTGCCCAACACCCATGGGTGTGAAAGGGAGGAAAGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  408  AGCTCTTCCTCCGGTGCCTGATGACTGCCCAACACCCATGGGTGTGAAAGGGAGGAAAGA  467

Query  121  GCTTCCTGATTCAAAAGAAGTTGTaaaaaaaGTACTTCTAAGAAGAAAGTTCATTCCTGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  468  GCTTCCTGATTCAAAAGAAGTTGTAAAAAAAGTACTTCTAAGAAGAAAGTTCATTCCTGA  527

Query  181  TCCCCAGGGCACAAATCTGATGTTTGCATTCTTTGCCCAGCACTTCACCCATCAATTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  528  TCCCCAGGGCACAAATCTGATGTTTGCATTCTTTGCCCAGCACTTCACCCATCAATTTTT  587

Query  241  CAAGACAGATTTTGAACGAGGACCAGCTTTCACTAAGGGAAAGAACCATGGGGTGGACTT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  588  CAAGACAGATTTTGAACGAGGACCAGCTTTCACTAAGGGAAAGAACCATGGGGTGGACTT  647

Query  301  AAGTCACATTTATGGTGAATCTTTAGAGAGACAGCATAAGCTGCGCCTTTTCAAGGATGG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  648  AAGTCACATTTATGGTGAATCTTTAGAGAGACAGCATAAGCTGCGCCTTTTCAAGGATGG  707
```

genetic variability. Naskar et al. (2012) cloned and characterized MHC (*Bubu*)-*DRB* cDNA in water buffalo. Gul et al. (2023) performed molecular cloning, expression and structural characterization of growth hormone-receptor (GHR) and its extracellular domain as growth hormone binding protein (GHBP) from the liver of Nili-Ravi buffalo. Medina et al. (2023) conducted molecular characterization of TLR4 genes of swamp and riverine types of water buffaloes to determine unique genotypic characteristics specific to each type of water buffalo and provide baseline information for explaining differences in disease resistance between each type. Pramanik et al. (2022) conducted characterization of α^{s1}-casein gene in buffalo at the molecular level to determine complete αs1-casein cDNA sequence.

Conclusion

In the present study, *COX-2* gene was characterized in the endometrial epithelial cells of buffalo. The purified PCR product (449 bp) was sequenced using buffalo *COX-2* gene specific primers that was subjected to sequence analysis. Nucleotide sequence analysis of PCR amplified product exhibited 100 percent identity with the reference sequence of *Bubalus bubalis COX-2* gene. The optimized PCR conditions and the buffalo *COX-2* gene specific primers used in the study can be useful in pursuing further gene expression studies to explore various functions of the *COX-2* gene in buffalo.

Acknowledgements

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RESEARCH ARTICLE

Colostrum quality and the neonatal calf nutrition and growth with or without a source of PUFA supplement

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Abstract: The calf acquires immunity from the colostrum only after birth, which protects for a few weeks but developing innate immunity after birth is essential for its lifetime performance. The present study aimed to supplement flaxseed oil (FSO) in neonatal calves as polyunsaturated fatty acids (PUFA) source. Twenty male and female Deoni calves were randomly distributed into four groups where a group of male (TGM) and female (TGF) calves were drenched with FSO daily from day 6 to 28 and compared with control; CGM, and CGF. After that, a bolus consisting of ground flaxseed was fed till 90 d besides mixed green fodder and concentrate mixture. Digestibility trial in neonates was carried out by indirect method. The colostrum quality or immunoglobulins (Ig) were reduced from 93 to 39 mg/mL on the second day. The total diet CP in neonatal age was 22%, and digestible CP was 13.2%. It was 18% and 9%, respectively, during preweaning. Weight gain in the male calves was higher than in the female calves, although their intake was statistically comparable. The weight gain in the TG was significant until the first fortnight and subsequently comparable, but the relative risk of disease in males and females of CG was 6.57 folds more than TG (P< 0.001). The study concluded that the FSO supplementation at neonatal age followed by supplementing ground flaxseed as bolus had no adverse influence on the diet intake, digestibility of nutrients, and energy balance. The marginal improvement in weight gains and reduced disease risk prompts the recommendation of the supplement/drench FSO 30 mL/d for neonatal calves.

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Keywords: Colostrum, Fatty acids, Growth, Health, Nutrition, Resilience

Introduction

The newborn calf's growth until puberty is the foundation and basis for the future production of the herd. The neonatal calf is born with little or no detectable immunoglobulins (Ig) in blood serum and is highly susceptible to many infectious diseases and retard growth (Arthur et al. 1996), particularly when their serum IgG levels are <10 g/L (Furman-Fratczak et al. 2011). According to Radostits (2007), 75% of the mortality of dairy heifers occurs during the first month of life. The daily gains in neonatal calves with higher diseases affect the weight gains during the first year (Trilk and Münch, 2010). The major challenge in newborn calf nutrition is at the time of the diet change from a high protein liquid milk to a solid diet with lesser quality crude protein (CP). Even when the neonatal calf is offered unlimited milk, begin to chew solid feed from the second week of the birth. Terré et al. (2007) reported an inverse relationship between milk and solid feed intake. A better health and quality diet translate into an efficient average daily gain (ADG) per unit feed consumption (Bishop et al. 1991). In neonates, ADG in the first two weeks has a cascading effect on gains at six, nine, and 12 mo of age, body weight at first insemination, first lactation milk yield, and lifetime efficiency (Volkmann et al. 2019). In the context of health, polyunsaturated fatty acids (PUFA) in general and omega-3 fatty acids in particular influence inflammation through various mechanisms known at least for three decades. PUFA or their derivatives' play role in signaling molecules in the immune system worth noting from the perspective of ameliorating symptoms in many diseases (Gutiérrez et al. 2019). Flaxseed oil (FSO), rich in PUFA, is known for its anti-inflammatory, antipyretic, antioxidant, and analgesic effect (Kaithwas et al. 2011). The body fat reserves in a newborn calf are just 3 to 4%; hence, dietary fat intake play an important role (Bascom et al. 2007). The present study aimed to supplement FSO to newborn calves orally, taking advantage of esophageal groove closure and dilation stimuli (Kaba et al. 2018) to ascertain better growth until preweaning such as 90 d of age.

Materials and methods

Experimental location and weather

The experiment was conducted at the Livestock Research Centre, Southern Regional Station, ICAR-NDRI, Bangalore, India. The location latitude, longitude, and elevation are 12.947014°N (12° 56' 49.2504" N), 77.607679 (77° 36' 27.6444" E), and 921m MSL, respectively. The tropical climate is considered Aw (Savanna, wet) according to the Köppen-Geiger climate classification, with a mean temperature of 23.6°C and 831mm annual rainfall. The variation in temperatures throughout the year is 6.4°C. During the experimental period, the relative humidity ranged from 59 to 72%, mean ambient temperature was as low as 15ºC and high as 31ºC. The experiment was carried out for three months (mo) with the approval of the Institute Animal Ethics Committee (IAEC) and reared as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Experimental calves care, groups and diets

Neonatal age

The calves born to Deoni (*Bos indicus*) breed of cows in fourth parity or less were selected for the study. As and when born on the farm, calves were randomly allocated into experiment based on birth weight and sex (male 21.88±0.68 kg, and female 19.49±0.85 kg). Soon after the birth, tincture iodine was applied to the naval card region and left the calf with the dam in a pen. Colostrum quality was tested according to Fleenor and Stott (1980) on the principle of specific gravity and IgG concentration using clostrometer (Kruus colostrum density meter, Denmark). Newborn calves were drenched colostrum *ad lib* 45 min of the birth. After the fifth day of calf birth, a total of ten female (F) and ten male (M) calves were randomly divided into four groups; control female (CGF) and male (CGM), and treatment female (TGF) and male (TGM) calf groups. All calves were kept as per the group under loose housing in calf pens. TGF and TGM calves were orally supplemented with 30 mL/d of FSO just before milk suckling in the morning. Calves were allowed suckling dam 3 min at the beginning and end of the manual milk at 6h00 and 17h00 daily for 28 d. The milk intake of calves was derived mathematically by multiplying milk flow rate (mL/strip), calf suckling rate (Numbers/min), and calf suckling duration (min). All the calves were offered weighed quantities of fresh mixed green fodder (MGF) consisted tender maize (*Zea mays*) fodder, para (*Brachariamutica*), and guinea (*Megathyruses Maximus*) grass in equal proportion and chaffed manually to 10 cm length.

Pre-weaner age

After a neonatal age of 28 d, the oral supplementation of FSO stopped, and milk suckling was restricted to 1 min before milking and 5 min after milking till 90 d. During the preweaner age, TGF and TGM were fed a bolus of 25 g of ground flaxseed mixed with 25 g of jaggery at 8h00 every day until 90 d of age. All calves were offered a concentrate mixture (CM) having 17% CP. All the calves fed CM 200 g/d at 9h00. Chaffed tender MGF was offered to calves at free choice during the neonatal and preweaning periods. When claves were 60 d old, they were confined in individual stalls for 2 wks but manually taken twice daily at the time of milking for suckling stimulus in their dams. All calves were offered drinking water four times daily at 9h00, 13h00, 18h00, and 21h00.

Digestibility trial by the indirect method in neonates

A digestibility trial was conducted on the neonates after 18 d age by the indirect double indicator method (Krishna et al. 1981). Chromic oxide (Cr_2O_3) was used as an external indicator for measuring the fecal output. Calves were dosed with 1 g of Cr_2O_3 daily at 9h00 for five days as a cellulose paper capsule, followed by collecting fecal samples from the rectum of the calves at 8h00 and 20h00 daily from day 2 of dosing to till 10 d. The fecal samples were collected daily for 10 d. Each day a 20 g sub-sample from pooled fecal samples was drawn after mixing separately for each calf. A part of the fecal sub-sample was dried in a drought oven at 100±1ÚC for DM (AOAC, 2012). Another sub-sample of the feces was kept in a glass bottle, acidified with 10% H_2SO_4 ($^v/_v$) to estimate the total N. A dried fecal sample of about 1 g was transferred to a 100 mL Kjeldahl flask to which 10 mL of concentrated nitric acid (N_2O) was added and kept overnight, followed by the addition of 10 mL of digestion mixture consisting of 10 g sodium molybdate dissolved in 150 mL of distilled water, 150 mL of concentrated H_2SO_4 and 200 mL of 70% perchloric acid (v/v). The contents in the flask were digested till a light yellowish tint appeared and then transferred to a 100 mL volumetric flask. Cr_2O_3 concentration in the sample was determined by the colorimetric method by reading the absorbance at 430 nm using a UV-visible spectrometer (LMSP-UV1200, M/s Labman Scientific Instruments Pvt., Ltd., India). A stock solution of 10 mg Cr_2O_3 was used to draw the standard curve. The fecal quantity excreted by the calves was estimated depending on the Cr_2O_3 dose and concentration in the fecal sample. Total DMI and digestibility were estimated using lignin as an internal marker.

Digestibility trial by the direct method in pre-weaner calves

The digestibility trial during the pre-weaning at 75 d age was carried for 5 d under confinement in individual calf pens. Daily faces excreted during the trial were collected, weighed, and sampled at 8h00. A part of the fecal sample was kept for oven drying at 100±1ÚC overnight, and another part was acidified with 10% H_2SO_4 (v/v) in a glass bottle for total N estimation. We collected 100 mL/d spot urine sample from each calf in a bucket acidified with 10% H_2SO_4 (v/v). Acidified urine samples were diluted with distilled water uniformly to 1.2 L, mixed thoroughly, filtered through glass wool, and stored 50 mL at –20^{Ú}C in polypropylene bottles till further analysis.

Feed, faces, and serum TVFAs analysis

Samples of MGF, CM, orts, and feces were processed through a cutting Willey mill (fitted with four steel knives) using a 1-mm sieve to screen the ground sample particle size. These samples were analyzed for DM, OM, CP, and EE (AOAC, 2012). The fat-free samples were treated with heat-stable α-amylase followed by reflex unit extraction for 60 min to estimate NDF. ADF and H_2SO_4 lignin in samples were analyzed sequentially to NDF (AOAC, 2012). Non-fibrous carbohydrates (NFC), hemicelluloses, and celluloses were mathematically calculated (Van Soest et al. 1991). The energy value of the diet was predicted from the amounts of digested nutrients, viz., CP, EE, and TCHO using the East German System for cattle (ARC, 1980).

Average daily gain (ADG) and feed efficiency

ADG during neonatal age was calculated based on the weekly weight gains till 28 d of age, followed by ADG during the preweaning period based on the fortnightly weight gains. Growth efficiency terms such as Kleiber ratio (KR), feed conversion ratio (FCR), relative growth rate and residual feed intake (RFI) were calculated (Arthur and Herd, 2008).

Statistical analysis

The weekly and fortnightly ADG in different groups were subjected to the MANOVA model, including the repeated measure. Data were subjected to variance tests using a completely randomized block design (CRD). Group means were compared by Duncan multiple range tests (DMRT). A significant difference between parameters was denoted by alpha superscripts when $0.10 > P > 0.01$ and validated against the null hypothesis (H_0). All analyses were made using a statistical package for social science (SPSS, Ver. 19.0. M/s IBM India Pvt., Ltd.).

Results and Discussion

Table 1 shows the colostrum quality. The IgG levels reduced to one-third on second day, whereas the protein content in colostrum declined 7%. All calves were left with the dam for five days with the liberty to suckle the mother at will. In our Deoni herd, the average colostrum production is only two kg/d and seldom beyond it. The mean birth weight of calves was 20 kg, and they could consume less than 10% of their body weight. The colostrometer estimates IgG concentration by measuring specific

Table 1: Colostrum composition

Parameter (%)	Day Zero	Day 1	Day 2	Day 3	Day 4
Specific gravity*	1063±1.05	1042±0.52	1038±0.40	1035±0.49	1032±0.45
Immunoglobulins mg/mL	92.51±2.88	39.12±1.35	28.95±0.86	21.68±1.25	14.16±1.05
Total solids, %	23.96±0.06	17.81±0.04	14.17±0.04	13.83±0.04	13.48±0.02
Protein, %	14.07±0.04	8.43±0.03	5.22±0.03	4.23±0.03	4.10±0.03
Fat, %	6.79±0.04	5.86±0.03	4.78±0.02	4.55±0.03	4.39±0.03
Lactose, %	2.63±0.02	3.90±0.02	4.29±0.03	4.53±0.03	4.61±0.02
Total ash, %	1.16±0.01	0.95±0. 01	0.85±0.01	0.81±0.01	0.79±0.01

* Kruus colostrum density meter reading is equivalent to specific gravity 1/1000

Table 2: Chemical composition of the diet (on a DM basis)

Parameter (%)	Green fodder	Concentrate Supplement	Whole Flax Seed	Whole milk
Moisture	74.07±0. 32	7.44±0. 19	7.00±0. 15	88.14±0.06
Dry Matter	25.93±0. 32	92.56±0.19	93.00±0.15	12.76±0.06
Organic Matter	93.00±0.06	90.40±0.21	95.63±0.15	12.05±0.05
Crude Protein	10.60±0.37	16.91±0.17	21.65±0.24	3.18±0.03
Ether Extract	1.80±0. 08	3.12±0. 04	37.67±0. 39	3.98±0.07
Total Carbohydrates	80.60±0.17	70.07±0.15	36.47±0.23	4.91±0.10
Neutral detergent fiber	76.87±0.20	33.07±0.15	35.07±0.32	
Acid detergent fiber	51.10±0. 68	13.33±0.03	19.33±1.04	
Hemicelluloses	26.37±0.70	19.73±0.20	18.90±0.17	
Celluloses	36.90±0.17	8.20±0.18	14.93±0.37	
Nonfibrous carbohydrates	4.10±0.11	37.23±0.09	1.40±0.11	

All the values were an average of 6 fortnightly samples in duplicates (N=12)

Table 3: Nutrient intake from the solid and liquid diet in Deoni neonates, g/kgw$^{0.75}$

Parameter	Female		Male		SEM	P-Value
	CG	TG	CG	TG		
Metabolic Body weight, kgW$^{0.75}$	12.01^{a}	12.69^{ab}	13.92^{b}	13.97^{b}	0.55	0.07
Solid feed intake: Green fodder, g/kgw$^{0.75}$						
Dry matter	18.81	18.41	16.65	16.73	0.78	0.16
Organic matter	17.69	17.30	15.64	15.73	0.78	0.16
Crude protein	4.08	3.99	3.61	3.63	0.17	0.15
Ether extract	3.54	3.47	3.14	3.15	0.15	0.15
Total carbohydrates	10.16	9.94	8.99	9.03	0.42	0.15
Neutral detergent fiber	5.07	4.96	4.49	4.51	0.21	0.15
Acid detergent fiber	3.36	3.28	2.97	2.98	0.14	0.16
Hemicelluloses	1.71	1.67	1.52	1.52	0.07	0.15
Cellulose	2.54	2.45	2.25	2.17	0.12	0.16
Nonfibrous carbohydrates	5.23	5.12	4.60	4.48	0.20	0.06
Liquid diet: Milk feeding, g/kgw$^{0.75}$						
Total milk intake	112	121	99	112	10.75	0.57
Dry matter	14.33	15.43	12.67	14.32	1.37	0.58
Organic matter	13.53	14.57	11.96	13.52	1.30	0.58
Protein	3.57	3.85	3.16	3.57	0.34	0.58
Fat	4.47	4.81	3.95	4.46	0.43	0.58
Carbohydrates	5.51	5.94	4.87	5.51	0.53	0.58
Total diet intake, g/kgw$^{0.75}$						
Dry matter	33.15	33.84	29.32	31.05	1.83	0.33
Organic matter	31.22	31.88	27.61	29.25	1.73	0.33
Crude protein	7.66	7.84	6.77	7.20	0.44	0.35
Ether extract	8.01	8.28	7.09	7.62	0.51	0.41
Total carbohydrates	15.67	15.88	13.86	14.54	0.80	0.28
Nonfibrous carbohydrates	10.74	11.06	9.48	9.99	0.63	0.32

Total diet PDF and the source of its constituents was only green fodder and similar to total intake from the diet.

Table 4: Digestible nutrient intake, nutritive value, and energy balance in Deoni neonates

Parameter	Female		Male		SEM	P-Value
	CG	TG	CG	TG		
Metabolic Body weight, kgW$^{0.75}$	12.01^{a}	12.69^{ab}	13.92^{b}	13.97^{b}	0.55	0.07
Dry matter, g/kgW$^{0.75}$	25.59	26.62	22.73	24.33	1.61	0.39
Organic matter, g/kgW$^{0.75}$	24.52	25.57	21.85	23.89	1.52	0.39
Crude protein, g/kgW$^{0.75}$	5.95	6.18	5.30	5.76	0.40	0.49
Ether extract, g/kgW$^{0.75}$	7.52	7.84	6.67	7.21	0.49	0.42
Total carbohydrates, g/kgW$^{0.75}$	11.24	11.64	9.92	10.41	0.65	0.28
Neutral detergent fiber, g/kgW$^{0.75}$	0.68	0.71	0.64	0.61	0.63	0.74
Acid detergent fiber, g/kgW$^{0.75}$	0.35	0.37	0.34	0.32	0.04	0.77
Hemicelluloses, g/kgW$^{0.75}$	0.26	0.30	0.31	0.33	0.04	0.55
Cellulose, g/kgW$^{0.75}$	0.24	0.28	0.27	0.25	0.02	0.51
Nonfibrous carbohydrates, g/kgW$^{0.75}$	10.37	10.84	9.23	9.81	0.60	0.31
Digestible CP%	17.93	17.99	18.00	18.36	0.21	0.50
Nutritive ratio	4.73	4.77	4.71	4.65	0.04	0.21
Energy Balance						
Gross energy, Kcal/kgW$^{0.75}$	165.44	169.40	146.31	155.50	9.45	0.35
Digestible energy, Kcal/kgW$^{0.75}$	143.37	148.99	127.14	136.20	9.05	0.39
Metabolizable energy, Kcal/kgW$^{0.75}$	118.12	123.02	104.03	111.89	7.85	0.39
Q-value (ME/GE)	0.71	0.72	0.71	0.71	0.01	0.79
Heat increment, Kcal/kgW$^{0.75}$	90.62	93.02	86.27	89.08	2.78	0.41
Energy retained, Kcal/kgW$^{0.75}$	27.51	30.00	17.77	22.81	5.08	0.38
Energy efficiency, %	22.97	25.27	19.36	21.75	1.93	0.23

gravity to differentiate high-quality having IgG >50 g/L with low-quality colostrum (Godden et al. 2019). After that, there was a decline in the milk protein but lactose increased. On day 5, we confirmed colostrum transition to milk from the composition. The fat and lactose digestibility in colostrum is more than 95% in neonates, but protein digestibility is only 83 to 86% but increases later to 93% (Kertz et al. 2017). The calves that suckle the dam for a long time have early puberty (Volkmann et al. 2019); thus, calves in TGF and TGM have a better edge than CGF or CGM.

The chemical composition of MGF and other diet components, including the whole milk fed to calves in neonatal and preweaning age, is shown in table 2. The results were expressed on a metabolic body weight basis to compare intake on the active tissue mass. The birth weight of female calves was lesser than male calves by 1.5 kg. The milk or MGF total or digestible nutrient intake in CGM or TGM during neonatal age was lesser than CGF or TGF, but differences were statistically insignificant (Table 3). The total diet CP intake in neonatal age was 22%, and digestible CP intake was 13.2%. High CP diets were recommended for neonates and preweaning calves than postweaning (Chapman et al. 2017). Maximizing the growth potential of calves in preweaning would determine the subsequent growth rate in postweaning (Volkmann *et al.* 2019). The CP intake of neonates was 7 to 8 g/kg $W^{0.75}$ and 5.3 to 6.2 in preweaning calves. Sharma et al. (2020) suggested a CP requirement of 5.20 g/kg $W^{0.75}$ and 0.40 g/g of ADG for maintenance and growth of Sahiwal (*Bos indicus*) breed calves.

Energy intake was comparable between CG and TG but higher in female than male calves. Heat increment was more than energy retained during neonatal age irrespective of gender, and noticed no significant difference between CG and TG groups (Table 4). Heat increment in newborn calves has been reported to be higher and decreases by 12 kcal/kg $W^{0.75}$ from the second week of birth

Table 5: Nutrient intake from the solid and liquid diet in preweaning Deoni calves

Parameter	Female		Male		SEM	P-Value
	CG	TG	CG	TG		
Metabolic Body weight, $kgW^{0.75}$	15.48^{a}	16.09^{ab}	17.56^{b}	17.57^{b}	0.68	0.12
Green fodder, $g/kgw^{0.75}$						
Dry matter	24.36	24.02	22.26	21.96	1.19	0.41
Organic matter	23.12	22.70	21.05	20.71	1.12	0.37
Crude protein	2.76	2.71	2.51	2.48	0.13	0.36
Ether extract	0.48	0.48	0.44	0.44	0.02	0.35
Total carbohydrates	19.85	19.50	18.08	17.79	0.96	0.38
Neutral detergent fiber	18.12	17.96	16.63	16.47	0.90	0.46
Acid detergent fiber	12.04	12.03	11.12	11.10	0.60	0.52
Hemicelluloses	6.08	6.28	5.75	5.94	0.32	0.68
Cellulose	8.21	8.18	7.58	7.52	0.42	0.52
Nonfibrous carbohydrates*	1.75^{b}	1.55^{ab}	1.46^{a}	1.32^{a}	0.08	0.02
Concentrate mixture, $g/kgw^{0.75}$						
Dry matter	10.22	9.82	9.02	9.20	0.45	0.25
Organic matter	9.25	8.89	8.16	8.32	0.40	0.25
Crude protein	1.77	1.70	1.56	1.59	0.08	0.26
Ether extract	0.32	0.31	0.29	0.29	0.01	0.25
Total carbohydrates	7.16	6.88	6.31	6.44	0.15	0.25
Neutral detergent fiber	3.39	3.26	2.99	3.06	0.15	0.26
Acid detergent fiber	1.37	1.32	1.21	1.23	0.06	0.26
Hemicelluloses	2.02	1.94	1.78	1.82	0.09	0.26
Cellulose	0.83	0.80	0.74	0.75	0.04	0.27
Nonfibrous carbohydrates	3.77	3.61	3.32	3.38	0.17	0.34
Liquid diet: Milk feeding, $g/kgw^{0.75}$						
Total milk intake	81.49	86.64	75.38	74.67	7.91	0.69
Dry matter	10.40	11.06	9.62	9.53	1.01	0.68
Organic matter	9.82	10.44	9.08	9.00	0.95	0.68
Protein	2.59	2.76	2.40	2.37	0.25	0.68
Fat	3.25	3.45	3.00	2.97	0.32	0.68
Carbohydrates	4.00	4.26	3.70	3.67	0.39	0.69

Values bearing different alpha superscripts differ significantly; *P< 0.05

(Arieli et al. 1995). The heat increment observed in the Deoni neonatal calves was lesser than in the *Bos taurus* calves reported, and the ME recommendation was 120 to 140 kcal/kg $W^{0.75}$ (Arieli et al. 1995). The ME intake was 118 to 123 kcal/kg $W^{0.75}$ in female calves of CG and TG but in males, it was about 10 kcal/kg $W^{0.75}$ lesser than female calves. The energy efficiency in neonatal age in Deoni calves was 20 to 25%, which was better than preweaning age.

During preweaning, total diet intake included MGF, concentrate mixture, and whole milk. Their intake was comparable between groups. Total milk intake was reduced by 30 to 50% in the preweaning compared to the neonatal period (Table 5). Nutrient intake in female calves was higher than in males even during preweaning age. The CP% of the diet in calves during preweaning was 18%, and digestible CP was 8 to 9%. Although no difference was observed in the ME intake in calves during neonatal and preweaning age, the heat increment was increased marginally in the preweaning. The energy efficiency in preweaning was less than 10% in CG or TG groups (Table 6). The 4th to 12th week after birth was the most critical period for the calves than the initial four weeks of the birth. At this age, immune cell functions are slower and lesser than in adults and depend on colostrum feeding (Trilk and Münch, 2010). If calves suffer from diseases due to their fragile immune system in the early stages, their growth at least lasts until 6 to 8 months and affects their lifetime performance ((Trilk and Münch, 2010; Furman-Fratczak et al. 2011; Volkmann et al. 2019). We observed that the disease incidence risk in TGM and TGF was reduced by 42%, and the relative risk of disease in CG groups was 6.57 folds more than TG groups ($P< 0.001$). Diarrhea, skin diseases, anorexia, eye infection, and profuse lacrimation were more frequently observed in CGF and CGM than TGF or TGM. The odds ratio for calf diarrhea in CG was 0.9 in contrast to 0.3 in TG.

Table 6: Total diet, Digestible nutrient intake, nutritive value, and energy balance in preweaning Deoni calves

Parameter	Female		Male		SEM	P-Value
	CG	TG	CG	TG		
Total diet and nutrient intake, g/kg$W^{0.75}$						
Dry matter	60.65	60.68	55.23	54.39	2.74	0.25
Organic matter	56.92	56.88	51.77	50.91	2.57	0.24
Crude protein	11.37	11.24	10.14	10.03	0.55	0.23
Ether extract	8.33	8.40	7.54	7.26	0.56	0.42
Total carbohydrates	37.22	37.27	34.11	33.68	1.58	0.26
Neutral detergent fiber	21.51	21.52	19.82	20.00	1.03	0.52
Acid detergent fiber	13.41	13.52	12.44	12.60	0.66	0.57
Hemicelluloses	8.10	8.39	7.65	8.03	0.41	0.65
Cellulose	9.03	9.12	8.04	8.48	0.45	0.59
Nonfibrous carbohydrates•	15.74	15.74	14.28	13.64	0.77	0.19
Digestible nutrients intake, g/kg$W^{0.75}$						
Dry matter	28.24	29.82	26.85	28.48	1.47	0.58
Organic matter	27.93	29.80	26.83	28.13	1.48	0.58
Crude protein	4.82	4.78	4.37	4.83	0.25	0.53
Ether extract	4.05	4.18	3.79	4.12	0.23	0.65
Total carbohydrates	19.03	20.21	18.11	18.67	0.97	0.50
Neutral detergent fiber	9.03	9.87	8.86	9.27	0.59	0.65
Acid detergent fiber	4.73	5.16	4.70	4.96	0.32	0.72
Hemicelluloses	4.27	5.05	4.36	4.75	0.38	0.46
Cellulose	3.47	3.96	3.63	3.61	0.27	0.64
Nonfibrous carbohydrates	8.27	8.46	7.60	7.84	0.45	0.54
Protein value and Energy Balance						
Digestible CP%	7.98	7.93	7.91	8.87	0.34	0.20
Nutritive ratio	5.84	6.21	6.12	5.79	0.13	0.12
Gross energy, Kcal/kg$W^{0.75}$	269.29	269.65	244.91	240.11	12.82	0.27
Digestible energy, Kcal/kg$W^{0.75}$	141.64	147.41	132.98	140.75	7.19	0.58
Metabolizable energy, Kcal/kg$W^{0.75}$	114.60	119.65	107.29	113.89	6.15	0.58
Q-value (ME/GE)	0.43	0.45	0.44	0.47	0.02	0.39
Heat increment, Kcal/kg$W^{0.75}$	107.69	108.24	102.84	102.37	2.56	0.27
Energy retained, Kcal/kg$W^{0.75}$	6.92	11.40	4.46	11.52	4.66	0.36
Energy efficiency, %	5.26	9.24	3.85	9.27	3.62	0.64

Values bearing different alpha superscripts differ significantly; •$P< 0.10$

Table 7: Average daily gain during neonatal and preweaning age in Deoni calves

Parameter	Female		Male		SEM	P-Value
	CG	TG	CG	TG		
Neonatal age (Birth to 28 d), g/d						
Birth weight	20.24	20.20	21.58	21.78	1.04	0.59
0 to 7 d (Wk1)*	245[a]	485[ab]	537[b]	720[b]	90	0.02
7 to 14 d (Wk2)*	392[a]	444[a]	629[b]	516[ab]	50	0.03
14 to 21 d (Wk3)	405	422	545	465	48	0.19
21 to 28 d (Wk4)	421	416	515	451	48	0.47
Preweaning age, g/d						
29 to 45 d (FN3)	367	365	413	400	31	0.62
45 to 60 (FN4)	386	380	401	426	26	0.62
60 to 75 d (FN5)	412	412	405	444	24	0.67
75 to 90 d (FN6)	431	450	414	463	21	0.42
Feed efficiency						
Kleiber ratio, ADG, g/kgW$^{0.75}$						
Neonatal age	30.29	34.87	39.67	38.09	2.84	0.15
Preweaning age	25.63	24.98	23.32	24.61	0.87	0.34
Residual feed intake (Regression method)						
Neonatal age	5.31	40.77	37.40	65.44	15.89	0.12
Preweaning age	-15.53	28.44	32.73	-5.62	22.74	0.38

Values bearing different alpha superscripts differ significantly; *P< 0.05; •P< 0.10

Weight gain in the male calves was higher than in the female calves, although their intake was statistically comparable (Table 7). The weight gained in the TGM and TGF was significant until the first fortnight but later comparable. Kertz et al. (2017) also explored fat supplements in neonates by adding 3.5% vegetable oils in milk replacers or skim milk, which aided in the improvement of growth, hair coat, and health of claves. The ADG recorded in Deoni calves was better than the Sahiwal calves reported by Sharma et al. (2020). The ADG was 1.17 in neonates and 1.07 g/g DMI in preweaning Deoni calve compared to 0.40, g/g DMI in Sahiwal calves. The Kleiber ratio was comparable between CGs and TGs of male and female calves. The negative RFI in CGM or CGF indicated lesser feed intake for a unit of growth compared to calves in TGM or TGF.

Conclusion

Colostrum quality reduces by one-third after 24 h of birth and IgG levels below 40 mg/mL. FSO supplementation from day five followed by supplementing ground flaxseed from day 28 until 90 d age had no adverse influence on the diet intake, digestibility of nutrients, and energy balance, but marginal improvement in weight gains was observed. Since FSO has health properties, it supports improving the calf's resistance to diseases early and facilitates weight gains; hence, drenching FSO 30 mL/d is recommended for neonatal calves.

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RESEARCH ARTICLE

Understanding the association of milk yield with major milk constituents and somatic cell count in Jersey crossbreds

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Abstract: In the dairy industry, understanding the correlation between somatic cell count (SCC), milk constituents and milk production is essential for optimizing milk production, ensuring milk quality, and maintaining cow health. Studying relationships among them can guide dairy farmers to enhance the productivity of their animals by making informed decisions thus improving food and nutritional security. Milk samples (n=400) were collected weekly from the fifty Jersey crossbred cows over five months and analyzed for SCC, total solids, solids not fat (SNF), lactose, protein, and fat content using standard methods. The study found correlations between test day milk yield with SCC, total solids, Solid Not Fat (SNF), lactose, protein, and fat as -0.233, -0.092, -0.056, -0.131, and 0.092 respectively. Protein and lactose content showed a significant, inverse relationship with milk yield, with correlation coefficients below zero ($P<0.01$). However, total solids, fat content, and SNF displayed a weaker, non-significant negative correlation with milk yield. Moreover, test-day milk yield and SCC also exhibited a significant negative ($P<0.05$) correlation. These results indicate that lower protein and lactose levels, along with higher SCC, are associated with reduced milk yield, indicating the importance of managing these factors to optimize dairy production and enhance the overall quality of the milk.

Keywords: Jersey crossbred, correlation, test day milk yields, somatic cell count, milk constituents

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Introduction

The dairy industry has placed a significant emphasis on improving milk production and quality (Singh et al. 2021; Bokharaeian et al. 2023), particularly focusing on the relationship between milk production and its major constituents. As per 20th livestock census the exotic and crossbred animals' population is 50.42 million which is an increase by 26.9% over the previous 19th livestock census (DAHD, 2020) in India. Milk quality is an essential factor in the dairy industry, determined by its chemical composition and hygienic properties. The primary components of milk include fat, protein, lactose, solids-not-fat (SNF), and total solids (TS), which not only define the nutritional value but also influence the processing and economic value of dairy products (Hnini et al. 2018). Milk composition is significantly affected by genetic (heritability) and non-genetic (lactation phase, nutrition, and seasonal variations) factors (Chaudhary et al. 2017). The seasonal shifts significantly impact milk's antioxidant activity and fatty acid profiles, with organic milk showing higher levels of polyunsaturated fatty acids and lower atherogenic indices compared to conventional milk (Kasapidou et al. 2023).

Milk production and its components are crucial parameters in determining the profitability of dairy farming and the quality of milk products. The fat content provides energy and is essential for flavor, while protein, particularly casein, contributes to milk's functional properties, making it ideal for cheese production (Kayihura, 2024). Lactose, the primary carbohydrate, plays a role in milk sweetness and supports fermentation in dairy products (Ohlsson et al. 2017). High-quality milk also requires a low somatic cell count (SCC), as elevated SCC levels, often indicative of mastitis, degrade milk quality by altering its composition, reducing fat and protein, and increasing the risk of spoilage (Sumon et al. 2020; Kaskous et al. 2022). Moreover, the exact nature of these relationships can vary on basis of genetic factors, seasonal conditions, and herd management practices (Bari et al. 2022).

Although several studies have explored the correlations between milk yield, composition, and SCC, many are breed-specific and limited to particular environmental conditions (Kul et al. 2019; Ablondi et al. 2023; Kasapidou et al. 2023). However, no studies have comprehensively examined these relationships in Jersey

crossbred cows, particularly under Indian climatic and management conditions. Identifying these associations is essential for developing balanced breeding and management strategies that enhance both milk quality and productivity. It was hypothesized that there exists a significant relationship between milk yield, composition, and SCC in Jersey crossbred cows. The objective of this study was to evaluate these correlations to provide insights that could guide breeding and management decisions for optimizing milk production and quality.

Material and methods

The current research was performed on the Jersey crossbred herd on the dairy Farm situated in Anjora city of District-Durg (Chhattisgarh, India). To investigate the relationship between milk yield, milk constituents, and somatic cell count (SCC), a total of 400 milk samples were collected weekly over a five-month period from February to June 2018. The samples were obtained from 50 lactating cattle aged 3 to 8 years, with parity ranging from 1 to 5, and in lactation stages from the first to the third. A sample of 30-40 ml milk was taken for analysis and cold chain for those samples was maintained using an ice box. The Milkotester (milk analyzing device of model LM2, Milkotester, Bulgaria) was used to measure the protein, lactose, total solid, fat, and SNF content of the milk samples. SCC analysis was done by smearing 10µl of fresh milk on a clean glass slide using sterilized platinum loop. After air drying, the smear was stained using a modified Newman's stain for 1-2 minutes, rinsed, and allowed to dry again. Microscopic examination of the stained smear under oil immersion was done. Only those cells, which possess blue stained nucleus, were counted. Milk samples were taken from all healthy animals. The correlation coefficient, between various milk constituents, test-day milk yield (TDMY) and SCC were calculated using the standards method used by Snedecor and Cochran (1994).

Results and Discussion

The correlation co-efficient between TDMY, various milk constituents as well as SCC are presented in Table 1. The findings indicates that there was negative correlation between the TDMY with milk constituents (total solid, lactose, protein, fat, and SNF) and SCC in Jersey crossbred cows. The descriptive statistics for milk constituents are presented in Table 2.

In this study negative (-0.092) and non-significant correlation was found between fat and TDMY. The negative correlation can be attributed to the dilution effect, where higher milk production is often associated with lower concentrations of fat due to increased secretion of milk volume. Similarly, Bekele et al. (2023) found negative association between fat and TDMY. The correlations of TDMY with lactose and protein were found to be negative and highly significant ($P<0.01$), with values of -0.131 and -0.192, respectively. Additionally, negative and non-significant correlation was found between TDMY and SNF and TS as -0.056 and -0.092, respectively. Similarly, several studies found a negative correlation between TDMY and various milk constituents (Ghule et al. 2016; Dora et al. 2020). These investigations suggest that selecting for higher milk production may lead to a decrease in the percentages of total solids, fat, protein and various other milk components. As a result, if milk composition is overlooked in selection programs focused on increasing milk yield, the quality of milk may decline. Therefore, developing a milk selection index that balances both milk yield and composition traits will be essential for improving both the quantity and quality of milk in dairy cows.

Correlation between fat with other milk constituents

A statistically significant and strong correlation ($P<0.01$) was observed between fat and various milk components such as protein (0.357), lactose (0.353), SNF (0.282), and total solids (0.890). The findings suggest that an increase in fat content tends to be associated with a rise in lactose, SNF, total solids (TS) and protein levels. Similar findings are reported by Yogi et al (2017) and Kro et al (2020) who observed positive and significant correlation. Bondan et al. (2018), Chandrakar et al (2017) also found a positive association between milk fat with protein and TS. So, selecting for higher fat content in Jersey crossbred cows will leads to simultaneous improvements in TS, SNF, protein, and lactose levels of milk.

Correlation between TS with protein, lactose and SNF

The correlation between TS with protein (0.506) was highly significant and positive ($P<0.01$). Similar findings were reported by Yogi et al (2017) also reported the positive and highly significant correlation between TS and protein. Correlation between TS and lactose (0.593) was highly significant and positive. Yogi et al (2017) also reported positive (0.304) and highly significant correlation between TS and lactose. Correlation between TS and SNF was (0.675) found to be positive and highly significant. Similarly, Chernet et al. (2024) reported a strong and statistically significant positive association between TS and SNF. A study on Gir cows demonstrated a correlation coefficient of 0.76 between TS and SNF, emphasizing that higher levels of SNF directly elevate TS, irrespective of fat content (Dora et al. 2020). Similarly, research on Anatolian buffaloes confirmed a significant positive relationship, indicating that SNF contributes consistently to TS across different lactation stages and environmental conditions (Şekerden and Avşar 2012). The positive and significant correlations observed between total solids and SNF, protein, and lactose are anticipated, as these components contribute to the overall total solids content.

Correlation between SNF with lactose and protein

The correlation between SNF and protein was positive (0.514) and highly significant ($P<0.01$). Likewise, the association between SNF and lactose was also positive (0.709) and highly significant

Table 1: Correlation of TDMY, milk constituents and somatic cell counts of Jersey crossbred cows

Parameter	TDMY	Fat	Protein	Lactose	SNF	TS	SCC
TDMY	1	-0.092	-0.192**	-0.131**	-0.056	-0.092	-0.233*
Fat		1	0.357**	0.353**	0.282**	0.890**	-
Protein			1	0.669**	0.514**	0.506**	-
Lactose				1	0.709**	0.593**	-
SNF					1	0.675**	-
TS						1	-
SCC							1

** Significant at <0.01; *Significant at <0.05

Table 2: Overall Means of various milk constituents (%), TDMY (kg/day) and Somatic Cell Count (x 10^5cell/ml)

Variable	N	Mean ± Std. Error	Standard Deviation
Fat	400	4.929 ± 0.032	0.641
SNF	400	8.051 ± 0.019	0.394
TS	400	12.983 ± 0.042	0.847
Protein	400	3.1905 ± 0.007	0.151
Lactose	400	4.425 ± 0.009	0.195
Test Day Milk Yield	400	10.571 ± 0.212	4.251
Somatic Cell Counts	400	1.3 ± 0.031	0.266

(P<0.01). It is desirable association indicates that selection for both traits can be practiced at the same time. Gautam et al. (2023) reported positive but non-significant correlation between SNF and protein. Chandrakar et al (2017) similarly found that the correlations of SNF with lactose (0.345) and protein (0.333) were both positive and highly significant (P<0.01), aligning with the results of the current study.

Correlation between protein and lactose

A significant positive correlation (0.669, P<0.01) was found between lactose and protein, suggesting that an increase in protein content may result in elevated lactose levels in milk. A similar finding was also found by Yogi et al (2017) and Henao-Velásquez et al (2014) reported significant and positive correlation between protein and lactose content. According to Alessio et al (2021) they also found positive association between lactose and protein in factor analysis. On contrary, Bondan et al (2018) observed negative correlation between milk protein and lactose. This divergence might be attributed to differences in genetic makeup, dietary factors, or environmental conditions affecting the dairy populations studied.

Correlation between somatic cell count (SCC) and test day milk yield (TDMY)

In the current study, a significant negative correlation (P<0.05) was observed between SCC and TDMY. Correlation coefficient was noted as -0.233. Similarly significant and negative correlation was reported by Ouedraogo et al (2008) and Bokharaeian et al. (2023). Research consistently demonstrates a negative association between SCC and milk yield. Contrary, Syridion et al (2012) reported non-significant association between SCC and TDMY. The elevated SCC causes decline in the milk quality of dairy animals (Safak and Risvanli 2023). Somatic cell count monitoring is advised in dairy farms on a frequent basis to keep an seye on changes.

Conclusion

The findings of this study highlight the significant associations between milk yield, SCC, and major milk constituents in Jersey crossbred cows. The study identified strong and significant positive correlations among milk constituents, such as protein, lactose, SNF, and TS, emphasizing their interdependence in determining milk quality. These findings suggest that while selecting for increased milk yield, care must be taken to maintain the balance of milk constituents to ensure high-quality production. This study underscores the importance of genetic and management strategies tailored to Jersey crossbred cows under Indian conditions.

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Conflict of interests

The authors declare that they have no conflict of interests.

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RESEARCH ARTICLE

Post-partum supplementation of calcium salts of long-chain fatty acids and fibrolytic enzymes on reproductive performance and blood metabolites of lactating Surti buffaloes

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Abstract: Twenty-four lactating Surti buffaloes (BW=459.21±7.39 kg and parity of 2.58±0.15) after seven days of calving were randomly divided into four treatment groups. The control (CON) group was fed with basal diet (chaffed sorghum straw, green fodder hybrid napier and BIS Type-I compound cattle feed). The second group was fed the exogenous fibrolytic enzymes (EFE) supplement, while the third and fourth groups (Bypass Fat-1 and Bypass Fat-2)were fed EFE along with 1% and 2% of bypass fat of total DMI, respectively. Blood biochemical parameters were measured at the start (0 d), middle (75th d), and end (150th d) of the experiment. The treatments had no significant effect on the serum levels of glucose, total protein, T_3, T_4, urea, and lipid profile (including triglycerides, total cholesterol, HDL, LDL, VLDL and NEFA). However, animals that received these supplements reduced service period and fewer services per conception when compared to the control group. Thus, adding bypass fat and fibrolytic enzyme supplements to animals' ration could improve their reproductive performance without negatively impacting their blood biochemical indicators.

Keywords: Bypass fat, Buffaloes, Blood metabolites, Fibrolytic Enzymes

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Introduction

In tropical regions, buffaloes are often fed with cereal crop residues. However, these residues are not easily digestible due to the high content of crude fiber that is coated with lignin making them resistant to biological degradation (Mahesh and Mohini, 2013). As a result, the rumen microbes are unable to efficiently utilize the nutrients, leading to negative energy balance (NEB) and lower production and reproduction performance of the animals (Sirohi et al. 2010). To increase the productivity of lactating animals, energy supplementation is necessary. One way to do this is by incorporating fat into their diet which increases energy density. Small dairy farmers commonly supplement with locally available concentrate to improve energy density. Further NEB could be overcome by supplementation of calcium salts of long-chain fatty acids (Ca-LCFA) as bypass fat (BPF) to increase the energy density of the ration without adversely affecting the dry matter (DM) intake and nutrient digestibility (Naik et al. 2009). The National Dairy Development Board (https://www.nddb.coop/services/animalnutrition/bypass) recommends using bypass fat (BPF) in the diet of dairy animals 10 days before and 90 days after calving. The BPF can be added to the diet of dairy animals at a rate of 15-20 g/kg of milk production or 100-150 g per animal per day. It's important to note that fat does not hinder the digestion of fiber and is always a better option than feeding ghee or oil. Supplementation of calcium salts of long-chain fatty acids (Ca-LCFA) as BPF is partially resistant to biohydrogenation by the rumen microbes and also reduces the risk of metabolic acidosis (Naik et al. 2009). Calcium salts of long-chain fatty acids would be beneficial in dairy nutrition, but it couldn't completely overcome the challenge of fiber digestion inhibition, even with the addition of calcium. Beauchemin et al. (2003) have reported improvement in fiber utilization in animal diets by using exogenous supplementation of fibrolytic enzymes. Feed enzymes are active in the rumen in the presence of feed substrate, and the mechanism of effects includes direct hydrolysis changes in gut viscosity, complementary action with ruminal enzymes and change in the site of digestion. Most of the studies have been conducted either by using BPF or EFE individually, but the literature on its combined effects on lactating buffaloes is inadequate and scanty. Considering the importance of BPF supplementation with EFE in

milk production, an attempt was made to study the effect of EFE alone and in synergetic effect with BPF (1% and 2% of DMI) supplementation on productive performance through blood biochemical profile of lactating Surti buffaloes.

Materials and methods

Location of the study

The experiment was conducted at the Livestock Research Station, Kamdhenu University, Navsari, Gujarat, India. The research site is located at 202 92°N and 722 89°E, with an altitude and average elevation of 9 m above sea level. The area experiences heavy rainfall, with an average annual rainfall of 122 cm. The experiment was conducted from September, 2019 to February, 2020 on an elite herd of Surti buffaloes.

Experimental animals and treatments

Multiparous lactating Surti buffaloes (24 No.) were divided into four groups based on their average body weight (459.21±7.39 kg), parity (2.58±0.15) and previous lactation yield (960.81±28.07 kg). The control group (CON) that was given a basal diet consisting of chaffed dry fodder (sorghum straw), green fodder (hybrid napier) and BIS Type-I compound cattle feed. The EFE group was given exogenous fibrolytic enzymes along with the basal diet while BF-1 and BF-2 groups had bypass fat added at 1% and 2% of DMI respectively along with exogenous fibrolytic enzymes, in addition to the basal diet. An EFE mixture weighing 08 g containing equal proportions of cellulase (with a minimum of 100000 IU/g) and xylanase (with a minimum of 50000 IU/kg) enzymes were used in a study conducted over a period of 150 days after a two week adaptation period. All animals were fed according to the ICAR (2013) feeding standards and were kept in well-ventilated byres with free access to fresh, clean drinking water. Feed intake was assessed on a biweekly basis, conducted over two consecutive days. During the afternoon session, the animals were permitted to roam freely in an open paddock area. The calculation of fortnightly dry matter and nutrient intake was derived from the quantity of feed offered and the leftover remaining. Additionally, daily milk production was recorded following hand milking procedures.

Sample collection and analysis

The compound cattle feed, sorghum straw and hybrid napier grass samples, both offered and leftover, were dried and then ground to pass through a 1-mm sieve using a MAC® Willey grinder. The pooled samples were analyzed for proximate composition (AOAC, 2005) and fiber fractions (Van Soest et al. 1991).

Blood sampling and analysis

Blood samples were collected from each animal during the experimental period. The collection was done by puncturing the jugular vein on the 0^{th}, 75^{th} and 150^{th} day at 08AM. The serum samples were separated from the blood by centrifugation at 2000 rpm for 15 min. and stored at "40°C for subsequent analysis of the biochemical profile. Diagnostic kits from Sigma Diagnostics and Randox Laboratories Company, India were used for analyzing various blood biochemical parameters, i.e., glucose (GOD–POD method), total protein, tri-iodothyronine (T_3), thyroxine (T_4), serum urea modified (UV/IFCC method), total cholesterol (CHOD–PAP method), low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL) (Friedewald's equation), triglycerides (GPO-PAP), non-esterified fatty acids (NEFA).

Statistical analysis

The data were analyzed using a statistical model that estimates the least squares means of variables (Harvey, 1982) for the random effect of treatment and periods. The analysis of variance was performed using the PROC MIXED procedure of SAS with repeated measures (version 9.3; SAS Institute Inc., Cary, NC) and Tukey's HSD (honestly significant difference) multiple comparison tests. Differences were considered significant at $P<0.05$, and values of $P<0.10$ were interpreted as a trend towards significance.

Results and Discussion

Chemical composition of feeds and forage

The chemical composition of feeds has been shown in Table 1. The bypass fat had a total fat content of 82.10%. The concentrate mixture with or without the bypass fat was similar in chemical composition, except for the ether extract and total ash contents, which were non-significant ($P>0.05$) in the concentrate mixture fed to all the groups. The total ash content of the bypass fat (17.90%) was comparatively higher than other feeds and fodders, due to the high level of Ca contributed from the bypass fat. Although the diet was iso-nitrogenous, it was not iso-caloric because of the addition of bypass fat.

The total dry matter intake (DMI) showed no significant variation ($P>0.05$) among all groups, regardless of the treatment regimen, throughout the experimental period (Movaliya et al. 2021). The total DMI recorded was 11.43 kg/d for the control group (CON), 12.17 kg/d for the EFE group, 12.42 kg/d for the BF-1 group, and 11.27 kg/d for the BF-2 group. Notably, DMI was highest in the BF-1 group, followed by the EFE group, then the CON group, with the BF-2 group having the lowest intake.

Total milk and fat-corrected milk (6% FCM) yield (kg/d) were significantly (PÂ0.05) higher in BF-1 (5.41 & 6.44), followed by

EFE (4.48 & 5.40), when compared to the CON (3.93 & 4.65) and BF-2 (3.89 & 4.47) groups (Movaliya et al. 2021).

Blood biochemical profile

The levels of serum glucose, total protein, tri-iodothyronine, thyroxine and urea were not influenced by supplemental bypass fat and fibrolytic enzymes. However, they differed (P<0.05) with the advancement of lactation trials (Table 2) except for serum urea. The serum glucose level was similar in all the groups (Ramteke et al. 2014a; Singh et al. 2014; Raval et al. 2017, 2019). Similar findings were observed by Zilio et al. (2019) with fibrolytic enzyme supplementation were serum glucose levels. This might be due to high metabolic rate of glucose utilization in body during early lactation and developed homeostatic mechanism of animal body that does not allow noticeable changes in glucose level. In contrary to present findings, bypass fat (Nirwan et al. 2019 and Vala et al. 2020) and fibrolytic enzymes (El-Bordeny et al. 2015) supplementation significantly increased (P<0.05) serum glucose level.

Average serum total protein (g/dL) was comparatively similar amongst treatment groups. The serum total protein showed non-significant difference due to supplementation of bypass fat (Wadhwa et al. 2012; Raval et al. 2017). El-Bordeny et al. (2015) and Beigh et al. (2017) also reported normal serum protein level after EFE supplementation. The probable reason might be improved digestibility of nutrients which made no major changes in protein level but reflected in improvement of milk production.

The concentration of tri-iodothyronine and thyroxine was similar in the groups. Overall, periodically T_3 (mg/dl) significantly increased from 0.84 (0 d) to 0.92 (150 d) and T_4 (mg/dl) decreased from 2.75 (0 d) to 2.42 (150 d). Wadhwa et al. (2012); Theodore et al. (2017) and Vala et al. (2020) indicated that bypass fat supply during transitional period in lactating animal has increased (P<0.01) serum levels of T_3 while T_4 decreased (P<0.05) consistently. The serum thyroid hormone levels are also shown to be influenced significantly by the stage of estrous cycle (Borady et al. 1985) and stage of lactation (Garg, 1998).

Serum urea nitrogen revealed no significant (P>0.05) difference amongst treatment groups as well as periods. Overall periodical effect shown non-significant (P>0.05) decreasing trend without treatment effect. In contrary, Wadhwa et al. (2012); Ramteke et al. (2014a) and Katiyar et al. (2019) observed that postpartum bypass fat supplementation reduced the blood urea nitrogen level in buffaloes and Morsy et al. (2016) also concluded that the blood urea nitrogen level remained unaffected with EFE supplementation in buffaloes.

Blood lipid profile parameters (Table 3) like total cholesterol including LDL, HDL, VLDL cholesterol and NEFA were also not influenced by dietary treatment but affected by time (P<0.05) with advancement of lactation. Serum total cholesterol level (mg/dL) was non-significant (P>0.05) with dietary treatment. However, total cholesterol was also increased gradually with advancement of sampling time due to continuous intake of lipids for long time in basal diet in the form of bypass fat supplementation. Wadhwa

Table 1: Chemical composition of feed and fodder (% DM basis)

Attributes	Concentrate mixture	Hybrid napier	Sorghum straw	Bypass fat
Dry matter	90.50	26.04	94.50	-
Organic matter	95.59	88.23	93.00	-
Crude protein	21.72	05.54	02.83	-
Ether extract	02.93	01.24	01.15	82.10
Crude fiber	07.36	33.77	38.19	-
Neutral detergent fiber	27.58	51.97	73.20	-
Acid detergent fiber	17.01	40.90	44.70	-
Nitrogen free extract	63.58	47.68	50.80	-
Hemi-cellulose	10.57	11.04	28.50	-
Cellulose	15.33	36.21	33.60	-
Acid detergent lignin	01.68	04.69	11.10	-
Total ash	04.41	11.77	07.03	17.90
Calcium	00.92	01.44	01.04	12.15
Phosphorus	00.93	00.22	00.21	03.56

et al. (2012) and Raval et al. (2017) found that rumen protected fat supplementation improved serum triglycerides and cholesterol levels in crossbred cows and Surti buffaloes, respectively.

Serum HDL, LDL and VLDL cholesterol in treatment groups remained statistically (P>0.05) similar but overall periodically increased (P<0.05) from beginning to end of experiment. Greater concentration of lipid metabolites in animals supplemented with bypass fat can be explained by increased intestinal secretions of lipoprotein (especially HDL cholesterol). Higher level of cholesterol in portal circulation is dependent on how it is transported within lipoproteins (HDL or LDL). Shelke et al. (2012b) also reported increased level of HDL cholesterol using bypass fat supplementation. Likewise, Ranjan et al. (2012) found that supplementation of bypass fat to lactating buffalo's increased HDL cholesterol in fat supplemented group. Increased HDL cholesterol in bypass fat supplemented group might be contributed by long-chain fatty acids incorporation.

Average serum triglyceride was similar in all the groups, however there periodic increase (16.26 at 0 d to 20.20 mg/dL at 150 d). Raval et al. (2017) also showed that supplementation of rumen protected fat caused energy enrichment of diets for buffaloes at early stage of lactation, which improved serum triglycerides and

Table 2: Impact of adding fibrolytic enzymes and bypass fat on Surti buffalo blood biochemical parameters

Day	CON	EFE	BF-1	BF-2	Mean	SEM	P value		
							D	T	D x T
Glucose (mg/dl)									
0	26.60	31.50	29.00	29.94	29.26[b]				
75	45.43	50.10	45.31	48.30	47.28[a]				
150	44.61	42.66	38.97	44.37	42.65[a]				
Mean	38.88	41.42	37.76	40.87	39.73	2.84	0.781	0.0001	0.93
Total protein (mg/dl)									
0	6.91	7.00	6.76	6.54	6.80[b]				
75	7.05	7.13	7.42	7.32	7.23[a]				
150	7.19	7.50	7.29	7.07	7.26[a]				
Mean	7.05	7.21	7.16	6.98	7.10	0.16	0.714	0.007	0.618
T_3 (mg/dl)									
0	0.87	0.95	0.82	0.75	0.84[b]				
75	0.85	0.87	0.82	0.82	0.84[b]				
150	0.88	0.92	0.92	0.96	0.92[a]				
Mean	0.87	0.91	0.85	0.84	0.87	0.03	0.376	0.0012	0.051
T_4 (mg/dl)									
0	2.68	2.80	2.87	2.67	2.75[a]				
75	2.64	2.90	2.57	2.32	2.61[ab]				
150	2.21	2.66	2.62	2.19	2.42[b]				
Mean	2.51	2.78	2.69	2.39	2.59	0.11	0.03	0.003	0.351
Serum urea nitrogen (mg/dl)									
0	41.13	42.96	40.89	40.89	41.47				
75	37.70	38.83	39.76	40.93	39.30				
150	36.21	39.65	39.39	37.99	38.31				
Mean	38.34	40.48	40.01	39.94	39.69	1.73	0.833	0.289	0.983

[a,b]Means bearing different superscripts in a row differ significantly (P<0.05)
CON- control group that was given a basal diet
EFE- basal diet + exogenous fibrolytic enzymes
BF-1- basal diet + bypass fat added at 1% + exogenous fibrolytic enzymes
BF-2- basal diet + bypass fat added at 2% + exogenous fibrolytic enzymes

cholesterol concentration, respectively. In contrary to present findings, Kumar and Thakur (2007) reported significantly increased triglycerides in buffalo calves with bypass fat supplementation. Most of research showing positive energy balance might be responsible for normal serum triglycerides level.

Mean NEFA (mg/dL) concentration was non-significant (P>0.05) within the treatment but periodically it was declined significantly (P<0.05) from 0.69 (0 d) to 0.55 (150 d) indicating the utilization of NEFA for energy purposes, in addition to fatty acid synthesis. Shelke et al. (2012b) and Mohamed et al. (2013) reported positive energy balance with bypass fat and EFE supplementation and no effect on NEFA level in serum. In contrary to present findings, significantly increased level of serum NEFA level with bypass fat and EFE supplementation has been reported (Kumar and Thakur 2007, Ranaweera et al. 2019).

First post-partum heat (d) was observed earliest in BF-1% and last in CON but effect of treatment on it did not reach up to significant (P>0.05) level. Service period (d) and number of

Table 3: Consequence of supplementing bypass fat and fibrolytic enzymes on serum lipid profile of Surti buffaloes

Day	CON	EFE	BF-1	BF-2	Mean	SEM	P value		
							D	T	D x T
Cholesterol (mg/dl)									
0	83.13	86.15	84.90	90.59	86.19[b]				
75	115.37	126.11	115.33	108.84	122.84[a]				
150	118.04	132.43	120.89	119.99	116.41[a]				
Mean	105.51	114.90	107.04	106.48	108.48	8.85	0.868	<0.0001	0.755
LDL Cholesterol(mg/dl)									
0	29.10	34.07	31.51	32.67	31.84[c]				
75	43.17	51.26	51.07	48.87	48.59[b]				
150	52.69	59.76	51.16	51.37	53.75[a]				
Mean	41.65	48.36	44.58	44.30	44.72	4.62	0.784	<0.0001	0.613
HDL Cholesterol (mg/dl)									
0	56.11	64.8	55.17	63.92	60.00[c]				
75	73.17	73.98	71.98	77.31	74.11[b]				
150	81.87	93.00	78.04	81.35	83.57[a]				
Mean	70.38	77.26	68.40	74.19	72.56	0.270	0.695	<0.0001	0.748
VLDL Cholesterol (mg/dl)									
0	3.08	3.30	3.76	2.87	3.25[b]				
75	3.03	2.84	3.88	3.33	3.27[b]				
150	3.64	3.76	4.50	4.26	4.04[a]				
Mean	3.25	3.30	4.04	3.49	3.52	0.270	0.152	0.0004	0.655
Triglycerides (mg/dl)									
0	15.40	16.51	18.78	14.34	16.26[b]				
75	15.13	14.19	19.39	16.67	16.34[b]				
150	18.21	18.81	22.48	21.31	20.20[a]				
Mean	16.25	16.50	20.22	17.44	17.60	1.33	0.152	0.0004	0.655
Non-esterified fatty acids (mg/dl)									
0	0.67	0.70	0.61	0.77	0.69[a]				
75	0.66	0.68	0.58	0.74	0.66[a]				
150	0.55	0.54	0.54	0.58	0.55[b]				
Mean	0.70	0.64	0.63	0.58	0.64	0.05	0.461	0.0001	0.068

[a,b,c]Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4: Result of supplementing bypass fat and fibrolytic enzymes on reproductive performance

Attributes	CON	EFE	BF-1%	BF-2%	SEM	P value
Post-partum heat (d)	94.83	87.17	65.33	77.67	13.38	0.580
Numbers of services per conception	03.10[a]	02.80[b]	02.60[b]	02.10[c]	00.50	0.038
Service period (d)	112.65[a]	108.6[ab]	106.78[ab]	95.92[b]	23.72	0.027

services per conception were significantly (P<0.05) decrease in both bypass fat supplemented group as compared to control. Present findings revealed that positive energy balance with bypass fat supplementation may be responsible for better performance. Higher cholesterol level may have favourable effect on the synthesis of reproductive hormones and in turn may affect the reproductive performance of animals in the bypass fat supplemented group. Present findings are in line with the findings of Patel et al. (2020) and Sihag et al. (2020) that bypass fat supplementation, enhanced early onset of first postpartum estrus and reduced number of services required per conception with improved conception rate and significantly improve the postpartum fertility. The role of fatty acids in the reproductive performance of dairy animals, as noted by Patel et al. (2020), includes several key factors. Enhanced energy balance results in an earlier return to ovarian cycling after postpartum periods. An increase in linoleic acid may elevate PGF2α levels, stimulating ovarian cycling and promoting follicular recruitment. Additionally, an increase in progesterone secretion either from improved energy balance or altered lipoprotein composition due to dietary fat can further enhance fertility.

Conclusions

The supplementation of bypass fat and exogenous fibrolytic enzymes did not have any adverse effect on the total dry matter intake (DMI), blood biochemical and lipid profile, however, total milk and fat-corrected milk yield were significantly higher in BF-1 group of Surti buffaloes. Nevertheless, both types of supplementation improved their reproductive efficiency.

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Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Measuring the technical efficiency of milk production in Punjab: Frontier production function approach

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Abstract: An effort has been made to examine the technical efficiency in milk production and the determinants thereof for 80 dairy farmers, selected from Ludhiana and Patiala districts of Punjab state. The total number of milch animals on small, medium, and large farms was 2.71, 4.73 and 12.79, respectively with an overall average of 5.12 milch animals. Overall, of the total costs, variable costs and fixed costs accounted for 85.0 and 15.0 percent respectively. The net returns were observed to be Rs 22568.9, Rs 81784.2 and Rs 340827.5 per farm in case of small, medium and large dairy units, respectively. The net returns per litre of milk were estimated to be Rs 3.65, Rs 7.50 and Rs 10.51, respectively on the respective farms with an overall average of Rs 7.92. The farmers intended to realize 77.30 percent of the technical abilities and potential for improvement in technical efficiency in milk production was 21.3 percent. This implies that dairy farmers could enhance the milk production by 21.30 percent with existing level of technology and resources. The technical efficiency of dairy farmers determined by age of farmers, land holding, price of milk received by the farmers and training were found to be positive and significant. The study indicated that dairy farmers should be trained on a regular basis for appropriate feeding practices, rearing optimal herd size with quality animals, and new technologies in milk production to attain maximum milk production and thus achieve more benefits. Besides, strong and effective linkage of farms to market could provide incentives towards increasing their efficiency in milk production. Dairy farmers can gain considerable higher profits by increasing the efficiency in their operations.

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Key words: Cost and returns, Dairying, Determinants, Technical efficiency, Tobit analysis

Introduction

Animal husbandry and dairying have been part of human life since the start of civilization. They have contributed not only in providing low cost and nutritious food to millions of people but also in providing animal power and maintaining ecological balance (Dhawan and Kashish 2016). There exists a close link between livestock and agriculture sector. In India, livestock sector produced 230.58 million tonnes of milk, 9.77 million tonnes of meat and 138.38 billion eggs in 2022-23. The share of Gross Value Added (GVA) of livestock sector to agriculture sector has increased to 30.19 percent during the year 2022-23 while its share in total GVA was 5.73 percent in the said year (Anonymous, 2023). Livestock sector in general and dairy sector in particular provides cushion to overall agricultural growth (Kashish et al. 2014; Dhawan and Kashish 2016; Dadhich 2017). Dairying is one of the oldest professions pursued by mankind. It came up as the complementary activity to agriculture and now become a full-fledged business. The dairy industry is a very important part of the global food system (Singh et al. 2022). India has been the leading producer and consumer of dairy products worldwide since 1998 with a sustained growth in the availability of milk and milk products (Kashish et al. 2016). Dairying has always been quoted as one of the means for poverty alleviation and improvement of nutritional security (Kumar and Shah, 2016; Kashish et al. 2017). It has been proud to be the largest milk producing country, accounting for 23.1 percent of world production with an annual output of 230.58 million tonnes at an annual growth rate of 3.83 percent achieved during 2022-23 (Anonymous, 2023).

As Punjab is one of the major milk producing state with about 7 percent share in total milk production of India and ranked sixth with annual milk production of 13.34 million tonnes (Anonymous 2020). Dairy farming is one of the alternatives to the wheat-rice system in Punjab, which offers regular income and employment to families, particularly small and marginal (Elumalai and Pandey 2004; Kashish et al. 2017). To stimulate milk production in Punjab, an ambitious program has been set up to include the genetic

improvement of local bovine and buffalo breeds through breeding and crossbreeding. Despite the efforts of the Government, Punjab Dairy Development Board and the various cooperatives to improve milk production there are still high input costs for milk production compared with low milk prices, which together reduce profit margins. Productivity has to be increased in order to sustain milk production in Punjab. Productivity can be improved in two ways: technological progress and to improve technical efficiency (Gerber and Franks 2001; Karanja et al. 2012). In a developing country like India, it is important to know what policies and measures should be taken to improve productivity before investing scarce capital to achieve technological progress (Saha and Jain, 2004). In this context, the analysis of efficiency assumes fundamental importance, since the improvement of technical efficiency implies that inefficient farmers adopt technologies and practices and, therefore, save scarce capital to obtain better results (Nizam and Armagan 2006). Furthermore, the analysis of the factors that cause (in) efficiency provides important insights on key variables that could be worthy of consideration in the formulation of policies to ensure optimal use of capital and resources. Increasing efficiency is an important factor for productivity growth and can be increased by using better technologies together with better management of all essential inputs available to farmers (Gunden et al. 2006; Lovell 1993; Jaforullah and Whiteman 1999). Efficiency analysis in milk production becomes all the more important in underdeveloped production environments of developing countries like India which are basically low-input and low-output environments characterized by subsistence holdings, resource poor locations with milch animals of low production potential and having poor infrastructural support system.

Available studies have shown that farmers in developing countries fail to exploit full potential of technology and make allocative errors (Gelan et al. 2010; Otieno et al. 2012 and Rao 2012). Thus, increasing the efficiency in production assumes greater significance in attaining potential output at the farm level. The consequence of technical inefficiency is the increased production cost, which make dairy farms less competitive and the viability of dairy farming is questioned. A clear understanding of farm level inefficiency in milk production and identification of their determinants would provide the clue for making this sector competitive and viable. In view of the above, the present study was carried out to examine the technical efficiency in milk production along with influence of various factors on the efficiency in central plain region of Punjab state.

Materials and Methods

To achieve the stipulated objectives, the primary data pertaining to crop year 2017-18 were collected by using well-structured and pre-tested interview schedule. The central plain region of Punjab was purposively selected being developed and highest milk producing region of the state. Multistage simple random sampling technique was used for sample selection. In first stage two districts namely Patiala and Ludhiana having highest milk production above the state average were selected purposively. In next stages, one block from each chosen district and two villages from each chosen block were selected randomly. After the selection of villages, a list of all the dairy farmers with number of milch animals (cows and buffalos) maintained on each farm was prepared. Using Cube root frequency method of stratification, the size distribution of herds of these farms was then transformed to identify the size ranges of small (1-5 animals), medium (6-10 animals) and large (>10 animals) units. A sample of 20 respondents from each selected village was selected randomly, making a total sample of 80 farmers. The details of sample selection are presented in Table 1.

To calculate the cost and returns from milk production, primary data regarding quantity of dry fodder, green fodder and concentrates fed to the dairy animals per day and the purchase price per unit were collected from selected dairy farmers. Furthermore, the data on their expenditure on veterinary and health care services were also collected from the dairy farmers. To examine fixed cost per farm, data regarding value of dairy animals, value of equipments, dairy buildings and different machineries were also compiled. In order to evaluate the gross returns, data regarding amount of milk sold, quantity of dung sold and young stocks sold were also collected from different dairy farmers to know the exact picture of dairy farmers at a field level.

Technical efficiency in milk production

Of the various approaches to the estimation of technical efficiency, the parametric Stochastic Frontier Production function (SFP) (Aigner et al. 1977, Meeusen and van den Broeck 1977), and non-parametric Data envelopment analysis (DEA) (Charnes et al. 1978) are the two most popular approaches. Each approach has its own advantages and disadvantages. Although the advantage of DEA lies in its general nonparametric limit, its limitations are due to the fact that, using the DEA model, the efficiency values are contaminated by omitted variables, measurement errors, and other sources of statistical noise. On the other hand, the strength of SFP lies in its ability to separate the term error into two components, namely, inefficiency and random noise can only be implemented by introducing a specific functional form, and therefore the resulting efficiency indicators can be sensitive to the selected functional form his (Gelan and Muriithi 2010). The frontier production function defines the potential output that can be produced by a farm/firm with the given level of inputs and technology.

The stochastic frontier production function was used in this study to estimate the technical efficiency in milk production. In the general form the stochastic frontier production function can be written as:

$Y_k = f(X_{ik}) \exp(v_k - u_k)$

Where, Y_k is the output of the k^{th} farm, X_i's are the inputs in the production process, v_k is a random variable representing statistical noise and other stochastic shocks entering into the definition of the frontier. It is almost universal to specify this random term as independent normally distributed with zero mean and constant unknown variance σ_v^2, and independent of X_i, i.e., $v_k \sim N(0, \sigma_v^2)$. u_k is a non-negative random variable representing technical inefficiency and is assumed to be distributed independently of v_k and X_i. It can be measured by the difference between maximum output Y* (estimated through the stochastic frontier production function) and observed output, Y_i. Thus, farm-specific inefficiency is the distance below the frontier ($Y_i - Y^*$). The above stochastic frontier production function can be estimated by maximum likelihood once a density function for u_k is specified.

The stochastic frontier production function of Cobb-Douglas type has been specified for this study:

$\ln Y_i = \beta_0 + \beta_1 \ln X_1 + \beta_2 \ln X_2 + \beta_3 \ln X_3 + \beta_4 \ln X_4 + \beta_5 \ln X_5 + \beta_6 \ln X_6 + (v_i - u_i)$

Where

Subscript i, denotes the i^{th} farmer in the sample

ln = the natural logarithm (i.e., to base e)

Y_i = Returns from milk (in Rs)

$\beta_0 \ldots\ldots\ldots \beta_6$ = parameters to be estimated

X_1 = Expenditure on green fodder

X_2 = Expenditure on dry fodder

X_3 = Expenditure on concentrates

X_4 = Veterinary expenses

X_5 = Value of labour

X_6 = Fixed cost

$v_i - u_i$ = random error term

The model is estimated by using stochastic production function and the Maximum Likelihood Estimates (MLE) . The model was estimated using the computer program FRONTIER 4.1 (Coelli, 1996) to estimate simultaneously the parameters of the stochastic production frontier and the technical inefficiency effects.

Potential for increasing milk production

The average potential to measure milk production was determined using the following formula.

$$= \left(1 - \frac{mean\ technical\ efficiency}{\text{maximum technical efficiency}}\right) * 100$$

Determinants of technical efficiency

The observed differences in technical efficiency may be due to numerous factors including the degree of sample homogeneity, the methods employed and differences in farm specific characteristics. The present study analyzed the variation in technical efficiency in milk production due to farm specific characteristics such as age of farmer, herd size, proportion of milk sold, price received and dairy training of farmer. In order to know the contribution of each factor, the level of technical efficiency of the milk producers considered was regressed in these factors. Tobit model, also known as censored regression model or limited dependent variable regression proposed by Tobin, 1958 was used to examine the determinants of technical efficiency. A censored sample is a sample in which information on dependent variable is available for only some observations. If we use OLS on censored data set, estimates obtained will be inconsistent meaning coefficients will not necessarily approach the true population parameters as sample size increases (Gujarati, 2003). In such cases, Tobit model is used for analyzing censored sample.

Table 1: Sample selection of dairy farmers in Ludhiana and Patiala districts of Punjab

Districts	Blocks	Villages Selected	Herd size group			Overall
			Small	Medium	Large	
Ludhiana	Raikot	Kalsiyan	11[#]	5	4	20
		Lohatbadi	8	7	5	20
Sub-total (a)			19	12	9	40
Patiala	Nabha	Laloda	12	6	2	20
		Sangatpura	9	8	3	20
Sub-total (b)			21	14	5	40
Grand Total (a+b)			40	26	14	80

[#]*Number selected*

$$Y = \beta X + \mu \ if \ \beta' X + \mu > 0;$$

= 0 otherwise

Such that $\mu \sim N(0, \sigma^2)$

Thus, Tobit's analysis of factors influencing the technical efficiency of selected farms is specified as:

$$TE_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + e_i$$

Where

TE_i = Technical Efficiency

X_1 = Herd size

X_2 = Land holding

X_3 =Age of farmer

X_4 =Square of age

X_5 = Price received

X_6 = Proportion of milk sold

X_7 = Training in dairy sector

e_i = Error term

Results and Discussion

The purpose of this section is to study the crucial characteristics of the sample dairy farmers that may have an impact on the decision-making process, efficiency and profitability of the dairy enterprise. In the present section, the study has focused on the demographic profile of sample farmers, variable and fixed cost incurred in milk production and technical efficiency of sample farmers and determinants thereof in the study area.

Socio-economic profile of selected dairy farmers

The socio economic profile of surveyed households is presented in Table 2. The average age of households was 53.1 years. The family size varied among different categories of households. The average size of family on small, medium and large size categories was 5.33, 5.77 and 7.00 respectively (Table 2). Overall, the family size came out to be 5.76 members. The herd strength and the number of milch animals in the households affect the economic position of milk producers. The total number of milch animals on small, medium and large dairy units was 2.71, 4.73 and 12.79 respectively. The proportion of buffaloes was found evidently higher on all size categories as compared to crossbred cows and indigenous cows collectively. On an average, out of total milch animals the percentage of buffaloes was 69.14. The proportion of crossbred cows (26.36 %) and indigenous cows (4.50 %) were found to be the highest in case of large farms.

Cost structure in dairy farming

To understand milk production from its economic perspectives, it is vital to examine the costs, explicit or implicit being used into its production. The cost structure in dairy farming on different categories of dairy units is presented in Table 3. The different costs endured on rearing the milch animals were categorized as fixed and variable costs. A perusal of table reveals that overall per farm total cost of rearing the milch animals was estimated to be Rs 438285.4 and total cost was found increased with the increase in herd size which ranged from Rs 257847.2 on small and Rs1013700.8 on large dairy units. Overall, the fixed and variable costs accounted for 14.53 percent and 85.4 percent respectively. Further, breakup of the variable costs revealed that a sizeable portion of variable costs i.e. 79.5 percent was accounted by the feed cost component as also found by Kumawat et al. 2014; Kashish et al. 2016. Amongst the feed and fodders, concentrates like oil cakes, cotton seeds, gram husk and wheat bran, etc. together constituted 48.5 percent while across the different categories 49.8, 46.5 and 48.9 percent of the total variable costs was incurred on small, medium and large dairy farms, respectively. Labour being the second highest component of cost accounted

Table 2: Socio-economic profile of sample households in Punjab, 2017-18

Particulars	Small	Medium	Large	Overall
Average age (years)	52.9	54.5	50.8	53.1
Average family size (No.)	5.33	5.77	7.00	5.76
Operational Land-holding (acres)	8.72	12.39	20.07	11.90
Land used for dairying (acres)	0.32	0.39	1.13	0.48
Total Herd size	3.44	6.28	17.35	6.80
Total Milch Animals	2.71	4.73	12.79	5.12
Buffalo	*2.10*	*3.35*	*8.00*	*3.54*
Crossbred cattle	*0.53*	*1.26*	*3.86*	*1.35*
Indigenous cattle	*0.08*	*0.12*	*0.93*	*0.23*

for 17.6, 17.1 and 18.0 percent of the total variable costs in the case of small, medium and large sized dairy units respectively. It is pertinent to mention here that the proportion of expenditure on hired labour was increasing with the increase in herd size and the share of family labour was higher on small sized dairy units in comparison to other categories. Overall, the contribution of veterinary charges, transportation charges, electricity charges and interest on working capital accounted for 0.8 percent 0.7 percent, 0.3 percent and 1.2 percent, respectively of the total variable costs. Amongst the fixed costs, interest on fixed capital was the major item of fixed cost and overall accounted for 50.7 percent of the total fixed costs followed by depreciation on milch animals (37%). The examination of Table 3 revealed that the highest proportion of fixed costs in total costs were found on small sized dairy farms (15.4%) and that of variable costs were observed on large dairy farms (87.1%).

Returns structure in dairy farming

The perusal of Table 4 shows that per farm gross returns on small, medium and large farms were to the tune of Rs 280416.1, Rs 487791.7 and Rs 1354528.6 respectively. Overall, the gross returns were found to be Rs 535782.9 out of which the returns from sale of milk, dung and young stock were estimated to be Rs 497086.5 (92.8%), Rs 23911.6 (4.5%) and Rs 14784.8 (2.8%) respectively.

The returns to fixed farm resources (RFFR) indicate the level of profitability resulting from the existing use of variable resources

Table 3: Cost structure in dairy farming on different size categories of dairy units in Punjab, 2017-18

Particulars	Small		Medium		Large		Overall	
	Rs/farm	%	Rs/farm	%	Rs/farm	%	Rs/farm	%
A. Variable Cost								
Feed and Fodder								
Green Fodder	26745.4	12.3	50012.0	14.6	113938.7	12.9	49565.9	13.2
Dry Fodder	37435.3	17.2	64498.0	18.9	154196.2	17.5	66663.8	17.8
Concentrates	108678.8	49.8	158775.0	46.5	431742.9	48.9	181496.3	48.5
Total Feed Costs	172859.5	79.2	273285.0	80.0	699877.8	79.3	297726.0	79.5
Human Labour								
Hired Labour	4200.0	1.9	12646.2	3.7	90428.6	10.2	22035.0	5.9
Family labour	34218.8	15.7	45625.0	13.4	68437.5	7.8	43914.1	11.7
Total labour	38418.8	17.6	58271.2	17.1	158866.1	18.0	65949.1	17.6
Veterinary & insemination charges	1572.5	0.7	2746.2	0.8	7692.9	0.9	3025.0	0.8
Electricity	2000.0	0.9	2500.0	0.7	4519.0	0.5	2603.3	0.7
Transportation charges	800.0	0.4	900.0	0.3	1500.0	0.2	955.0	0.3
Interest on working capital	2515.9	1.2	3939.9	1.2	10178.7	1.2	4319.7	1.2
Total Variable Cost	218166.7	100.0	341642.2	100.0	882634.3	100.0	374578.1	100.0
B. Fixed Cost								
Depreciation								
on milch animals	12636.0	31.8	23413.5	36.4	55252.8	42.2	23596.6	37.0
on dairy buildings	2466.7	6.2	3569.2	5.5	4628.6	3.5	3203.3	5.0
on equipments	3863.1	9.7	4241.5	6.6	7585.7	5.8	4637.5	7.3
Interest on fixed capital	*20714.8*	*52.2*	*33177.0*	*51.5*	*63599.4*	*48.5*	*32269.8*	*50.7*
Total fixed cost	39680.5	100.0	64401.3	100.0	131066.5	100.0	63707.3	100.0
Total Cost (A+B)	257847.2		406043.5		1013700.8		438285.4	

in dairy business. It was calculated by deducting the total variable costs from gross returns. The perusal of Table 4 shows that per farm RFFR were Rs 62249.4, Rs 146149.5 and Rs 471894.3 on small, medium and large size of dairy farms respectively while per farm net returns were estimated to be Rs 22568.9, Rs 81748.2 and Rs 340827.8 on above said farm categories respectively. This revealed that RFFR and net returns increased with increase in herd size. On an average, RFFR and net returns were Rs 161204.8 and Rs 97497.5 respectively. The gross returns per litre of milk produced were estimated highest on small farms (Rs 45.38) followed by medium (Rs 44.71) and large (Rs 41.75) dairy units. The cost per litre of milk production was estimated to be Rs 41.73, Rs 37.21 and Rs 31.24 on the respective farm categories. It revealed that cost per litre of producing milk decreased with increase in herd size indicating prevalence of economies of scale on large farms. This might be due to fact that large dairy farmers were rearing better milch animals and following better management practices as compared to small and medium dairy owners.

Overall, net returns per litre of milk produced came out to be Rs 7.92 while these were estimated to be Rs 3.65, Rs 7.50 and Rs 10.51 on small, medium and large size dairy farms, respectively. This revealed that net returns per litre were highest on large farms and lowest on small sized dairy farms.

Technical efficiency in milk production

The level of technical efficiency of a particular farm is characterized by the relationship between observed production and ideal or potential production. The measurement of the specific technical efficiency of the operation is based on the deviation of the observed production from the best production or the efficient production frontier. If the actual production point of a farm is at the frontier, it is completely efficient. If it is below the frontier, it is technically inefficient, with the ratio of actual production to potential determining the efficiency of each farm.

The results of the maximum likelihood estimates of the parameters in the stochastic production frontier for milk producers are presented in Table 5. The comprehensive likelihood ratio (LR) statistic for testing the null hypothesis for the absence of inefficiency effects in the Cobb-Douglas stochastic frontier production was 7.06. The calculated LR statistics were statistically significant, suggesting that the null hypothesis i.e. there were no technical inefficiency effects in the Cobb-Douglas stochastic production function was rejected. The estimated gamma parameter (γ) for production function was 0.982, indicating that about 98.2 percent of the variation in the output of milk among the farmers was due to differences in their technical efficiencies and remaining 1.8 percent variation is due to random errors. Saha and Jain (2004) reported a relatively lower gamma value (0.723) from their study on milk production efficiently in Haryana.

The concentrates proved to be a significant factor that positively influenced milk production in the MLE model, which means that there is potential to enhance the profitability through this input. One percent increase in the value of concentrates would raise the milk production by 0.90 percent. The estimates of dry fodder (-0.20) found significant and negative indicating excessive use of this input suggesting in its reduction in order to increase the

Table 4: Returns structure in dairy farming on different size categories of dairy units in Punjab, 2017-18

Particulars	Small	Medium	Large	Overall
Returns				
(i) Per farm				
Milk	*259830.5 (92.7)*	*438628.8 (89.9)*	*1283525.0 (94.8)*	*497086.5 (92.8)*
Dung	*12560.0 (4.5)*	*31036.5 (6.4)*	*43112.6 (3.2)*	*23911.6 (4.5)*
Young Stock	*8025.6 (2.9)*	*18126.4 (3.7)*	*27891.0 (2.1)*	*14784.8 (2.8)*
Gross Returns	280416.1	487791.7	1354528.6	535782.9
RFFR	62249.4	146149.5	471894.3	161204.8
Net Returns	22568.9	81748.2	340827.8	97497.5
(ii) Per litre of milk				
Gross returns	45.38	44.71	41.75	43.51
Total variable cost	35.31	31.31	27.20	30.42
Total fixed cost	6.42	5.90	4.04	5.17
Total cost	41.73	37.21	31.24	35.59
Net returns	3.65	7.50	10.51	7.92

Figures in parentheses indicate percentage to the gross returns

technical efficiency of milk production on the selected farms. Human labour was significant variable and the effect was was positive implying that there is scope devoting more labour hours for taking care of animals.

Distribution of dairy farms on the basis of technical efficiency level

To determine the technical efficiency of the dairy households the mean technical efficiency indices of milk production were established and are presented in Table 6. Considering the entire sample of 80 farmers, the mean technical efficiency was observed to be 77.30, ranged between as high as 98.22 to as low as 27.39. Farmers still had a room to increase the efficiency in their farming activities by 21.30 percent to fill the efficiency gap. They could substantially improve their income levels through better farming practices. It is pertinent to mention here that more than one fourth of the respondents were operating in technical efficiency range of more than 90 percent while 20 percent of the selected respondents were operating within the technical efficiency range of 60-70 percent.

Factors influencing technical efficiency in milk production

Given a particular technology to transform the physical inputs into output, some farmers are highly efficient while others are inefficient. The efficiency of the farmers is determined by various socio-economic and demographic factors. The perusal of Table 7 shows the results of tobit regression model performed to see the factors that affect the technical efficiency of dairy farms. The variable herd size was found to be significant but negative which clearly indicating that the larger herd size containing more number of unproductive animals will decrease the technical efficiency of dairy farms. The land holding variable has positive and significant effect ($p<0.05$) on technical efficiency which clearly indicating that farmers having more land are technically more efficient in comparison to the other categories of dairy farms. The age variable has a significant ($p<0.05$) influence on technical efficiency of dairy farmers in the study area. This may be attributed to the fact that more experienced farmers would have come across more problems and found their solutions through their skills and knowledge. Price received was found to be a significant and positive determinant of technical efficiency due to increased investment on improved breeds of milch animals and improved cash flow. The proportion of milk sold had significant and positive influence on milk production efficiently implying that farmers with high degree of intensity of market participation were more efficient in milk production.

Table 6: Distribution of dairy households according to technical efficiency level

Technical efficiency levels	Overall
<60	8 (10.0)
60-70	20 (25.0)
70-80	14 (17.5)
80-90	15 (18.7)
90-100	23 (28.8)
Number of observations	80
Minimum	27.39
Maximum	98.22
Mean Technical efficiency	77.30
Average Potential to increase in efficiency	21.30

Figures in the parentheses are the percentages to the total

Table 5: Maximum likelihood estimates of parameters for milk production frontier functions

Variables	Overall
Constant	3.51** (0.80)
Green Fodder	-0.14 (0.11)
Dry Fodder	-0.20** (0.08)
Concentrates	0.90** (0.08)
Veterinary expenses	0.08 (0.07)
Human Labor	0.32** (0.13)
Depreciation	-0.18** (0.06)
σ^2	0.13** (0.02)
γ	0.982** (0.02)
LR test of one-sided error	16.28**
Log likelihood function	7.06
Number of observations	80

*** indicates significant at 5% level*

Conclusions

It is concluded that feed and fodder cost become an important component of variable cost and accounted for about 80 percent of the total variable cost. Among all the components of dairy sector, sale of milk accounted for more than 90 percent of the gross returns from milk production. Further, the results brought out that the presence of technical inefficiencies significantly affected milk

Table 7: Factors influencing technical efficiency of milk production in selected farms, 2017-18

Variables	Overall
Intercept	-0.9076 (0.4251)
Herd size	-0.0080* (0.0028)
Land holding	0.0035** (0.0016)
Age	0.0245** (0.0090)
Square of age	-0.0002** (0.0001)
Price received	0.0223*** (0.0063)
Proportion of milk sold	0.0019** (0.0006)
Training	0.1406*** (0.0305)
Log Likelihood	65.92
Pseudo R^2	-0.608

*Significant at *1%, **5% and ***10% level of significance.*

production. The estimated gamma parameters for production function was 0.982 indicating that about 98.2 percent of the variation in the output of milk among the farmers was due to differences in their technical efficiencies and remaining 1.8 percent variation is due to random errors. The mean technical efficiency of the dairy farms was estimated to be 77.3 percent. The average potential for improvement in technical efficiency in milk production was 21.3 percent which implies that dairy farmers could enhance the milk production by 21.3 percent with existing level of technology and resources. The factors such as age of farmers, land holding, price of milk received by the farmers and training were found to be positive and significant. The results of technical efficiency indicated that dairy farmers can gain significantly higher profits by increasing their efficiency as there is a room to increase the efficiency to fill the gap. Strong and effective linkage of farms to the market could provide incentives towards increasing their efficiency in milk production and thus greater returns.

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RESEARCH ARTICLE

Economic impact of COVID-19 pandemic on milk unions and milk vendors

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Abstract: In this present study, an attempt was made to identify and compute the scale of the economic impact of the COVID-19 pandemic occurred to the two most important players of the milk supply chain in India, i.e., cooperative milk unions (3 milk unions, 18 dairy cooperative societies) and milk vendors (60 numbers). The study was conducted in Howrah, Nadia and N-24 Parganas districts of West Bengal state, India. The data collected from the respondents covered three periods of the first wave of the COVID-19 pandemic i.e. pre-lockdown (January 01 2020 to March 23 2020), lockdown (March 24 2020 to May 31 2020) and post-lockdown period (June 01 2020 to December 31 2020). The analysis revealed that the total marketing margin of cooperative milk unions from the sale of single-toned milk increased by 6 to 7 per cent and total marketing cost decreased by 8 to 11 per cent. During the lockdown period, there was a sudden hike in sales of single-toned milk (5 to 25%), curd (5 to 56%), paneer (11 to 26%) and ghee (40 to 80%) but the sale of lassi (-25 to -50%) and peda (-50 to -80%) decreased. The sale of milk was reduced by around 64 to 100 per cent during the lockdown period and net returns decreased substantially (70 to 680%) for all types of vendors during the pandemic. Vendors supplying milk to hotels and small processors were the most affected during the lockdown period.

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Introduction

The dairy sector provides huge employment opportunities both directly and indirectly. Approximately 20.5 million people across the country depend on cattle for their livelihood. It employs around 8.8 per cent of India's population (DAHD&F, 2019). The cooperative milk unions are the backbone of the Indian dairy sector and are the only organised structure of the milk supply chain run by the State Governments. The cooperative milk unions covered around 1,90,516 village dairy cooperative societies (DCS), with a cumulative membership of 16.93 million milk producers. The cooperative milk unions collectively procured an average of 507.69 lakh kg of milk per day (NDDB, 2019). Of the total milk produced in the country only 20 per cent of milk is sold to the organised sector, 34 per cent to the unorganised sector i.e. milk vendors, private dairies, halwai (sweet-maker) etc. and the remaining 46 per cent of milk is consumed locally (Mahida et al. 2022).

Milk vendors are individuals or businesses that sell milk to consumers. They play an important role in the dairy industry and ensure that people have access to a nutritious and essential food source (Das et al. 2024). One of the main benefits of milk vendors is that they provide a convenient and accessible source of milk for consumers. Many milk vendors sell milk door-to-door, which can be particularly beneficial for people who live in rural areas or who have difficulty in getting to a grocery store. Additionally, milk vendors can provide a source of fresh milk, which can be more nutritious than milk that has been sitting on a grocery store shelf. Many milk vendors source their milk from small, local dairy farmers, which can help to support the local economy and ensure that farmers are paid a fair price for their milk (The dairy site, 2021). However, as compared to the milk unions, the challenges associated with milk vendors are more. One challenge is that milk vendors may not have the same level of food safety and sanitation standards as larger dairy processors and retailers. This can increase the risk of foodborne illness and can be a concern for consumers. Additionally, milk vendors may not have the same level of regulation and oversight as larger

dairy processors and retailers, which can make it more difficult to ensure that they are operating safely and ethically (Kumar, 2021; Munshi et al. 2024).

Historically, global pandemics like smallpox, plague, Spanish flu and cholera not only affected the health of people across the world but also caused severe impacts on the global economy. The Coronavirus disease that surfaced in the year 2019 is a challenge to mankind globally where advancements in the technological and economic spheres have been sharp and unparalleled in the past. The first infection was reported in Wuhan city, the People's Republic of China and in no time spread across the world like wildfire. The World Health Organisation (WHO) declared the novel coronavirus as a pandemic and pronounced it as "COVID-19". To contain the virus, many countries declared a lockdown on all social and economic activities. India too observed a nationwide lockdown from March 24, 2020, to May 31, 2020, followed by conditional unlocking of social and economic activities in a phased manner from June 1, 2020, and with strict lockdown in the containment zones (GOI, 2020).

Due to lockdown restrictions businesses, all modes of transport and the movement of individuals were severely affected. The dairy sector, which deals with highly perishable products affected severely due to disruption in demand and supply chain caused by restricted business hours, and shutdown of bulk consumers such as hotels, the confectionary industry, marriages, parties, schools and inter-state transport (Chechi, 2020). The COVID-19 pandemic has not only reduced the income of dairy farmers but also made a significant impact on cooperative milk unions and milk vendors. Hence, the present study was undertaken to understand the constraints and economic impact of the COVID-19 pandemic on cooperative milk unions and milk vendors.

Materials and methods

Study area and data sources

The present study was conducted in Howrah (22.5604° N, 88.0510° E), Nadia (23.4710° N, 88.5565° E) and N-24 Parganas (22.6168° N, 88.4029° E) districts of West Bengal (22.9868° N, 87.8550° E), India (Fig. 1).

West Bengal has twelve cooperative milk unions and 1627 DCSs (WBCMPF, 2023). Three milk unions namely Howrah Cooperative Milk Union Ltd. (HOMUL), Howrah; Kishan Cooperative Milk Union Ltd. (KIMUL), Nadia; and Ichhamati Cooperative Milk Union Ltd. (IMUL), N-24 Parganas, are working actively in the study districts, were selected for the study. From each milk union, six DCSs were selected randomly.

A total sample of 60 milk vendors was selected randomly from the study districts. After the collection of data, milk vendors were classified into three categories based on their end customers as Vendor-A (Supplies milk to consumer households), Vendor-B (Supplies milk to hotels and small processors) and Vendor-C (Supplies milk to both). The data from cooperative milk unions and milk vendors were collected using a standard interview schedule. The study covers three periods of the first wave of the COVID-19 pandemic, i.e. pre-lockdown (January 01, 2020, to March 23, 2020), lockdown (March 24, 2020, to May 31, 2020) and post-lockdown periods (June 01, 2020, to December 31, 2020).

Analytical tools

Gap in number of active milk pourers and milk procurement quantity

The gap in the number of active milk pourers (%ΔMP) and milk procurement quantity (% ΔMPQ) was estimated using the following formula and compared across three periods.

$$Gap\ (\%) = \left[\frac{(MP/MPQ\ during\ period\ t) - (MP/MPQ\ during\ period\ (t-1))}{MP/MPQ\ during\ period\ (t-1)}\right] x\ 100$$

t = lockdown or post-lockdown period

t-1 = pre-lockdown period

Wilcoxon signed-rank test was used to test the significant gap in active milk pourers and milk procurement quantity during the lockdown and post-lockdown periods as compared to the pre-lockdown period. The level of statistical significance was fixed at 5 per cent ($P \leq 0.05$).

Estimation of cost of milk collection, reception, chilling and processing for milk unions, and cost and returns for milk vendors

The total cost of milk collection, reception, chilling and processing for milk unions, and cost and returns for milk vendors per litre of milk per day was estimated for pre-lockdown, lockdown and post-lockdown periods of the COVID-19 pandemic using standard methodology.

1. Fixed costs: These costs do not vary with the output level and remain unchanged in the short run. These costs include costs due to depreciation and interest on fixed capital. Annual depreciation of buildings, machinery and equipment used by cooperative milk unions and milk vendors was included for calculation using Capital Recovery Cost (CRC) method (Singh and Datta, 2016).

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

Where,
R = capital recovery cost
Z = initial value of the capital asset
r = interest rate
n = useful life of the assets

2. Variable costs

a. Cooperative milk union: Variable costs are those costs that vary with the output level and can be altered in the short run. Variable costs include the cost of raw materials, electricity charges, labour wages, water and steam charges, store and maintenance charges, refrigeration charges, cost of testing, weighing, packaging and quality control expenses.

$$Cost\ of\ milk\ collection\ (Rs./litre/day) = \frac{Total\ cost\ of\ milk\ collection}{Total\ quantity\ of\ milk\ collected}$$

$$Cost\ of\ milk\ reception\ (Rs./litre/day) = \frac{Total\ cost\ of\ reception}{Total\ quantity\ of\ milk\ received}$$

Procurement cost of milk (Rs./litre/day) = Cost of collection + Cost of reception

$$Chilling\ cost\ of\ milk\ (Rs./litre/day) = \frac{Total\ cost\ of\ chilling}{Total\ quantity\ of\ milk\ chilled}$$

Total cost of processing of single-toned milk= Total fixed cost + Total variable cost

b. Milk vendor: Variable costs include purchasing cost of milk, fuel cost, labour cost and miscellaneous cost.

$$Milk\ handling\ cost\ (Rs./litre/day) = \frac{Total\ cost}{Quantity\ of\ milk\ handled}$$

Milk marketing cost (Rs./litre/day) = Milk handling cost + Milk cost

Returns (Rs./litre) = Consumers' price - Milk marketing cost

Results and Discussion

Constraints faced by cooperative milk unions during the COVID-19 pandemic

The major constraint faced by the cooperative milk unions was shortage of labours in the processing units. Two out of three milk unions reported moderate constraints on this account. Some of the permanent workers were also not able to join their workplaces due to the unavailability of transportation during the lockdown period. Jitendra (2020) and Khairnar (2020) also reported such observations. The demand for single-toned milk, curd, paneer and ghee was not affected during the lockdown period, however, the demand for other dairy products such as lassi and peda decreased during the lockdown period. Haritha et al. (2022) and Thejesh et al. (2022) also reported a similar observation. The majority of milk unions reported that inventory costs increased during the lockdown period due to lower demand for some dairy products, which caused wastage of such products and ultimately

Fig. 1 Physical map of West Bengal showing Nadia (red border), N-24 Parganas (blue border) and Howrah (green border) districts

caused revenue loss to milk unions. These findings corroborate with CRISIL (2020), DPAC (2020), Mukherjee (2020), Munshi et al. (2023) and Raghu and Deb (2020).

Cost of milk collection, reception, chilling and processing for milk unions during the COVID-19 pandemic

a. Cost of milk collection by DCSs

During the pre-lockdown period, DCSs of KIMUL procured 91.11 litres of milk daily, which increased to 97.41 and 103.33 litres per day, respectively, during the lockdown and post-lockdown periods. DCSs of IMUL (Pre-lockdown: 104.03 litres per day, Lockdown: 114.64 litres per day and Post-lockdown: 104.69 litres per day) and HOMUL (Pre-lockdown: 80.97 litres per day, Lockdown: 93.59 litres per day and Post-lockdown: 95.75 litres per day) also showed a similar trend of results (Supplementary Table 1). During the lockdown and post-lockdown periods, gap analysis revealed that DCSs were able to collect more quantity of milk due to the increasing number of milk pourers (Table 1) with their existing labour, machinery and other fixed resources, which significantly reduced per-litre milk collection costs. It was found

to reduce by around 8 to 14 per cent and 0.27 to 14 per cent during the lockdown and post-lockdown periods, respectively, across different DCSs. Srivatsa (2020) and Das et al. (2021) also reported such observations.

b. Cost of receiving liquid milk by milk unions

Milk received at the processing plants of milk unions was tested and chilled before processing. During the lockdown period, DCSs received around 7 to 16 per cent more milk (Table 1), therefore the per-litre cost of receiving liquid milk by KIMUL, IMUL and HOMUL decreased significantly during the lockdown period by 13, 18 and 7 per cent, respectively, as compared to pre-lockdown period. During the post-lockdown period, the decreasing trend was found in HOMUL, but in KIMUL and IMUL it again increased to the pre-lockdown level.

c. Total procurement cost of liquid milk by milk unions

The total procurement cost of milk is the sum of the cost of collection of milk by DCSs and the cost of receiving milk by milk unions (Table 2). The milk procurement cost decreased during the lockdown and post-lockdown periods as compared to the pre-lockdown period for all the milk unions. The highest decline was found in HOMUL, where the total milk procurement costs decreased by 13.6 and 14.3 per cent during the lockdown and post-lockdown periods, respectively.

d. Cost of chilling liquid milk

After transportation from dairy cooperative collection centres to the milk processing plant, the liquid milk undergoes a chilling process before further processing activities. The chilling cost incurred by processing plants of milk unions was estimated and presented in Supplementary Table 3. The average quantity of milk chilled during the lockdown period increased and that caused low milk chilling costs. In HOMUL per litre milk chilling cost decreased from Rs.1.49 in the pre-lockdown to Rs.1.40 and Rs.1.37 in the lockdown and post-lockdown periods respectively. In the case of KIMUL and IMUL, milk chilling costs decreased during the lockdown period but than again increased and reached pre-lockdown levels. Mohapatra (2022) estimated the chilling cost in the case of milk union as Rs. 0.53 per litre during normal period before the COVID-19 pandemic.

e. Processing cost of single-toned milk

The total cost incurred by the processing plants of milk unions for the conversion of liquid milk into single-toned milk along with its manufacturing and packaging forms the total processing cost of single-toned milk. The processing cost incurred by the dairy plants of milk unions is presented in Supplementary Table 4. The milk processing cost per litre for IMUL and HOMUL decreased during the lockdown period (IMUL: Rs. 2.08 per litre, HOMUL: Rs. 1.52 per litre) and post-lockdown period (IMUL:

Table 1: Gap analysis of the average number of active milk pourers (%ΔMP) and milk procurement quantity (%ΔMPQ) in selected DCSs under different milk unions during the COVID-19 pandemic

DCS	ΔMP (%)		ΔMPQ (%)	
	LD vs PRLD	POLD vs PRLD	LD vs PRLD	POLD vs PRLD
KIMUL	-0.98	5.28	6.91	11.83*
IMUL	14.47*	8.87**	10.20*	0.63
HOMUL	11.18**	10.93**	15.59	15.43**

Statistically significant at *P<0.05; **P<0.01; ***P<0.001
PRLD- Pre-lockdown, LD- Lockdown, POLD- Post-lockdown

Table 2: Total procurement cost of liquid milk for different milk unions during the COVID-19 pandemic (Rs./litre)

Particulars	KIMUL			IMUL			HOMUL		
	PRLD	LD	POLD	PRLD	LD	POLD	PRLD	LD	POLD
A. Cost of collection of liquid milk	8.19	7.52	7.24	7.37	6.67	7.35	9.18	7.87	7.82
B. Cost of receiving liquid milk	0.30	0.26	0.42	0.51	0.42	0.51	0.70	0.65	0.64
Total procurement cost of liquid milk (A+B)	8.49	7.78	7.66	7.88	7.09	7.87	9.87	8.53	8.46

PRLD- Pre-lockdown, LD- Lockdown, POLD- Post-lockdown

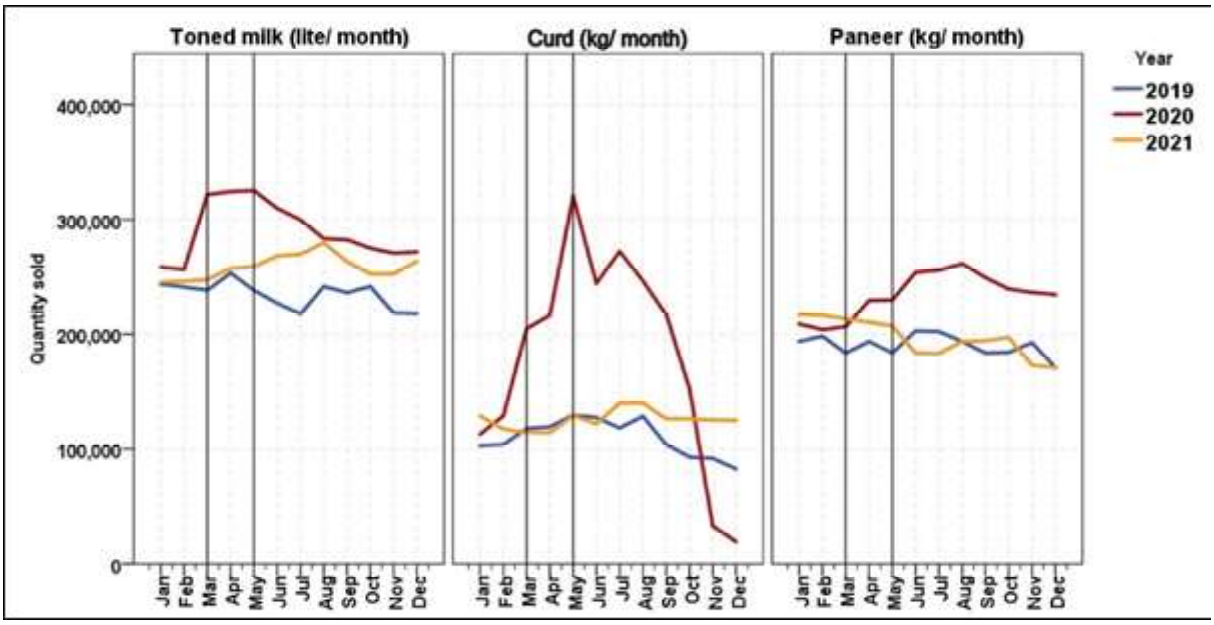

Fig. 2 Trends in the monthly sale of milk products by KIMUL (2019 – 2021)

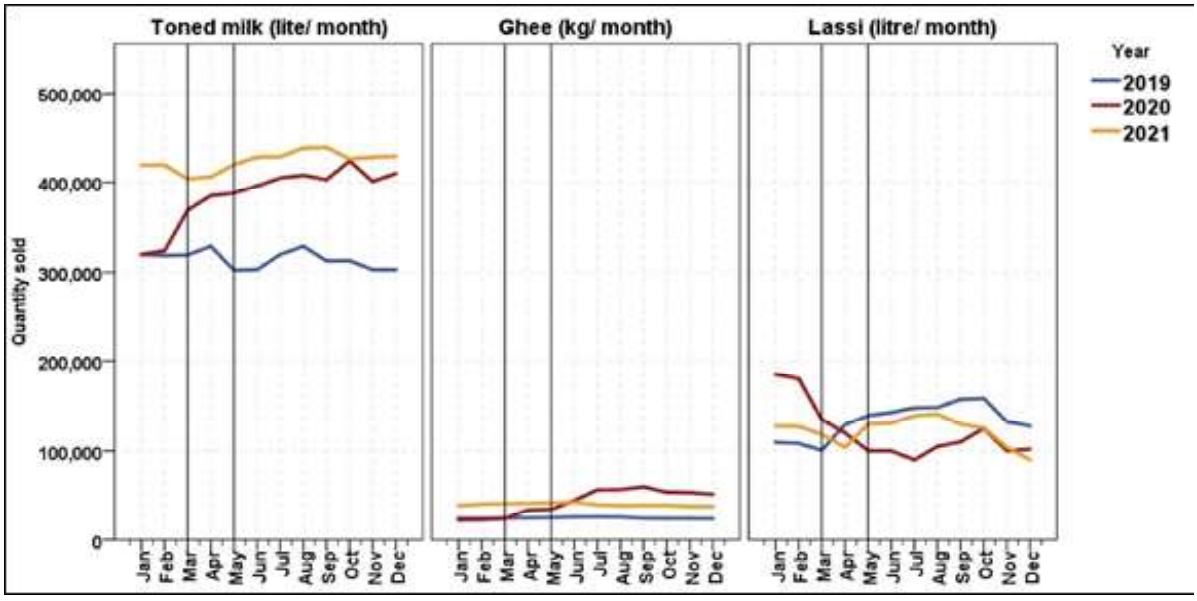

Fig. 3 Trends in the monthly sale of milk products by IMUL (2019 – 2021)

Rs. 2.69 per litre, HOMUL: Rs. 1.48 per litre) periods as compared to the pre-lockdown period (IMUL: Rs. 2.73 per litre, HOMUL: Rs. 1.56 per litre). In the case of KIMUL, milk processing cost declined during the lockdown period (Rs. 1.30 per litre) but during the post-lockdown period (Rs. 2.06 per litre) it increased more than the pre-lockdown level (Rs. 1.38 per litre) because in KIMUL during the post-lockdown period, amount of milk processed to make single-toned milk declined as compared to the pre-lockdown level. Mohapatra et al. (2022) found the processing cost of full cream milk by milk unions as Rs. 2.98 per litre during a normal period before the COVID-19 pandemic.

f. Marketing margin and marketing cost

Table 3 revealed that during the lockdown period marketing margin surged by around 6 to 7 per cent, whereas total marketing cost decreased by around 8 to 11 per cent across the milk unions. During the lockdown period, milk procurement from dairy farmers increased and unions were able to reduce the cost of milk processing and marketing. Along with that by keeping the selling price same (Rs. 48 to Rs. 49 per litre) they were able to generate more marketing margin.

The concept of "economies of scale" stated that if a farm can produce more with its existing infrastructure, its efficiency increases. In the case of milk unions also this theory appears to

be applicable. Milk unions were getting an excess quantity of milk from dairy farmers with their existing facilities leading to a reduction in the cost of total milk procurement, milk chilling/ processing cost, and thus a decrease in total marketing cost and an increase in marketing margin.

Trends in monthly sale of milk and milk products by milk unions during the COVID-19 pandemic

Figure 2, 3 and 4 present the monthly trends in the quantity of milk and milk products sold by KIMUL, IMUL and HOMUL, respectively, for the period 2019 to 2021. From the figures, it can be seen that there was an unusual pattern in the number of products sold by milk unions during 2020 as compared to the years 2019 and 2021.

During the lockdown period, the sales of toned milk increased by around 5 to 25 per cent as compared to the pre-lockdown period in all the milk unions. Along with toned milk, sales of curd (5 to 56%), paneer (5 to 56%) and ghee (40 to 80%) were also found to increase. Due to lockdown restrictions, local milk vendors or other private players and hotels were not able to operate in the study region, therefore consumers shifted their consumption preferences from local unpackaged milk products to packaged milk products to boost their immunity against Corona virus. Due to changes in consumers' consumption preferences, the sales of mentioned milk products increased suddenly during the lockdown and post-lockdown periods. However, Figure 3 and 4 revealed that during the lockdown period, sales of lassi (25 to 50%) and sweets like peda (50 to 80%) decreased substantially for IMUL and HOMUL, respectively. During the lockdown period, consumers mainly preferred to consume dairy products that are more beneficial to their health. Therefore, by analysing the consumers' preferences, milk unions decided to produce a very less quantity of products like lassi and peda, and ultimately sales of these products were found to be decreased in the year 2020.

Constraints faced by milk vendors during the COVID-19 pandemic

During the lockdown period, milk vendors reported that they faced constraints concerning milk procurement quantity and

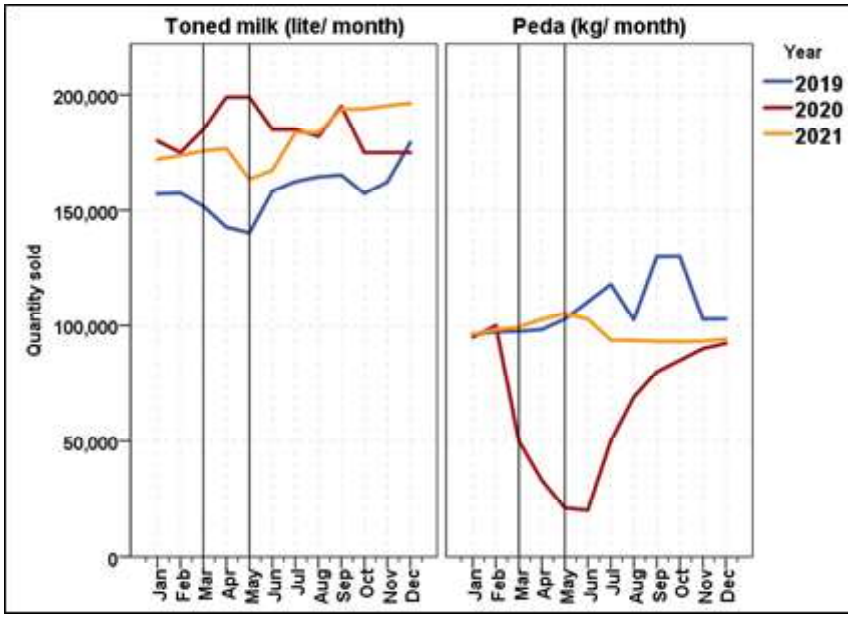

Fig. 4 Trends in the monthly sale of milk products by HOMUL (2019 – 2021)

Table 3: Marketing margin and marketing cost of single-toned milk (Rs./ litre)

Particulars	KIMUL			IMUL			HOMUL		
	PRLD	LD	POLD	PRLD	LD	POLD	PRLD	LD	POLD
Total cost (TC) (IC+PC+CC+PrC)	40.06	39.58	40.90	40.19	39.44	41.77	42.08	41.08	41.96
Selling price (SP)	48.00	48.00	48.00	48.00	48.00	48.00	49.00	49.00	49.00
Marketing margin (TC-SP)	7.94	8.42	7.10	7.81	8.56	6.23	6.92	7.92	7.04
Total marketing cost (PC+CC+PrC)	10.48	9.63	10.64	11.82	10.15	11.77	12.92	11.44	11.31

IC = Input cost, PC = Procurement cost, CC = Chilling cost, PrC = Processing cost

revenue due to the lockdown restrictions, they faced difficulties in milk procurement causing a reduction in total revenue. All the vendors of Vendor-A and Vendor-B types and 95 per cent of Vendor-C reported that the demand for milk in the market reduced drastically (Supplementary Fig. 1).

During the post-lockdown periods, the majority of restrictions were removed and it was seen that the number of vendors who faced constraints also decreased. In terms of milk procurement quantity and milk demand, severe constraints were faced by Vendor-A, because the consumption preferences of consumers shifted to packaged milk, and due to that their procurement quantity was reduced as compared to other vendors. In terms of revenue, a severe constraint was faced by Vendor-B, because during the post-lockdown period, hotels and small processors were not working at full strength.

Costs and returns incurred by milk vendors during the COVID-19 pandemic

The costs and returns incurred by milk vendors during the pre-lockdown, lockdown, and post-lockdown periods of the COVID-19 pandemic are presented in Table 4. The details of cost and returns analysis are presented in Supplementary Tables 5, 6 and 7.

During the pre-lockdown and post-lockdown periods, the share of TVC (76 to 86%) to total cost was found to be higher for all the types of milk vendors. In contrast, during the lockdown period, the TVC for Vendor-B was found to decrease drastically and its share was reduced from 11 to 14 per cent. For the other two vendors i.e., Vendor-A and C the share of TVC was also found to be decreased to around 62 to 80 per cent. Among the different components of TVC, the share of transportation and labour costs was found to be higher. During the lockdown period, the cost of transportation and labour was reduced, so the total cost was also reduced significantly. The TFC remained constant for each milk vendor throughout the pre-lockdown, lockdown and post-lockdown periods.

The total cost incurred by all the categories of milk vendors decreased during the lockdown period due to reduced costs of transportation, labour and vehicle maintenance cost. In contrast, miscellaneous costs increased during the lockdown and post-

Table 4: Costs and returns incurred by milk vendors during the pre-lockdown, lockdown and post-lockdown periods of the COVID-19 pandemic

Districts	Particulars	Vendor-A			Vendor-B			Vendor-C		
		PRLD	LD	POLD	PRLD	LD	POLD	PRLD	LD	POLD
Howrah	Total variable cost (TVC)	194	156	184	234	9	240.35	206	144	218
	Total fixed cost (TFC)	37	37	37	50	50	50	43	43	43
	Total cost (TC=TVC+TFC)	231	194	222	285	59	290	249	188	261
	Net returns (Rs./ litre)	9.74	-0.76	10.28	12.45	-59.05	15.82	11.52	1.06	14.46
			(-107.8)	(5.5)		(-574.3)	(27.1)		(-90.7)	(25.50)
Nadia	Total variable cost (TVC)	185	93	174	222	8	239	219	164	228
	Total fixed cost (TFC)	57	57	57	62	62	62	33	33	33
	Total cost (TC=TVC+TFC)	243	151	232	284	70	301	252	197	261
	Net returns (Rs./ litre)	10.06	2.67	11.63	12.06	-70.25	13.38	12.19	3.61	13.59
			(-73.4)	(15.6)		(-682.6)	(10.9)		(-70.3)	(11.4)
N-24 Parganas	Total variable cost (TVC)	188	132	183	241	6	252	236	133	231
	Total fixed cost (TFC)	38	38	38	43	43	43	50	50	50
	Total cost (TC=TVC+TFC)	227	170	222	284	49	295	287	184	282
	Net returns (Rs./ litre)	9.41	0.03	11.32	10.28	-49.26	15.08	13.76	2.32	15.34
			(-99.6)	(20.4)		(-579.2)	(46.7)		(-83.1)	(11.4)

Figures within parentheses indicate percentage differences in net return during the lockdown and post-lockdown periods as compared to the pre-lockdown period. PRLD- Pre-lockdown, LD- Lockdown, POLD- Post-lockdown

lockdown periods as vendors purchased masks and sanitizers, and took other Corona virus preventive measures such as hygiene, and sanitization of vehicles and equipment. During the lockdown and post-lockdown periods, vendors offered a low price to dairy farmers and charged a higher price to customers to balance the marketing costs of milk. However, they failed to reduce the total marketing costs due to higher milk handling costs (per litre). Milk handling cost was found to be increased because the total milk handled per day decreased severely during the lockdown period. Due to the above-mentioned reasons, vendors faced severe economic losses during the lockdown period. Net returns decreased from Rs. 9.74, Rs. 12.45 and Rs. 11.52 to Rs. -0.76, Rs. -59.05, and Rs. 1.06 per litre during the lockdown period for Vendor-A, Vendor-B and Vendor-C, respectively, in Howrah district. Govindaraj et al. (2022) reported similar findings in their study. Mohapatra et al. (2022) estimated the returns of the two categories of milk vendors as Rs. 12.22 and Rs. 9.45 per litre during a normal period before the COVID-19 pandemic.

Vendor-B faced the highest economic loss as all the hotels and small processors remained closed, followed by Vendor-A and Vendor-C. However, due to higher demand and reduction in COVID-19 restrictions during the post-lockdown period, their net returns increased as compared to the pre-lockdown period. Nadia and N-24 Parganas districts also showed similar trend as that of the Howrah district.

Vendor-B of Nadia district suffered the highest economic loss during the lockdown period, as their net return (per litre of milk) decreased by 682.5 per cent, followed by Vendor-B of N-24 Parganas (-579.2%) and Howrah (-574.3%) districts. However, the net returns of all the categories of milk vendors improved during the post-lockdown period than the pre-lockdown period due to increased selling prices of milk.

Conclusion

During the COVID-19 pandemic, the total marketing margin of milk unions from the sale of milk increased by 6 to 7 per cent and total marketing cost decreased by 8 to 11 per cent due to reduction in the cost of milk collection, reception, chilling and processing. During the lockdown period, there was a sudden hike in sales of toned milk (5 to 25%), curd (5 to 56%), paneer (11 to 26%) and ghee (40 to 80%) but the sale of lassi (-25 to -50%) and peda (-50 to -80%) decreased. Though cooperative milk unions were in a slightly gainful position during the pandemic, on the other hand, milk vendors suffered the worst. Due to strict lockdown restrictions, milk vendors were unable to operate at full strength and their sale of milk was reduced by around 64 to 100 per cent during the lockdown period and which ultimately caused the reduction of net returns by around 70 to 680 per cent Vendors supplying milk to hotels and small processors (Vendor B) were the most affected during the lockdown period. Milk vendors should diversify their customer base and scale operations to minimize loss during a pandemic like COVID-19. The government may also devise policies for private vendors to produce, process and supply dairy products efficiently.

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RESEARCH ARTICLE

Efficiency of resources use in cattle milk production in the lower Brahmaputra valley zone of Assam, India

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Abstract: From the perspective of profit-making in dairy farming, it is essential to understand how to make the most of limited resources for milk production. It is essential to know if the resources owned and utilized by the farmers were utilised effectively or not. The study was undertaken to analyze the resource use efficiency in the cattle milk production in the Lower Brahmaputra Valley Zone of Assam. Multi stage random proportionate sampling technique was undertaken from a cluster of villages and a total of 172 dairy farmers were randomly selected from the two districts, namely Kamrup and Barpeta for the study. Data was collected through the interview method using a pre tested semi structured interview schedule. To estimate the productivity of resources in milk production, Cobb Douglas Production Function was used. The co-efficient of multiple determinations was 0.825 and 0.796 for the local and crossbred cattle, indicating that the five variables selected for the analysis had explained 82.5 per cent and 79.6 per cent variation in local and crossbred cattle milk production respectively. The results of the Cobb-Douglas Production Function revealed that variables like concentrate, green fodder and labour had a positive and highly significant influence on milk production of local cattle whereas for crossbred cattle green fodder, concentrate, dry fodder and veterinary charges had a significant influence. The result of allocative efficiency of resources in milk production revealed that concentrates, labour and veterinary charges were under-utilized, whereas green fodder and dry fodder were over-utilized.

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Keywords: Cobb-Douglas Production Function, Concentrate, Green Fodder, Labour, Milk Production, Resource

Introduction

The efficiency of resource use in cattle milk production refers to how effectively resources such as feed, water, land, and energy are utilized to produce milk. Efficient resource use is important for sustainable and profitable milk production while minimizing environmental impacts. India is the largest producer of milk in the world accounting for an annual milk output of 209.96 Mt in 2020-21 (NDDB, 2020). India accounts for 21 per cent of the global milk production. The organized sector comprises of only 34 per cent of the total milk production in the country. During the last few decades, India's milk producers have transformed Indian dairying from stagnation to world leadership. In India, dairying is recognized as an instrument for social and economic development. Dairy sector plays a significant role in supplementing family income and generating gainful employment in rural areas besides providing cheap nutritional food to millions of people. The growth of the dairy sector during the last three decades has been impressive. The nation's milk supply comes from millions of small producers. India ranks first in the world in milk production. The per capita availability of milk is 444 grams in 2020-2021. Milk emerged as one of the biggest contributors to the value of agricultural output in the country. Increase in milk production with limited resources like quality and quantity of feed, labour, genetic potential of the animal and to ensure the optimal use of various inputs used by the milk producers is matter of primary concern. It is important to know whether the inputs owned by milk producers are used efficiently or not. Resource use efficiency comprised the distribution of a given amount of scare factor among the set of alternatives in the production so as to maximize the profit (Ganeshkumar et al. 2000). Education of farmers plays a significant role in both technical and allocative efficiency (Kumbhakar et al. 1991). An empirical assessment of determinants of milk production and resource use efficiency is important for planning, dairy development policies in a particular region. This study estimates the various factors of milk production and their levels of allocative efficiency in milk production for local and crossbred cows in the region. Efforts to improve resource efficiency in cattle milk production are ongoing and involve advancements in man-

agement practices, technological innovations, and sustainable farming systems. By optimizing resource use, dairy farmers can enhance productivity, reduce costs, and promote environmental sustainability in the dairy industry in the country.

Materials and Methods

For the present study, Lower Brahmaputra Valley Zone of Assam was selected purposively owing to highest milk production from the region in the state. In the Lower Brahmaputra Valley Zone of Assam, the dairy farmers were selected randomly from the two districts of Kamrup and Barpeta for the present study. From the selected districts, a total of 172 dairy farmers were selected randomly using the multi stage random proportionate sampling technique from a cluster of villages. The field survey for this study was conducted during the months of November and December 2022 and the data was collected from the sample units related to the year 2022-23.

Information relating to various aspects of dairy farming was collected from selected farmers by survey method with a well-designed and pre-tested standardized interview schedule. Details of inputs used like green fodder, dry fodder, concentrates with their quantities and price, labour employed with wage particularly veterinary and breeding expenses, miscellaneous expenses and data on outputs were also collected from the dairy farmers. The data collected were analyzed with a view to achieve the sole objective of the study.

Analytical Framework

The log- log regression analysis was used to study the relationship between milk and different factors influencing it. The Cobb-Douglas production was used to obtain the parameters for the measurement of productivity of resources in milk production. Various studies are available on the use of Cobb-Douglas production function for the measurement of productivity of resources in milk production (Kumar and Shukla, 2017; Meena et al. 2012 ; Venkatesh et al. 2011).

The Cobb-Douglas production function for milk production was specified and defined as follows:

$$\ln Y = ?0 + b1\ln X1 + b2\ln X2 + b3\ln X3 + b4\ln X4 + b5\ln X5 + \mu$$

Where,

Y = Milk produced per annum (lit)

?0 = constant

X1 = Concentrates fed per annum (Kg)

X2 = Green fodder fed per annum (Kg)

X3 = Dry fodder fed per annum (Kg)

X4 = Labour employed per annum (man-days)

X5 = Veterinary services per year (?)

μ = error term

Determining the Efficiency of Resource Use of Cattle Milk

The following ratio was used to estimate the relative efficiency of resource use (r):

$$r = MVP/(MFC)$$

Where:

MFC = Marginal Factor Cost (cost of one unit of a particular resource)

MVP = Marginal value product, calculated by multiplying the MPP by the price of output

$$MVP = biY/X\ Py$$

Where,

Y = geometric mean of total output

X = geometric mean of particular input

bi = regression coefficient of that input

Py= price per unit of output

Decision rule

If r = 1, resource is efficiently utilized,

If r > 1, resource is under-utilized

If r < 1, resource is over utilized.

Economic optimum takes place where MVP = MFC ,i.e, production is said to be efficient when MVP =MFC (Rajendran and Prabaharan, 1989). If r is not equal to 1, it suggests that resources are inefficiently utilized. Adjustments could be therefore made in the quantity of inputs used and costs in the production process to restore r = 1. Any deviation of MVP of ith inputs from its unit price are termed as resource use inefficiency.

Results and Discussion

In a production process, the objective is to co-ordinate and utilize resources in such a manner that they together yield the highest net returns. This is optimum use of resources in production. To study the resource use efficiency of factors in milk production, a log- log production function was fitted to the data. The independent variables used were concentrate feed (X1), green fodder(X2),

dry fodder(X3), labour(X4), and veterinary charges(X5). The result of the regression analysis in respect of the production function is presented in Table 1 and Table 2.

From the Table 1, it could be observed that for local cattle the co-efficient of multiple determination was 0.825, which indicated that the five variables selected for the analysis have explained 82.5 per cent variation in total milk production. Among the five variables, the green fodder and labour variables were significant at one percent level and concentrate fed was significant at five percent level. The above analysis clearly indicated the importance of concentrates, green fodder and labour input for higher milk production and profits. Similar findings were reported by Singh et al. (2010). The variable veterinary charges and dry fodder were not having any significant influence on milk production in the study area. The marginal productivity of concentrates, green fodder and labour was 0.259, 0.346 and 0.258 respectively explaining that one percent increase in these variables would increase the milk production by 0.259, 0.346 and 0.258 per cent respectively ceteris paribus.

From the Table 2, it could be observed that the co- efficient of multiple determinations for the crossbred milch cattle was 0.796, which indicated that the five variables selected for the analysis have explained 79.6 per cent variation in total milk production. Among the five variables, the concentrate feed and green fodder were significant at one per cent and five per cent level whereas dry fodder and veterinary charges were significant at ten per cent level. The above analysis clearly indicated the importance of concentrates, green fodder, veterinary charges and dry fodder for higher milk production and profits. Similar findings were reported by Singh et al. (2010). The labour input was not having any significant influence on milk production of crossbred milch cattle in the study area. The marginal productivity of concentrates, green fodder, dry fodder and veterinary charges was 0.461, 0.257, 0.017 and 0.173 respectively explaining that one per cent increase in these variables would increase the milk production by 0.461, 0.257, 0.017 and 0.173 per cent respectively ceteris paribus. Previous studies by Sharma and Singh (1993) and Ahuja et al. (1999) observed the strong relationship between the concentrates and milk production.

Table 3 revealed that for the local milch cattle, the difference between marginal value product (MVP) and marginal factor cost (MFC) for green fodder and concentrate were positive indicating that both the inputs were under-utilized in the study area. The study's findings revealed that the milk productivity of milch animals could be increased by the increase in the quantity of green

Table 1: Regression co-efficients for milk production of local milch cattle in the study area

Variables	Regression co-efficient	t value	p-value
Constant	0.148	0.729	0.147
Concentrate feed(X_1)	0.259**	5.897	0.010
Green fodder(X_2)	0.346***	1.871	0.000
Dry fodder(X_3)	0.029	1.602	0.472
Labour(X_4)	0.258***	3.378	0.004
Veterinary charges(X_5)	0.048	1.392	0.124
N	172		
R^2	0.825		
Adjusted R^2	0.818		

Note: *** and ** indicates ($p<0.01$) and ($p<0.05$)

Table 2: Regression co-efficients for milk production of crossbred milch cattle in the study area

Variables	Regression co-efficient	t value	p-value
Constant	0.326	0.635	0.354
Concentrate feed(X_1)	0.461***	4.317	0.001
Green fodder(X_2)	0.257**	1.940	0.040
Dry fodder(X_3)	0.017*	1.613	0.078
Labour(X_4)	0.328	2.279	0.273
Veterinary charges(X_5)	0.173*	1.348	0.015
N	172		
R^2	0.796		
Adjusted R^2	0.781		

Table 3: Resource use efficiency for local and crossbred milch cattle

Particulars	MVP	MFC	Difference	SE of MVP	MVP/MFC	t-value
			Local cows			
Green fodder	3.84	2.00	1.84	2.15	1.92	4.57
Concentrate	12.41	10.00	2.41	23.57	1.24	1.96
Labour	0.84	1.00	-0.16	1.43	0.84	0.76
			Crossbred cows			
Green fodder	3.42	2.00	1.42	2.12	1.71	1.96
Dry fodder	3.16	4.00	-0.84	3.76	0.79	1.58
Concentrate	7.03	10.00	-2.97	0.86	0.70	4.07
Veterinary charges	9.39	8.00	1.39	28.72	1.17	2.46

fodder and concentrate. The results also revealed that the difference between marginal value product (MVP) and marginal factor cost (MFC) for labour was found to be negative indicating that the labour were over utilised in the region. It shows that there is no possibility of augmenting local cow milk production by using more of this input. Thus, there is a potential to increase the milk production by judicious feeding of green fodder and concentrate in the study area. The results corresponds with the findings of Lalrinsangpuii and Malhotra (2016) who revealed positive marginal value productivity of green fodder and concentrate for local cow milk production in Mizoram and the findings of Kumar and Shukla (2017) in western Uttar Pradesh.

Also from the Table 3 it could be observed that unlike the local cattle, in case of the crossbred cattle the difference between marginal value productivity of inputs and their marginal factor cost was found as positive and statistically significant for the inputs, namely, green fodder and the veterinary charges indicating that green fodder and veterinary expenses were under-utilised in the study area. Thus, there is a potential to increase the milk production by judicious feeding of green fodder. Also, increasing veterinary expenses would help to boost milk production of crossbred cows in the study area. The findings of Singh et al. (2007) in Imphal West district of Manipur and Mahajan and Chauhan (2011) in Ludhiana district of Punjab reported that concentrates were being efficiently utilised for crossbred cows. On the contrary, the results were in contrast with the findings of Kaur and Toor (2022) in rural Punjab who reported that green fodder, dry fodder, concentrates and human labour were over-utilised with negative and statistically significant difference between marginal value productivity of inputs and their marginal factor cost.

Conclusions

This paper is an attempt to estimate the resource use efficiency of cattle milk production in Lower Brahmaputra Valley Zone of Assam. The study of productivity of resources in milk production revealed that the variables like green fodder and concentrate the difference between marginal value productivity of inputs and their marginal factor cost was positive in case of local milch cattle indicating that both inputs were under-utilized in the study area while the labour variable was insignificant. Moreover, in case of the crossbred milch cattle, the difference between marginal value productivity of inputs and their marginal factor cost for the variables like green fodder and veterinary charges were positive indicating that it was under- utilized and variables like dry fodder and concentrates were over utilized. Since feed and fodder resources encompass a major chunk of the cost of milk production, therefore good care should be taken to use these resources optimally. Further, now a days, labour is a very important resource in milk production, therefore this resource should be used optimally in the milk production system. The study concludes that the technology augmentation in the form of strategic mix of additional inputs, viz. green fodder and concentrates in feed ration optimally.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the content of the paper.

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SHORT COMMUNICATION

Exploring the compatibility between *Kluyveromyces lactis* and probiotic *Lactobacillus* spp. and their In-vitro antimicrobial potential

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Abstract: This study investigated symbiotic interactions between dairy yeast and probiotic *Lactobacillus* spp., where stable coexistence occurs through the shared utilization of common metabolites and environmental conditions. The primary objective was to evaluate the compatibility between the potential probiotic yeast *Kluyveromyces lactis* (*K lactis*) and probiotic *Lactobacilli* strains, namely *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* subsp. *plantarum*, and *Lactobacillus helveticus*. Additionally, the antibacterial activity was explored. The spot assay on MRS agar confirmed equal growth intensity and the absence of incompatibility between *K. lactis* and probiotic *Lactobacilli* spp. Their cultures mutually promoted each other's growth. Most probiotic *Lactobacillus* strains exhibited significant antibacterial activity against tested pathogens, while *K. lactis* showed no antibacterial activity. Among six strains of *Lactobacillus*, *L rhamnosus* displayed the maximum zone of inhibition (cm) with 3.063 ± 0.071, 2.754 ± 0.133, 2.818 ± 0.125 against *B cereus*, *S aureus* and *E coli*, respectively. The minimum activity was observed in *L acidophilus* and *L bulgaricus* with no zone of inhibition against *S aureus* by both bacteria. The findings of this study on the symbiotic interactions and compatibility of dairy yeast and probiotic *Lactobacillus* strains, as well as their enhanced growth and significant antibacterial activity, provide important insights for the development of innovative probiotic products with potential health benefits.

Keywords: Compatibility, Antimicrobial, Yeast, *Lactobacillus*, Probiotic

Introduction

Probiotic-based products represent the future of the food market. It is evidenced from the fact that the worldwide probiotics market, which reached a valuation of USD 77.12 billion in 2022, is projected to experience a compound annual growth rate of 14.0% from 2023 to 2030 (Grand View Research, 2023). The *Bifidobacteria* and *Lactobacillus* spp. are the most commonly employed for the development of probiotic based food products. Nonetheless, due to numerous research' findings about the possible health advantages of yeasts, the market for probiotic formulations based on yeast has grown (Czerucka et al., 2007). Although, *Sacchromyces* is the only proven yeast as a probiotic, the recent studies report evidence that yeast spp. like *Kluyveromyces marxianus* and *Pichia kudriavzevii* may have probiotic properties (Staniszewski & Kordowska-Wiater, 2021). This is primarily attributed to their enhanced resistance to diverse environmental challenges, reduced likelihood of acquiring and transmitting antibiotic resistance, and distinct immune signaling to the host in comparison to probiotics based on lactic acid bacteria (Oliveira et al., 2017).

The widely recognized advantages of probiotics are frequently ascribed to the interaction between the probiotic that is delivered and the microbiota of the gastrointestinal tract (GI). The diversity and makeup of gut microbes have a significant impact on host health because they can affect the synthesis of enzymes, the generation of metabolites like vitamins and short-chain fatty acids,

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the control of cell-to-cell interactions, the immune system and neuroendocrine responses, and the digestion and absorption of nutrients (Washburn et al., 2022). However, just as no single food can fully meet the needs of the human body, likewise, no individual probiotic is sufficient to deliver comprehensive health benefits to consumers. According to Washburn et al. (2022), microbial gastrointestinal diversity is not substantially impacted by the administration of a single species probiotic in healthy people. Consequently, the emerging trend in the market is the utilization of a combination of probiotic-based products. Primarily, the blending of probiotic *Lactobacillus* varieties is employed to offer a diverse range of health advantages. Nevertheless, the combination of yeast and *Lactobacillus* spp. has been employed historically in the production of cheese and other food products. However, formulations of probiotics containing both yeast and *Lactobacillus* spp. have been relatively underexplored.

Kluyveromyces, particularly *Kluyveromyces lactis*, has emerged as a pivotal yeast genus in both research and industrial biotechnology. Frequently found in milk and cheese, these yeasts are naturally ingested along with these foods (Andrade et al., 2017; Ceugniez et al., 2017; Fadda et al., 2017). This genus exhibits resistance to passage through the gastrointestinal tract (GI) and demonstrates potential for adhesion to the intestinal epithelium. Additionally, it possesses functional properties, including the production of short-chain fatty acids, immune modulation, inhibition of pathogens, and pro-apoptotic activity in cancerous epithelial cells (Kumura et al., 2004; Maccaferri et al., 2012; Ceugniez et al., 2017; Saber et al., 2017). Most recently, Gut et al. (2019) has studied the probiotic properties of *K lactis* and stated that it has potential probiotic characteristics comparable with established probiotic yeast i.e., *Saccharomyces boulardii*.

Therefore, this study attempted to evaluate the mutual compatibility between probiotic *Lactobacillus* and *Kluyveromyces lactis*, with the overarching objective of establishing a harmonious interaction between yeast and probiotic *Lactobacillus* strains. The investigation also encompasses an assessment of their respective antibacterial properties, particularly in relation to potential pathogenic microorganisms.

The probiotic yeast culture *Kluyveromyces lactis* (MTCC 458) was purchased from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The *Lactobacillus* strains viz., *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lactobacillus acidophilus*, and *Lacticaseibacillus rhamnosus* of probiotic cultures were procured from Chr. Hansen A/S, 10-12 Boege Alle, DK-2970 Hoersholm, Denmark. The other probiotic strains i.e. *Lactiplantibacillus plantarum* subsp. *Plantarum, Lactobacillus bulgaricus* and *Lactobacillus helveticus* were obtained from NCDC, NDRI, Karnal. *Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), and Bacillus cereus (B. cereus)* cultures were procured from Department of Dairy Microbiology, College of Dairy Science and Technology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana**.**

Compatibility between different strains of probiotic cultures was checked by spot assay on MRS Agar. The probiotic cultures were taken in active log phase of their growth. An aliquot of 3 µL broth from both cultures was spotted adjacent to each other on agar to check the compatibility between two cultures. Plates were incubated at 37 ! for overnight and after incubation intensity of growth in spots was observed to check any inhibition or spontaneity into the growth zone of bacteria.

Antagonistic activity of probiotic strains against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Bacillus cereus* (*B. cereus*) was assessed by the cut well diffusion assay as per the protocol of Zhang et al., (2011) with partial modifications. Briefly, cell free supernatant (CFS) from each of the probiotic strains was collected by centrifugation of overnight grown culture at 8,000 g for 10 min. An aliquot of 100 µl of actively growing individual strains *E. coli* and *S. aureus* in BHI broth (log phase culture) containing 10^7 cfu/ml of pathogen were seeded in 7 mL of molten soft BHI agar, (0.5 %) mixed gently and poured in BHI agar plates. 1.25 cm diameter wells were punched in agar plates using a sterile borer and an aliquot of 200 µl of the supernatants from each probiotic strain was added separately in their respective wells. Un-inoculated MRS broth adjusted at pH 6.8 served as the negative control. Supernatants were allowed to diffuse into agar for few minutes by storing the plates in a refrigerator (7°C) and the plates were then transferred in an incubator set at 37°C in an inverted position till growth free inhibition zones appeared around the wells. The zone diameters (in cm) reflecting the antibacterial activity was measured with the help of an imageJ software. One way analysis of variance (ANOVA) was performed using minitab 18 statistical software to find significant differences in the zone diameters. Means with p-value < 0.05 were considered statistically different.

Figure 1 shows the compatibility between *K lactis* and probiotic *Lactobacilli strains.* After being incubated overnight at 37 !, both cultures in the spot exhibited nearly equal growth intensity. These findings are supported by the growth pattern of the cultures which clearly demonstrates the absence of any incompatibility between *K lactis* and the probiotic *Lactobacilli* spp. Moreover, it was observed that *K lactis* and the probiotic *Lactobacilli* spp. mutually promoted each other's growth. This is evident from the thicker colony observed at the intersection of their cultures compared to the individual cultures (Fig. 1). The results are in agreement with the previous studies reported by Shimizu et al. (2006) and Menezes et al. (2018). Shimizu et al. (2006) studied the symbiotic relationship between *Lactococcus lactis* and *K lactis* and reported harmonious relationship between each other. Furthermore, Menezes et al. (2018) specified that yeasts contribute to LAB growth, and vice versa, since they provide

Table 1: Antibacterial activity of probiotic *Lactobacillus* strains against *B cereus*, *S aureus* and *E coli* using cut well assay

Strain	Zones of inhibition (cm) including well diameter (1cm)		
	B cereus	*S aureus*	*E coli*
LA	1.987 ± 0.302^{d}	ND	2.444 ± 0.198^{bc}
LB	2.197 ± 0.217^{cd}	ND	2.397 ± 0.138^{bc}
LC	2.723 ± 0.29^{abc}	2.043 ± 0.077^{b}	2.511 ± 0.141^{abc}
LH	2.353 ± 0.19^{bcd}	2.009 ± 0.199^{b}	2.31 ± 0.115^{c}
LG	3.063 ± 0.071^{a}	2.754 ± 0.133^{a}	2.818 ± 0.125^{a}
LP	2.829 ± 0.415^{ab}	2.572 ± 0.199^{a}	2.74 ± 0.285^{ab}

Zone of inhibition including well diameter (1 cm); ND- Not detected; Means with small letter superscripts within a column shows significant ($p<0.05$) differences in the zone diameters

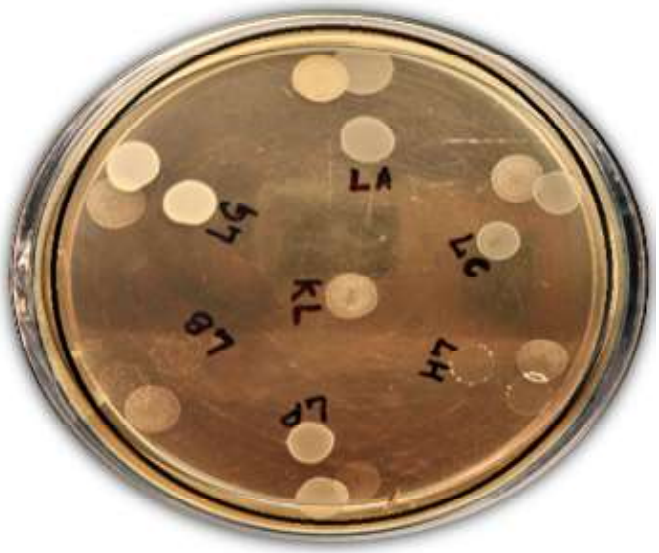

Fig. 1 Compatibility between *K lactis* and probiotic *Lactobacilli* spp.; LA- *L acidophilus*; LC- *L casei*; LH- *L helveticus*; LP- *L plantarum*; LB- *L bulgaricus*; LG- *L rhamnosus*

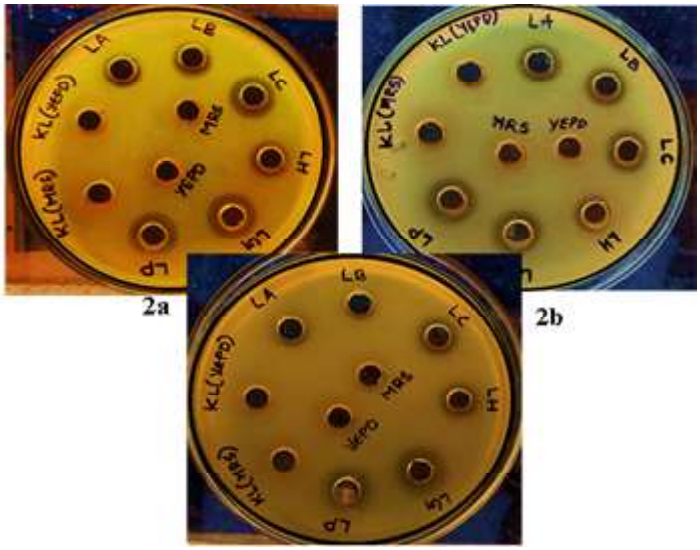

Fig. 2 Agar plates showing inhibitory activity of probiotic *Lactobacillus* strains against tested pathogens; 2a) *B cereus*; 2b) *E coli*; 2c) *S aureus*

some nutrients, such as amino acids and vitamins to LAB. The compatibility between dairy yeast *K Lactis* and probiotic *Lactobacilli* needs to be further explored in different type of food matrices to confirm their symbiotic attributes that can be harnessed in the developments of novel functional foods.

Table 1 shows the zone of inhibition of probiotic *Lactobacillus* strains against *B. cereus*, *S. aureus* and *E. coli* using cut well assay. It could be observed from the table 1 that among six different strains of *Lactobacillus*, *L rhamnosus* displayed the maximum zone of inhibition (cm) with 3.063 ± 0.071, 2.754 ± 0.133, 2.818 ± 0.125 against *B. cereus*, *S. aureus* and *E. coli*, respectively. The minimum activity was observed in *L acidophilus* and *L bulgaricus* with no zone of inhibition against *S aureus* by both bacteria. The results of the study indicated that most of the probiotic *Lactobacilli* strains displayed significant antibacterial activity against the tested pathogens. However, the inhibition spectra varied among the different strains, as evident from the diverse sizes of the zones of inhibition shown in Figure 2. The results are in accordance with the previous studies where authors have reported antibacterial effect of different *Lactobacillus* spp. against various pathogens (Fayol-Messaoudi et al., 2005; Zhang et al., 2011). The antibacterial effect of *Lactobacillus* spp. could be due to excessive acid production and other microbial component including bacteriocins which become active in acidic pH conditions.

However, it is noteworthy that the cut well assay method employed to evaluate the antimicrobial properties of *K lactis* yielded no growth inhibition against *E. coli*, *S. aureus*, and *B. cereus*, as illustrated in Figure 2. These findings align with the results reported by Gut et al. (2019), where no antibacterial activity

against *E. coli* ATCC 43895 and *Enterobacter aerogenes* VUN 00025 was observed. Conversely, Gut et al. (2022) reported antimicrobial potential of *K lactis* against *Salmonella*, comparable to *Saccharomyces boulardii* strains, emphasizing the need for further exploration of *K lactis'* antibacterial potential due to the limited studies available on this aspect.

Conclusion

The exploration of the compatibility between *K lactis* and probiotic *Lactobacillus* has revealed symbiotic characteristics, suggesting the potential of *K lactis* as a probiotic yeast alongside established probiotic *Lactobacillus* species. The mutual enhancement of their growth was evident at the intersection of their cultures, resulting in denser growth compared to individual cultures. In terms of antimicrobial potential, *K lactis* did not exhibit antibacterial activity. Conversely, *L rhamnosus*, and *L plantarum* demonstrated the highest antibacterial activity among the various *Lactobacillus* species. On the other hand, *L acidophilus*, *L helveticus*, and *L bulgaricus* exhibited relatively lower activity compared to the former strains. This study suggests that *K lactis* can be effectively combined with other probiotic *Lactobacillus* species to produce novel functional products with enhanced benefits.

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SHORT COMMUNICATION

Genetic analysis of body weights and average daily weight gains of Black Bengal goats

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Abstract: The impact of environmental factors on growth traits was evaluated by least-squares analysis of variance followed by estimation of variance components along with heritability of different growth traits and average daily weight gain using simple animal model. Average body weights of Black Bengal kids at birth, 3, 6, 9 and 12 months of age were 1.05±0.01, 3.13±0.05, 6.16±0.13, 9.12±0.46 and 11.28±0.40 kg., respectively. Average daily weight gains at 0-3 months (ADG0-3M), 3-6 months (ADG3-6M), 6-9 months (ADG6-9M) and 9-12 months (ADG9-12M) of age were 23.68±0.59, 31.80±1.21, 23.72±2.83 and 26.68±2.73 g respectively. The study revealed that birth-year of kids significantly (P<0.05) influenced the body weight traits and average daily weight gains (ADG) at different age stages and age intervals of growth, except for 9 months of age. Season of birth showed significant (P<0.05) effect on body weights and ADGs except for 12 months body weight and ADG9-12M. Significant variations for birth weight, 3-and 6-month weights as well as ADG0-3M of kids were observed in different parities of does. Male kids exhibited significantly higher body weights than their female counterparts at all ages except at birth and ADG9-12M. Birth status of kids showed significant effect on 6 months, ADG0-3M and ADG6-9M of animals. Direct heritability estimates of different growth traits ranged from 0.12 to 0.44 whereas ADG at different phases of growth varied from 0.22 to 0.32; indicating some scope for genetic improvement of these traits under study may be possible through selection.

Keywords: Environmental factors, Growth traits, Average daily gain, Heritability, Black Bengal goat

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Growth traits are important for profitability of goat production system mostly reared for meat purposes (Zhanget al. 2009), because rapid growth during the early part of life reduces maintenance costs and can be considered as an early indicator of post-weaning animal growth. Besides this, growth rate in terms of body weight at market weight is one of the main determinants of profit from goat farming for pastoral communities and poor villagers (Gautam et al. 2019). Thus, body weights and average daily gains are important selection traits for improving production performance by selective breeding (Rout et al. 2018). Various studies (Mandal et al. 2018, Gautam et al. 2019) showed that a number of environmental factors can affect the growth traits, which may directly obscure the recognition of the genetic potential of animals. Adjustment of data for different environmental/non-genetic factors is of utmost importance for obtaining the reliable estimates for the traits of interest and for precise estimation of genetic parameters of the traits to increase the accuracy of selection of breeding animals in any breeding program. Therefore, the present study was carried out to determine the important environmental factors affecting the growth performance and their genetic control in Black Bengal goats.

Data on body weights from 416 Black Bengal goats, descended from 21 sires and 61 dams born during the period of 7 years (2016 to 2022), maintained at ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia, West Bengal, were collected and used for the present study. Growth traits included for this study were birth weight and weights at 3, 6, 9 and 12 months of age. Pre-weaning average daily weight gains (ADG) at 0-3 months (ADG0-3M) and post-weaning ADG at 3-6 months (ADG3-6M), 6-9 months (ADG6-9M) and 9-12 months (ADG9-12M) were also considered for the present study.

The data were classified according to year of birth / kidding, season of birth, parity of dam, sex of kid and type of birth. Mixed model least-squares analysis was implemented for fitting constants (Harvey 1990) including all main effects as follows:

$$Y_{ijklmn} = \mu + Y_i + Sj + A_k + E_l + T_m + e_{ijklmn}$$

Where, Y_{ijklmn} is the record for the n^{th} kid, Y_i is the effect of the i^{th} year of birth, S_j is the effect of the j^{th} season of birth, A_k is the effect of the k^{th} parity of dam, E_l is the effect of the l^{th} sex of kid, T_m is the effect of the m^{th} birth status / type of kid born, e_{ijklmn} is the residual error element. The comparison of different sub-groups means was made by Duncan's multiple range test (DMRT) as described by Kramer (1957).

Variance components as well as heritability of different growth traits were estimated by simple animal model. Only significant fixed effects obtained from least-squares analysis for each growth trait were included in the final model used for genetic parameter estimation of growth traits by animal model. The components of variance were estimated by Restricted Maximum Likelihood (REML), through Average Information-REML algorithm on WOMBAT program using single-trait animal model (Meyer 2007).The following simple animal model was used:

$$Y=X\beta + Z_l a + e$$

Where, Y is the vector of observations for the dependent variable (growth traits); X is the incidence matrix of fixed effects for the dependent variable and β is the corresponding vector of fixed effects; Z_l is the incidence matrix of the direct additive genetic effects; a is the vector of direct additive genetic effects associated with the Z_l incidence matrix and e is the vector of residual random effects associated with the observations.

The average body weights of Black Bengal kids at birth, 3, 6, 9 and 12 months of age were 1.05±0.01, 3.13±0.05, 6.16±0.13, 9.12±0.46 and 11.28±0.40 kg., respectively (Table 1). The average values for ADG0-3M, ADG3-6M, ADG6-9M and ADG9-12M were recorded as 23.68±0.59, 31.80±1.21, 23.72±2.83 and 26.68±2.73 g, respectively (Table 2). The average body weights at various ages in the present study were well comparable with the findings of

Table 1: Least-squares means along with standard errors of different growth traits of Black Bengal Goats

Effects	Growth traits (Kg)				
	Birth wt.	3-month wt.	6-month wt.	9-month wt.	12-monthwt.
Overall mean	1.05±0.01 (326)	3.13±0.05 (281)	6.16±0.13 (234)	9.12±0.46 (148)	11.28±0.40 (117)
Year of Birth	**	**	**	NS	**
2016-17	1.26±0.03^{a} (36)	3.53±0.12^{a} (33)	7.02±0.27^{a} (35)	9.83±0.49 (34)	13.37±0.67^{a} (27)
2017-18	1.00±0.03^{bcd} (35)	3.31±0.11^{ab} (32)	6.56±0.28^{ab} (27)	8.79±0.49 (26)	10.26±0.72^{b} (19)
2018-19	1.03±0.03^{bc} (27)	3.14±0.13^{bc} (24)	6.20±0.30^{bc} (22)	8.93±0.79 (8)	11.40±0.91^{b} (10)
2019-20	0.94±0.02^{d} (83)	2.98±0.09^{c} (58)	6.21±0.20^{b} (62)	9.05±0.40 (51)	11.41±0.48^{b} (49)
2020-21	1.00±0.02^{cd} (78)	2.95±0.08^{c} (69)	5.83±0.21^{c} (47)	8.33±0.50 (23)	9.96±0.87^{b} (12)
2021-22	1.06±0.02^{b} (67)	2.89±0.09^{c} (65)	5.16±0.27^{d} (41)	9.71±0.31 (6)	
Season of Birth	**	**	**	**	NS
Summer	1.09±0.02^{a} (122)	3.56±0.08^{a} (91)	6.54±0.17^{a} (110)	8.68±0.48^{ab} (82)	10.68±0.56 (55)
Rainy	1.03±0.02^{b} (124)	3.01±0.07^{b} (118)	5.56±0.19^{b} (81)	8.48±0.59^{b} (35)	11.39±0.62 (30)
Winter	1.02±0.02^{c} (80)	2.83±0.08^{c} (72)	6.39±0.23^{a} (43)	10.15±0.59^{a} (31)	11.78±0.53 (32)
Parity of dam	**	**	*	NS	NS
1	1.01±0.02^{c} (93)	2.94±0.08^{c} (81)	5.57±0.20^{b} (65)	9.01±0.65 (34)	11.11±0.68 (27)
2	1.02±0.02^{c} (73)	2.83±0.09^{c} (66)	5.63±0.22^{b} (53)	9.06±0.62 (35)	11.73±0.60 (29)
3	0.99±0.02^{c} (50)	2.96±0.10^{c} (42)	6.07±0.22^{a} (41)	9.06±0.59 (28)	11.58±0.58 (24)
4	1.05±0.03^{bc} (26)	3.11±0.14^{bc} (23)	6.70±0.33^{a} (19)	9.89±0.77 (14)	12.77±0.93 (11)
5	1.05±0.03^{bc} (30)	3.09±0.12^{c} (27)	5.94±0.29^{a} (22)	8.90±0.74 (11)	11.51±0.78 (12)

6	1.15±0.03[a] (25)	3.46±0.14[ab] (20)	6.50±0.34[a] (18)	8.98±0.82 (13)	10.43±1.13 (6)
7	1.13±0.04[ab] (11)	3.77±0.23[a] (7)	6.96±0.56[a] (6)	9.20±1.11 (5)	10.35±1.66 (3)
8	0.98±0.04[c] (18)	2.91±0.17[c] (15)	5.94±0.45[a] (10)	8.75±1.03 (8)	10.77±1.30 (5)
Sex of kid	NS	*	**	**	**
Male	1.06±0.01 (182)	3.23±0.06[a] (155)	6.62±0.15[a] (121)	9.94±0.46[a] (80)	12.29±0.46[a] (53)
Female	1.03±0.01 (144)	3.04±0.07[b] (126)	5.70±0.17[b] (113)	8.28±0.52[b] (68)	10.28±0.50[b] (64)
Type of birth	NS	NS	*	NS	NS
Single	1.05±0.02 (115)	3.21±0.08 (94)	6.36±0.20[a] (79)	9.20±0.58 (54)	11.08±0.59 (41)
Twin	1.04±0.02 (164)	3.02±0.06 (145)	5.89±0.15[b] (120)	8.99±0.49 (68)	10.91±0.44 (61)
Triplet	1.04±0.03 (47)	3.17±0.11 (42)	6.27±0.26[b] (35)	9.13±0.70 (26)	11.86±0.84 (15)

Means with different superscripts between the rows in a column differed significantly
NS, Not significant; *, Significant (P<0.05); **, Highly Significant (P<0.01)

Kumar et al. 2021 and Alam et al. 2021 for this breed. However, higher body weights of Black Bengal kids at different stages of growth were reported by Solaiman et al. (2020) at 6-12 months age and Chakrabarti et al. (2022) at birth and 3 months of age.

Different environmental factors significantly (P<0.05) affected most of the growth traits of the kids in this study. Birth year had significant (P<0.01) effect on all the growth traits except for 9-months body weight. Significant effects of year of birth on body weights were also reported by Mandal et al. (2018) and Jasmine et al. (2022) for various breeds of goat. Variations in body weight of kids in this study across birth years may be due to variations in managemental condition and varied climatic conditions including humidity, rainfall, and temperature etc. in the flock over the years. Significant variations for all growth traits except weight at 12 months and ADG9-12M were observed among kids born in different seasons. Kids born in summer season had higher weight at birth and subsequent body weights until 6 months of age than kids born during rainy and winter seasons. The lower body weights at birth of the kids, born in rainy / winter season in this study may be an unfavorable effect of the temperature, since gestation period of the does would occur during hot period of the year. Parity of doe showed significant (P<0.05) effect on only ADG0-3 months and all growth traits except 9 and 12 month of age (Table 1 and 2). Kids born from does of later parities had significantly (P<0.05) higher body weights than kids born from does of earlier parities. Similar significant effect of parity of doe on different body weights of kids was observed by Singh et al. (2013) in Jamunapari and Bhusan & Dass (2015) in Jakhrana goats. Similarly, Amy (2020) and Jasmine et al. (2022) also observed the significant effect of parity on all body weight traits except weight at 9 and 12 months of age in Black Bengal goats.

Sex of kid had significant effect on all growth traits except weight at birth and ADG9-12 months of Black Bengal goats . Single born kids exhibited significantly (P<0.01) higher body weight only at 6 month of age and ADG 6-9M than kids born as twins or triplets. However, significantly heavier male kids than female at all the ages were reported by Haque et al. 2013 in Black Bengal Goats.

Variance components and heritability estimates of different body weights and average daily weight gains at different ages of Black Bengal goats are presented in Table 3. Estimates of direct heritability for birth weight, 3, 6, 9, and 12-months body weights were low to moderate in magnitude, which ranged from 0.12 - 0.44. The heritability of body weights exhibited a decreasing trend from birth to 6 months of age whereas a increasing trend was observed from 9 to 12 months. Several researchers (Rout et al. 2018 and Gautam et al. 2019) reported the heritabilities for body weights of kids at different ages in various goat breeds, which were in agreement with our findings. The decreasing heritability of kids' body weights at the later stages of developmental process except 12 months in this study indicates that environmental factors, in relation to additive genetic factors, had more influence on weights attained later in the developmental stages. This attributes to the maternal influence associated with kid performance at early stage of growth. Estimates of heritability of all body weight traits at different ages in this study were low to moderate in magnitude, indicating genetic progress is possible for these traits under prevalent management system. Direct heritability estimates for average daily weight gains at 0-3 months (ADG0-3M), 3-6 months (ADG3-6M), 6-9 months (ADG6-9M) and 9-12 months (ADG9-12M) of age were moderate to high in nature, which ranged from 0.22 – 0.32 (Table 3). The heritability estimate of AGD at 0-3 M was slightly increased in the subsequent

Table 2: Least-squares means along with standard errors of average daily gains (ADG's)

Effects	Average daily weight gains (g)			
	ADG0-3M	ADG3-6M	ADG6-9M	ADG9-12M
Overall mean	23.68±0.59 (282)	31.80±1.21 (212)	23.72±2.83 (140)	26.68±2.73 (105)
Year of Birth	**	**	**	*
2016-17	27.26±1.29^{a} (35)	35.01±2.43^{a} (34)	35.94±3.10^{a} (31)	35.86±3.85^{a} (27)
2017-18	25.68±1.28^{a} (32)	35.86±2.54^{a} (26)	27.43±3.03^{b} (26)	21.54±4.08^{b} (18)
2018-19	24.19±1.38^{ab} (25)	32.42±2.63^{a} (22)	24.27±4.82^{b} (8)	29.12±7.72^{ab} (4)
2019-20	22.77±0.93^{b} (58)	33.76±1.88^{a} (48)	27.72±2.35^{b} (50)	26.42±2.68^{b} (48)
2020-21	21.11±0.86^{b} (68)	31.95±1.95^{a} (41)	19.36±3.28^{c} (25)	20.44±5.77^{b} (8)
2021-22	21.08±1.03^{b} (64)	21.82±2.39^{b} (41)		
Season of Birth	**	**	*	NS
Summer	27.04±0.83^{a} (91)	32.07±1.54^{a} (91)	20.61±3.01^{b} (77)	25.01±3.43 (54)
Rainy	22.51±0.81^{b} (119)	27.00±1.72^{b} (78)	20.83±3.66^{b} (33)	29.63±3.93 (24)
Winter	21.50±0.90^{b} (72)	36.33±2.04^{a} (43)	29.70±3.66^{a} (30)	25.39±3.59 (27)
Parity of dam	**	NS	NS	NS
1	22.36±0.89^{c} (82)	29.10±1.80 (61)	28.64±4.02 (32)	30.65±4.27 (26)
2	20.56±0.95^{c} (66)	30.71±1.95 (49)	26.42±3.82 (32)	31.86±3.73 (26)
3	21.27±1.05^{c} (41)	33.16±2.01 (35)	22.96±3.63 (28)	31.93±3.41 (23)
4	23.91±1.49^{bc} (23)	37.77±3.14 (16)	27.99±4.77 (13)	34.29±5.53 (10)
5	22.67±1.33^{c} (27)	30.86±2.73 (19)	25.94±4.51 (11)	23.47±5.02 (10)
6	27.53±1.53^{ab} (21)	30.07±3.04 (18)	17.70±5.01 (13)	19.07±8.01 (4)
7	29.91±2.55^{a} (7)	33.11±5.29 (5)	18.70±7.37 (4)	20.24±10.74 (2)
8	21.28±1.80^{c} (15)	29.64±4.07 (9)	21.36±6.57 (7)	21.90±8.60 (4)
Sex of kid	*	**	**	NS
Male	24.59±0.68^{a} (157)	35.23±1.40^{a} (110)	28.01±2.86^{a} (74)	28.99±2.89 (47)
Female	22.78±0.74^{b} (125)	28.38±1.50^{b} (102)	19.42±3.23^{b} (66)	24.36±3.30 (58)
Type of birth	*	NS	*	NS
Single	23.96±0.89^{ab} (95)	33.23±1.81 (70)	19.30±3.62^{b} (50)	22.66±3.73 (39)
Twin	22.17±0.69^{b} (144)	30.27±1.41 (108)	27.12±2.99^{a} (65)	22.56±3.02 (52)
Triplet	24.92±1.19^{a} (43)	31.91±2.38 (34)	24.72±4.30^{ab} (25)	34.81±5.43 (14)

Means with different superscripts between the rows in a column differed significantly

NS, Not significant; *, Significant (P<0.05); **, Highly Significant (P<0.01)

Table 3: Estimates of variance components and heritability along with standard errors of different body growth traits of Black Bengal Goats

Traits	σ_a^2	σ_e^2	σ_p^2	h^2
Birth weight	0.006	0.02	0.02	0.29±0.14
3-month weight	0.06	0.31	0.37	0.17±0.14
6-month weight	0.21	1.55	1.76	0.12±0.15
9-month weight	0.67	3.89	4.56	0.15±0.16
12-month weight	3.04	3.77	6.81	0.44±0.27
ADG0-3M	9.69	34.46	44.15	0.22±0.14
ADG3-6M	44.65	93.65	138.30	0.32±0.20
ADG6-9M	62.45	132.49	194.94	0.32±0.22
ADG9-12M	59.72	140.19	199.91	0.30±0.27

σ_a^2, additive genetic variance, σ_e^2, residual variance, σ_p^2, phenotypic variance; h^2, heritability

time and remained at constant upto 9-12 months. Similar to the findings of the present study, Rout et al. (2018) and Gautam et al. (2019) obtained moderate heritability estimates of average daily weight gains of kids at different ages/phases of growth in various goat breeds. The moderate to high estimates of heritability for all ADG under study suggested that there is ample scope of genetic improvement of these traits through genetic selection.

Conclusion

Body weights and average daily gains are essential traits for improving production performance by selective breeding in goat. The study revealed that environmental factors significantly impacted the growth traits of Black Bengal. The growth performance of the Black Bengal goats at the farm level showed that, despite its climate vulnerability, this breed can perform admirably at organized flocks.

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Tariff Plan of Membership									
S.no	Membership Form	Fee	Admission Fee	(GST @18%)	Without Late Fee Amount	Late Fee After 31 MAY	Total Amount	Period	Qr Code of Form
1	OM Form	1000	500	270	1770	NIL	1770	April To March (One Year)	
2	OM Renewal Form	1000	NIL	180	1180	118	1,298	April To March (One Year)	
3	LM Direct Form	10,000	500	1890	12,390	NIL	12,390	Life Time	
4	Convert Om to Lm Form	10,000	NIL	1800	11,800	NIL	11,800	Life Time	
5	Student Membership Form	700	NIL	126	826	NIL	826	Per Course	
6	Institutional Form Sustaining Membership	12,500	NIL	2,250	14,750	NIL	14,750	(One Year)	
7	Institutional Form Benefactor Membership	70,000	NIL	12,600	82,600	NIL	82,600	(Eight Year)	

Impact Factor: 0.2; NAAS Score: 6.20 (January 2025)

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