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RESEARCH ARTICLE

Quality attributes of ghee residue prepared using Deoni and Holstein Friesian crossbred cow milk as influenced by method of preparation

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Abstract: Ghee residue (GR), a dairy by-product, was found to be nutrient dense. The ghee residue composition could be affected by clarification temperature, method of ghee preparation and breed of the cow. So, the present study was conducted to know whether the clarification temperature (110°C and 120°C), methods of ghee preparation (direct cream and creamery butter), and breed of the cow (Indigenous Deoni and Holstein Friesian crossbred) can affect the composition of ghee residue. The moisture, protein, fat, lactose and ash content of various ghee residue samples prepared from the milk of Deoni and HF crossbred cows varied from 10.50±0.22 to 14.65±0.1, 13.49±3.37 to 34.17±3.37, 51.61±0.86 to 67.69±0.09, 9.44±0.06to14.15±0.22 and 1.89±0.03to 5.59±0.41%, respectively. Further, the phospholipid content and antioxidant activity varied from 3.18 to 15.09 and 27.04 to 67.86%, respectively. In this study, it was found that temperature of clarification had a significant ($p<0.05$) effect on moisture, lactose, ash, hydroxy methyl furfural (HMF) and browning index. The clarification temperature significantly affected the protein, fat, lactose, ash, phospholipids and antioxidant activity. The protein, fat and browning index of ghee residue were also significantly different for the breeds. Thus, it can be concluded that GR's composition was affected by breed and method of ghee preparation.

Keywords: Antioxidants, Ghee residue, Holstein Friesian crossbred, Indigenous Deoni, Phospholipids

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Introduction

Milk is either utilized directly for consumption or converted into various products which can result in by products. One of the dairy products by name ghee is manufactured by utilizing 30 to 35% (Gandhi et al. 2013) of the total milk collected which leads to production of huge quantity of ghee residue. Ghee residue accounted for more than 3MT per annum (Verma and Raju 2008). Ghee residue; a slightly brown to darker brown coloured by-product of dairy industry is obtained by the conversion of cream or butter into ghee after the process of clarification. Prahlad (1954) reported the differences in ghee residue composition obtained by different methods of ghee making such as desi method, creamery butter, direct cream. The ghee residue obtained after processing of cream/butter was found to be rich in nutrients such as lactose (2 – 14%), protein (12 – 39%), fat (32 – 70%), moisture (8 – 30%), ash (1 – 8%) (Prahlad 1954). Santha and Narayanan (1978) reported that hand pressed ghee residue obtained from direct cream method had higher fat content than the ghee residue obtained from creamery butter method. But moisture, ash, proteins were higher in the latter (Prahlad 1954, Santha and Narayanan 1978). However, it was found that the temperature and method of clarification can affect the nutrient composition of the ghee residue. Compositional variation in ghee residue was also linked to raw material (Mani 1952).

The ghee residue obtained after processing of cream/butter was found to be rich in nutrients such as protein (25.07%), fat (50.25%), moisture (13.28%), ash (8.24%) (Ranjan et al. 2010). Selvamani et al. (2017) also reported that ghee residue contains 40.69 % fat and 24.32% protein. According to Munirathnamma et al. (2017) ghee residue is composed of 35.99 % fat, 17.88 % lactose, 3.81 % ash, 25.29 % protein (dmb). Janghu et al. (2014) reported that ghee residue prepared from direct cream showed 26.64 % moisture, 33.13 % fat, 3.27 % ash, 30.91 % protein whereas the ghee residue obtained from creamery butter was found to have 17.71 % moisture, 41.83 % fat, 2.56 % ash, and 31.69 % protein. The wide variation for the amount of various constituents was observed due to variation in method of preparation.

Apart from major nutrients, the method of preparation is expected to affect the minor constituents as well. It has been reported that

heating time and temperature used for ghee clarification can also affect the phospholipid content, due to the movement of phospholipids from GR to ghee (in minute amounts due to their polar nature) when ghee is clarified for longer time (Santha and Narayanan 1979). Also, the antioxidant activity of GR is expected to be affected by ghee manufacture method as it depends on the intensity of the brown coloured pigments such as melanoidins and reductones (Kiriyaga et al. 1968).

So, these differences in composition, prompted us to undertake the work on the characterization of ghee residue, which is obtained from the processing of indigenous (Deoni) *vs* HF crossbred cow's milk concerning temperature and method of making. Also, there are limited reports on the properties of ghee residue obtained from these breeds (indigenous and HF crossbred). Thus, the present article explores the differences in the physico-chemical characteristics of ghee residue as affected by the method of preparation for both Deoni and HF crossbred cows.

Materials and Methods

Milk and Ghee residue

The pooled milk was collected from 16 Deoni cows and 11 HF crossbred cows from the Livestock research centre of Southern Regional Station, ICAR-NDRI, Bengaluru. The animals were in their 2nd, 3rd and 4thlactations. The cream obtained by separation of Deoni and HF milk was collected from the dairy plant using a cream separator (Noe Tech Int. Pvt. Ltd, Haryana, India). Ghee was prepared by direct cream and creamery butter methods. For the creamery butter method, the butter having minimum 80-85 % fat was prepared from aged cream. Ghee clarification was done at two different temperatures; 110°C and 120°C. Ghee was then filtered through muslin cloth and residual ghee was removed by gently pressing the ghee residue in muslin cloth. The ghee residue obtained in previous step was further pressed using a hydraulic press (Multipurpose machine, Milk Tech Engineers, Bangalore, India) at 4 kg/cm^2 for 5 min to remove the entrapped ghee from ghee residue. Eight different samples of ghee residue were obtained in the study *viz*. ghee residue obtained by processing of Deoni milk by direct cream and creamery butter method, and ghee residue obtained by processing of HF crossbred milk by direct cream and creamery butter method for two different clarification temperatures, respectively.

Proximate composition

The pressed ghee residue samples were pooled for three replicates for further analysis. The collected samples were evaluated for moisture, protein, fat, ash and lactose content following the AOAC (2003) methods. All the parameters were analyzed in triplicates.

Phospholipid estimation

The estimation of phospholipids was performed by the method of Sharma et al. (2007). The fat was extracted by the process of Rose Gottlieb method. The extracted fat was digested in kjeldahl flask by adding sulphuric acid (0.5%) till the colour of the sample was colourless to light yellow in colour. The 5 ml of the aliquot was taken from the digested sample, 4ml 0.44% ammonium molybdate solution was mixed and 0.4 ml of reducing agent was also added before determining the absorbance in the UV-Visible spectrophotometer (LABINDIA Analytical UV 3200XE, India) at 720nm. Phospholipids content was estimated using the equation 1.

$$\text{Phospholipid(mg/100g)} = \frac{\text{O.D of sample}}{\text{O.D of standard}} \times 0.1 \times \frac{1000}{W} \times 25.9 \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where, W= weight of fat extracted from ghee residue

Antioxidant activity

For determining the antioxidant activity by the method of Shimada et al. (1992), the stock solution of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) dye was prepared, from which 1 ml was taken and volume was made up to 100 ml to get working solution. Around 5000 mg of ghee residue sample was macerated using 10 ml of methanol, followed by centrifugation at 4000 rpm for 30 minutes. For measuring the absorbance using spectrophotometer (LABINDIA Analytical UV 3200XE, India) at 517 nm, 0.1 ml of supernatant and 3ml of working solution was mixed and incubated at 25 to 30°C for 30 minutes. Antioxidant activity, per cent DPPH free radical scavenging activity was measured using the formula given in equation 2.

$$\text{Antioxidant activity (\% scavenging of DPPH)} = A_0 - \frac{A_1}{A_0} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where, A_o = absorbance of blank; A_1= absorbance of sample

Hydroxy methyl furfural

Hydroxy methyl furfural (HMF) was determined by Keeney and Bassette (1959) method. In this method, 1g of ghee residue was mixed with 9.5 ml of distilled water,5ml of oxalic acid and boiled in waterbath for 60 minutes. To this, 5ml of 40% tricarboxylic acid was added and filtration was done using whatman filter paper No. 42. Then, 0.5 ml of supernatant and 1ml of 0.05M of TBA was mixed, followed by heating in water bath for 40 minutes at 40°C. The absorbance was measured at 443nm using UV-Visible spectrophotometer (LABINDIA Analytical UV 3200XE, India). The standard curve for HMF was prepared using concentrations from 0 to 35 µmole/ml and the amount of HMF in ghee residue

sample was determined using the absorbance values and standard curve.

Browning index

Ghee residue samples were scanned in a scanner (Canon) in triplicates. Browning index values were analysed using the colour values L, a, and b. The observed parameters were: L* (luminosity or brightness L* = 0 black and L*= 100 white), a* = (red green component – a* = greenness and a+ = redness) and b* yellow – blue component, b* blueness, and +b* yellowness. The formulas used to calculate L* a* b*, chroma values and browning index (Yam and Papadakis 2004) are mentioned below in equations 3 to 7.

$$L^* = 100 \times \frac{L}{255} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

$$a^* = \frac{240 \times a}{255} - 120 \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

$$b^* = \frac{240 \times b}{255} - 120 \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

$$\text{chroma} = [(a^*+b^*)]^{1/2} \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

$$\text{Browning index (BI)} = 100 \times \text{chroma} - \frac{0.31}{0.17} \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Statistical analysis

The entire experiments were performed in triplicates and means and standard deviations/errors were calculated. The pooled milk sample for each breed was used for ghee preparation by two different methods. For each method, ghee was prepared in triplicates and collected GR was pooled for estimation of various parameters. From this pooled GR, each parameter was further analysed in triplicates. All statistical analyses were performed using SPSS software and statistical significance was set at $p<0.05$. The least significant difference (LSD) test was used to find out significant differences between sample means. Analysis of variance (ANOVA) was used to determine differences among treatment means using the Post Hoc Test (Duncan).

Results and Discussion

The ghee residues (GR) were obtained from the Indigenous (Deoni) *vs* Holstein Friesian (HF) Crossbred cows' milk processing by direct cream and creamery butter methods. The clarification temperatures of 110°C and 120°C were used for ghee preparation and the obtained samples were subjected to proximate analysis. The results obtained are mentioned in the below sections.

Proximate composition of ghee residue prepared at 110°C clarification temperature

Among the analysed samples, highest moisture content was observed in ghee residue samples prepared by creamery butter method, i.e. CB-Deoni (14.65%) and CB- HFC (14.35%) followed by DC-Deoni (12.71%) and DC-HFC (10.91%) (Table 1). The presence of higher amount of protein in ghee residue samples obtained from creamery butter method might have the ability to hold more moisture. There were significant differences ($p<0.05$) in the samples for protein, fat, lactose and ash content. Protein (34.17%) and ash content (3.87%) of CB-Deoni was significantly highest ($p<0.05$) and this could probably be due to retention of curd particles of butter in the GR. The DC-HFC ghee residue had highest fat content (67.69%) which may be attributed to the losses of fat from ghee to ghee residue samples obtained by direct cream method. The DC-Deoni ghee residue had the highest lactose content (10.68%), which may be due to the presence of solids not fat in higher amounts (4.53%). Several authors (Relwani 1978, Santha and Narayanan 1978, Grewal 1979) have reported a wide variation in ghee residue composition *viz.* 12 to 39% protein, 1 to 8% minerals, 2 to 14% lactose, 32 to 70% fat.

Phospholipids in ghee residue samples prepared at 110°C clarification temperature

Phospholipids are the lipids which have phosphate groups in their structure. They are amphiphilic in nature with hydrophilic moiety as polar head and glycerol and fatty acid as hydrophobic tail (Krishnegowda et al. 2021). They are surface active due to their amphiphilic nature. In this study, significant differences ($p<0.05$) were observed among all the four samples (Figure 1).

Table 1 Proximate composition of ghee residue samples prepared at 110°C clarification temperature

Sample	Moisture (%)	Protein (%)	Fat (%)	Lactose (%)	Ash (%)
DC- Deoni	12.71±0.1^{b}	26.72±3.37^{a}	59.88±0.09^{b}	10.68±0.06^{a}	2.63±0.03^{b}
DC-HFC	10.91±0.1^{c}	13.49±3.37^{b}	67.69±0.09^{a}	10.23±0.06^{b}	1.89±0.03^{d}
CB-Deoni	14.65±0.1^{a}	34.17±3.37^{a}	51.99±0.09^{d}	9.97±0.06^{c}	3.87±0.03^{a}
CB-HFC	14.35± 0.1^{a}	28.89±3.37^{a}	59.33±0.09^{c}	9.44±0.06^{d}	2.33±0.03^{c}

(N=12, Results are expressed as Mean ± SE, with different small letters superscript (a,b,c) within row differ significantly ($P< 0.05$) among the samples. Where, DC- Deoni- ghee residue obtained from processing of Deoni cow's milk by direct cream method; DC- HFC – ghee residue obtained from processing of HF crossbred cow's milk by direct cream method; CB- Deoni – ghee residue obtained from processing of Deoni cow's milk by creamery butter method; CB-HFC- ghee residue obtained from processing of HF crossbred cow's milk by creamery butter method)

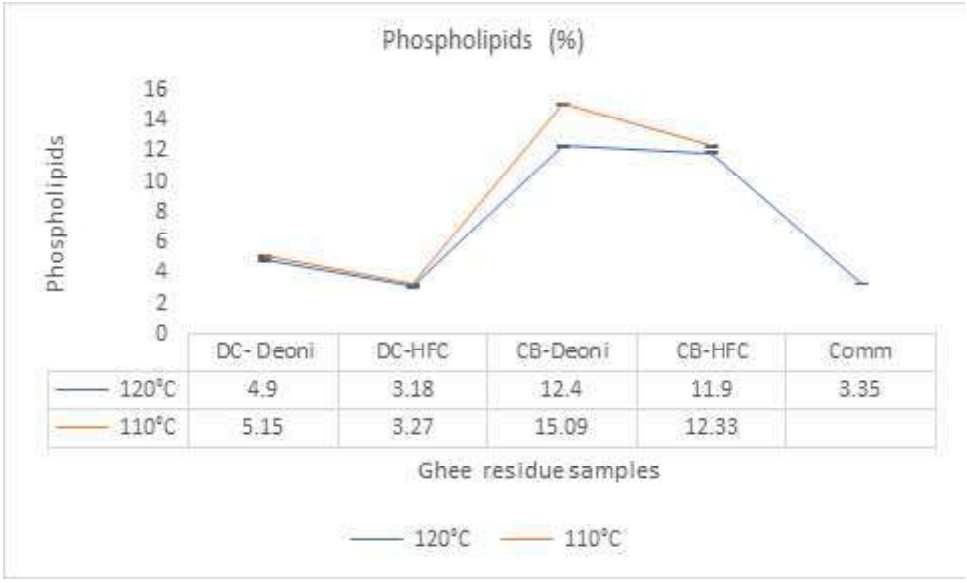


Fig. 1 Phospholipids content of ghee residue samples prepared at 110°C and 120°C clarification temperature

(n=12 for 110°C clarification, n= 15for 120°C clarification samples, results are expressed as Mean ± SE)

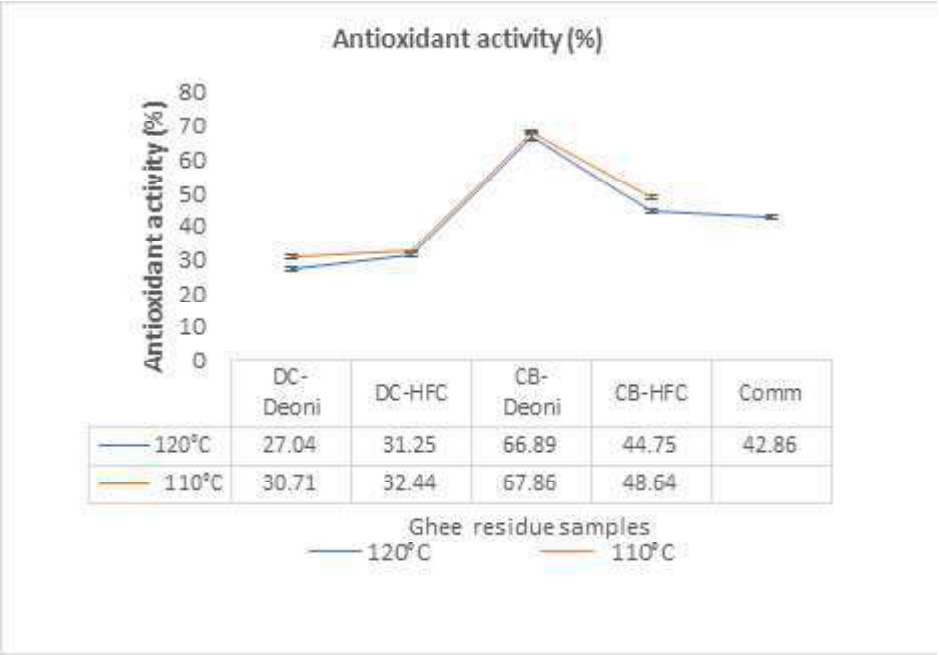


Fig. 2 Antioxidant activity of ghee residue samples prepared at 110°C and 120°C clarification temperature

(n=12 for 110°C clarification, n= 15for 120°C clarification samples, results are expressed as Mean ± SE)

CB-Deoni ghee residue sample had the highest phospholipid content (15.09%) compared to other samples such as CB- HFC (12.33%), DC-Deoni (5.15%) and DC-HFC (3.27%). This shows that ghee residue obtained by creamery butter method (13.71%) had higher content of phospholipids than ghee residues obtained by direct cream method (4.21%). Santha and Narayanan, (1978) also reported higher levels of phospholipids for the ghee residue prepared by creamery butter (17%) than direct cream method (1.5%). According to Jenness and Patton (1984), the phospholipid content in milk varies from 0.2 to 1%. The presence of phospholipids in the ghee residue might be due to the rupture of milk fat globule membranes owing to the leaching and hence, concentration of phospholipids in the ghee residue

Antioxidant activity of ghee residue samples prepared at 110°C clarification temperature

Antioxidants are substances that can delay or prevent the oxidation reactions of food product. The antioxidant activity of CB-Deoni (67.86%) sample was highest, followed by CB- HFC (48.64%), DC-HFC (32.44%), and DC-Deoni (30.71%). There were significant differences ($p<0.05$) in all the four samples (Figure 2). The antioxidant activity was significantly highest in ghee residue samples prepared from creamery butter method than direct cream method, which may be attributed to the higher presence of phospholipids in the creamery butter ghee residue (~15% phospholipids). According to Santha and Narayanan (1979), the

phospholipids also act as antioxidant. Other than phospholipids, presence of tryptophan, amino acids, cysteine hydrochloride lysine, proline etc. are also responsible for the antioxidant activity (Kiriyaga et al. 1971, Yamaguchi et al. 1981).

Hydroxy methyl furfural (HMF) content in ghee residue samples prepared at 110°C clarification temperature

The HMF compounds are those which are formed due to maillard reactions or dehydration of reducing sugars. HMF is most common in foods which contains sugars and have been exposed to high temperature during processing. The ghee residue samples such as DC-Deoni (38.1), CB- Deoni (37.47), CB-HFC (37.17) had significantly higher ($P<0.05$) HMF content (µmole/mg) than DC-HFC (34.03) (Figure 3). This shows that sugar caramelization and maillard reactions were least in DC-HFC compared to the other three samples. This could be correlated with least protein content in DC-HFC ghee residue than all other samples (Table 1), as it is well known that free amine groups are essential for forming maillard reaction products.

Browning index of ghee residue samples prepared at 110°C clarification temperature

The browning intensity of the product can be measured by browning index (BI). The browning of the product occurs due to maillard reactions and/or caramelization of sugars. CB-HFC (34.26), DC-HFC (33.75) samples had significantly ($p<0.05$) higher browning index than CB-Deoni (32.64), DC-Deoni (32.55) (Figure 4). Thus, it is evident that the BI of the ghee residue samples obtained from the processed Deoni cow's milk was lower than the HF crossbred cow's milk. This could be due to the presence of more free amine groups in the latter for maillard reaction.

Proximate composition of ghee residue prepared at 120°C clarification temperature

Ghee residues prepared in laboratory, clarified at 120° C were compared with commercial ghee residue sourced from a dairy plant (Table 2). Ghee residue samples obtained by creamery butter method i.e. CB- Deoni and CB- HFC had higher moisture content compared to the samples prepared by direct cream method which may be attributed to the presence of higher protein and their ability to hold water. But moisture content of commercial ghee residue samples was significantly ($p<0.05$) lowest. This may be due to the removal of fat from ghee residue during pressing for ghee recovery which might have resulted in oozing out of the moisture along with fat. Highest protein content (33.86%) was observed in CB- Deoni sample, which may be due to the presence of curd particles from butter. The DC- HFC had significantly higher fat (66.11%) compared to all the samples. Losses of fat occur from ghee to ghee residue samples in direct cream method which might have resulted in higher fat content. CB- Deoni and CB-HFC had significantly higher lactose content (14.15%, 13.60%) which can be due to presence of solids not fat (4.53%) in cream. The ash content (7.15%) was significantly high ($p<0.05$) in commercial ghee residue samples which can be due to the extreme treatments given to the ghee residue and low moisture content (Table 2). Several authors (Relwani 1978, Santha and Narayanan 1978, Grewal 1979) have reported on wide variation in ghee residue compositions *viz*.; 12 to 39% protein, 1 to 8% minerals, 2 to 14% lactose, 32 to 70% fat. Thus, the obtained ranges of composition of ghee residue are comparable with previous findings.

Phospholipids of ghee residue samples prepared at 120°C clarification temperature

Cow milk contains phospholipids in the range 0.2 to 1% (Jenness and Patton 1984). The concentration of phospholipids in the ghee residue may be due to the rupture of milk at globular membrane which leads to the leaching of phospholipids into the ghee residue. Significant differences were observed among all the ghee residue samples. However, highest phospholipid content ($p<0.05$) was observed in CB-Deoni (12.4%) compared to other samples *viz*; CB-HFC (11.9%), DC-Deoni (4.9%), DC-HFC (3.18%) and commercial sample (3.35%) (Figure1). Overall, ghee residue samples obtained by creamery butter and direct cream method had phospholipids content up to 12.15% and 4.04%, respectively. The results are comparable with the previous findings of Santha and Narayanan (1978) who reported 17% and 1.5% of phospholipids content in ghee residues obtained by creamery butter and direct cream method, respectively.

Table 2 Proximate composition of ghee residue samples prepared at 120°C clarification temperature

Sample	Moisture (%)	Protein (%)	Fat (%)	Lactose (%)	Ash (%)
DC- Deoni	11.92±0.22^{b}	25.73±0.21^{d}	59.41±0.86^{b}	11.92±0.22^{b}	5.16±0.41^{d}
DC- HFC	10.50±0.22^{c}	19.87±0.21^{e}	66.11±0.86^{a}	10.50±0.22^{c}	4.4±0.41^{e}
CB- Deoni	14.16±0.22^{a}	33.86±0.21^{a}	51.61±0.86^{d}	14.15±0.22^{a}	5.59±0.41^{b}
CB- HFC	13.6±0.22^{a}	27.22±0.21^{c}	58.67±0.86^{b}	13.60±0.22^{a}	5.31±0.41^{c}
Comm	8.77±0.22^{d}	29.44±0.21^{b}	55.33±0.86^{c}	8.14±0.22^{d}	7.15±0.41^{a}

(N=15, Results are expressed as Mean ± SE, with different small letters superscript (a,b,c) within rows differ significantly ($P< 0.05$) among the samples. Where, DC- Deoni- ghee residue obtained from processing of Deoni cow's milk by direct cream method; DC- HFC – ghee residue obtained from processing of HF crossbred cow's milk by direct cream method; CB- Deoni – ghee residue obtained from processing of Deoni cow's milk by creamery butter method; CB-HFC- ghee residue obtained from processing of HF crossbred cow's milk by creamery butter method; Comm- ghee residue obtained from a commercial dairy plant)

Fig. 3 Hydroxy methyl furfural (µmole/mg) in ghee residue samples prepared at 110°C and 120°C clarification temperature

(n=12 for 110°C clarification, n= 15for 120°C clarification samples, results are expressed as Mean ± SE)

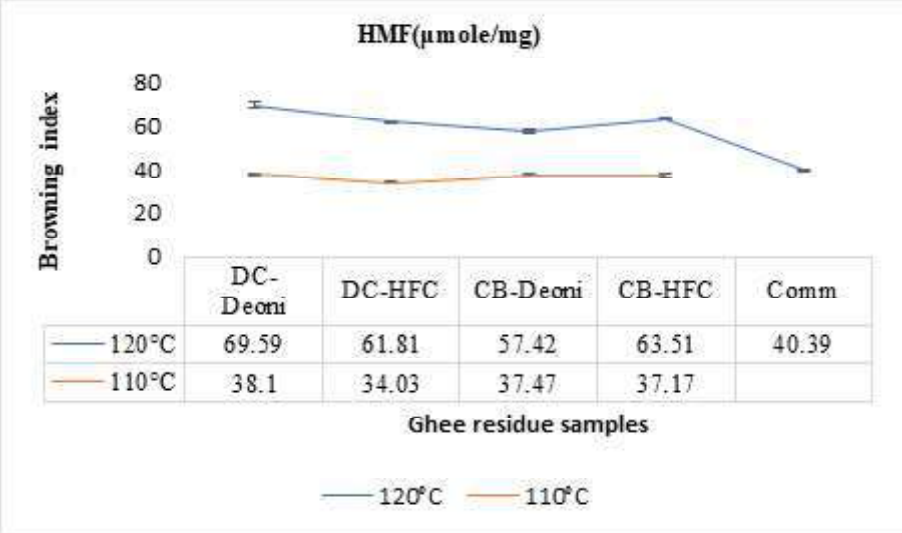
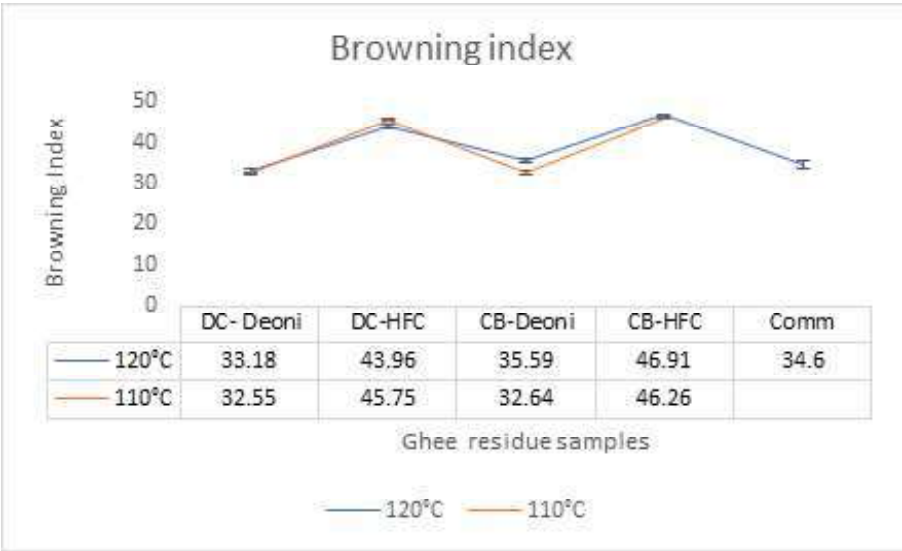


Fig.4 Browning index of ghee residue samples prepared at 110°C and 120°C clarification temperature

(n=12 for 110°C clarification, n= 15for 120°C clarification samples, results are expressed as Mean ± SE)



Antioxidant activity of ghee residue samples prepared at 120°C clarification temperature

The amino acids and condensation of sugars are one of the reasons for antioxidant activity (Kiriyaga et al. 1971, Yamaguchi et al. 1981). The CB-Deoni (66.89%) had the highest ($P<0.05$) antioxidant activity followed by CB-HFC (44.75 %), DC-HFC (31.25%), DC-Deoni (27.04%) and commercial sample (42.86%) (Figure 2). Significant ($p<0.05$) differences were observed among the five samples. Due to the higher presence of phospholipids in creamery butter ghee residue, the antioxidant activity tends to be higher than the ghee residue samples obtained by direct cream method. Santha and Nayrayanan (1979) reported that phospholipids were the main causes of antioxidant activity. Antioxidant activity decreases as temperature of clarification increases as there seems to be a movement of phospholipids from ghee residue to ghee. Since ghee residue contains both lactose and certain protein in the form of curd particles, this leads to development of brown colour pigments with antioxidant activity upon clarification of ghee. From the results obtained for the antioxidant activity, it can be inferred that the products obtained using DC-Deoni ghee residue may help in extension of shelf life owing to high antioxidant activity.

Hydroxy methyl furfural (HMF) content of ghee residue samples prepared at 120°C clarification temperature

The HMF compounds causes browning in the product, due to the reactions occurring between amino and carbonyl compounds (Keeney and Bassette 1959). The browning compound: 5-hydroxy methyl-2-furfural (HMF) also exhibits an antioxidant property. Among all the samples, the HMF content (65.59µmole/mg) in DC- Deoni was the highest (Figure3). The significant differences ($P<0.05$) were observed in all the five samples and HMF content ranged from 40.39 to 69.59 µmole/mg. The HMF content in ghee residues may be attributed to browning due to the degradation

Table 3 Quality characteristics of ghee residue samples as affected by the breed of cow, method followed for ghee preparation and clarification temperature

Parameter	Variation due to breed		Variation due to method of ghee preparation		Variation due to clarification temperature	
	Deoni (indigenous breed) cows	HF crossbred cows	Direct cream method	Creamery butter method	110°C clarification temperature	120°C clarification temperature
Moisture	13.36 ± 0.33^a	12.34± 0.51^a	11.51 ± 0.28^a	13.10 ± 0.53^a	13.15 ± 0.45^a	11.79± 0.44^b
Protein	30.12 ± 1.18^a	22.37± 2.34^b	21.45 ± 2.15^b	30.71 ± 1.25^a	25.82 ± 2.70^a	27.22± 1.50^a
Fat	57.2 ± 1.19^b	62.95± 1.23^a	63.27 ± 1.13^a	55.38 ± 1.27^b	59.72± 1.68^a	58.22 ± 1.29^a
Lactose	11.68 ± 0.47^a	10.94± 0.49^a	10.83 ± 0.22^b	11.06 ± 0.52^a	10.08± 0.13^a	11.66± 0.54^b
Ash	4.31 ± 0.34^a	3.48± 0.42^a	3.52 ± 0.39^b	4.85 ± 0.39^a	2.68 ± 0.22^b	5.52 ± 0.13^a
Phospholipids	9.38 ± 1.34^a	7.67± 1.34^a	4.12 ± 0.27^b	11.01 ± 1.06^a	8.96 ± 1.47^a	7.14± 1.10^a
scavenging activity)	48.13 ± 5.82^a	39.35± 2.31^a	30.36 ± 0.62^b	54.27 ± 2.90^a	44.91 ± 4.4^a	42.62 ± 3.72^a
Hydroxy methyl furfural (HMF) content	50.65 ± 4.10^a	49.13± 4.10^a	50.88± 4.56^a	47.19 ± 2.96^a	36.69 ± 0.49^b	58.54± 2.64^a
Browning Index (BI)	33.49 ± 2.62^b	39.72 ± 0.35^a	35.61± 1.82^a	39.91 ± 2.08^a	33.3 ± 2.02^b	42.26 ± 2.00^a

(N=12 for DC and N=15 for CB, Results are expressed as Mean ± SE, with different small letters superscript (a,b) within columns differ significantly (P< 0.05) among the samples. Where, DC- ghee residue obtained from processing of cow's milk by direct cream method; CB– ghee residue obtained from processing of cow's milk by creamery butter method)

of lactose or decomposition of sugars and proteins (Yamaguchi et al. 1981). Additionally, Sripad (1988) reported that as clarification temperature increases, the HMF content also increases. Temperature accelerates the browning reactions and it is well known that HMF is a product of high heat treatment or processing. Thus, HMF content was directly proportional to the increase in clarification temperature as the samples obtained at 120°C clarification temperature had higher HMF content than those obtained at 110°C (34.03 to 37.47 µmole/mg). Further, it is vital to note that the amount of lactose was the highest in the DC-Deoni samples (Table 2), which in turn could have contributed towards the browning reaction, thereby leading to higher HMF content in this sample.

Browning index of ghee residue samples prepared at 120°C clarification temperature

The browning in ghee residue could be either due to maillard reaction or caramelization of sugar. In the present study, the GR samples were analysed for BI and it was found that the browning index of CB-HFC (46.91) was significantly highest ($p<0.05$) followed by DC-HFC (43.96), commercial (34.6), CB-Deoni (35.59) and DC-Deoni (33.18) (Figure4).For both the methods of ghee preparation, higher browning index was observed for HF crossbred samples. The higher browning index in HF crossbred samples may be due to presence of free amine groups which might have been formed during processing of cream into ghee (Yamaguchi et al. 1981).

Properties of ghee residue samples as affected by the breed of cow

The effect of breed of cow on the properties of ghee residue has not been reported earlier, so in this study, the effect was evaluated on some properties, which are discussed here. The breed of the cow had significant effect (p<005) on protein, fat and browning index. There are reports in the literature, which suggest that the ghee residue composition is dependent on the raw material used for ghee processing (Santha and Narayanan 1979). On the contrary, there was no significant effect ($p>0.05$) of breed on moisture, lactose, ash, phospholipids, antioxidant activity and HMF (Table 3). However, higher fat content in ghee residue samples obtained from processing of HF crossbred cow milk (62.95 ± 1.23) may be due to the entrapment of fat moieties within the protein matrices or due to process conditions during cream separation such as speed of the bowl and rate of milk inflow. Ghee residue obtained from Deoni cream had higher protein content (30.12 ± 1.18) due to presence of higher total solids (Veeresh et al. 2019).

Browning index of ghee residue samples obtained from the HF crossbred cows was higher ($P<0.05$) than that of Deoni breed. This may be due to lactose degradation or reaction between amino acids and sugars. Further, as the HMF content and

phospholipids content were non-significantly ($p>0.05$) affected by the breed, the antioxidant activity was also found to be similar for both the breeds (Table 3). Since, antioxidant activity depends upon the phospholipids and certain processing products including HMF content, so the similar activity for both the breeds could be attributed to their (phospholipids and HMF) insignificant differences.

Properties of ghee residue samples as affected by the method of ghee preparation

The methods of ghee preparation are responsible for certain constituents in the ghee residue owing to the technological differences which results in fat globule membrane ruptures thereby leaching of certain molecules in the ghee residue from ghee. In the present study, ghee preparation method had significant effect ($p<005$) on proteins, fats, lactose, ash, phospholipids, antioxidant activity but non-significant effect ($p>0.05$) was observed for moisture, hydroxy methyl furfural, browning index (Table3). The protein content of ghee residue obtained by creamery butter method was higher (30.71 ± 1.25) than that obtained from direct cream (21.45 ± 2.15). The reason for higher protein may be due to more retention of curd particles in the butter. The fat of ghee residue obtained by direct cream method was higher than creamery butter method. This may be due to losses of butter fat into ghee residue from ghee, which is obtained from direct cream method, whereas not much fat losses occur in the creamery butter ghee residue. Higher lactose content (11.06 ± 0.52) was found in creamery butter ghee residue owing to higher solids not fat content (4.53%) in the cream itself. Another reason may be due to the higher lactose content (2.47%) in the cream. The lactose present in the raw material might have been the reason for higher lactose in direct cream ghee residue. Ghee residue obtained directly by clarifying cream showed higher fat content (63.27 ± 1.13) which may be due to losses of fat into ghee residue from ghee. Higher ash content was found in creamery butter ghee residue. According to Prahlad (1954) creamery butter ghee residues had higher moisture, ash and protein content whereas, direct cream ghee residues had higher fat and lactose content. Santha and Narayanan, (1978) reported similar results. From this, it can be inferred that the results are comparable with the previous findings.

Higher phospholipid content (11.01 ± 1.06) in creamery butter ghee residue may be due to higher phospholipid content in butter than in cream (Table 3). Similarly, Santha and Narayanan (1979) also reported higher phospholipid content of 17.39% for creamery butter ghee residue and upto 4.94% in direct cream ghee residue. Antioxidant activity increases as phospholipids content increases since phospholipids are responsible for the antioxidant property (Santha and Narayanan 1979). As far as the HMF content is considered, it's a product of maillard reaction, the samples were non-significantly different indicating independence on the method of ghee preparation. Contrary to this, the two major ingredients required for maillard reaction *i.e.*, lactose and amino acids were significantly affected by the method of ghee preparation. This could be attributed to the reaction complexity for formation of HMF, as it is controlled by various factors including type and nature of sugar and amino groups participating in the interaction, temperature, pH and any catalysts e.g., metal ions in the food substrate (Ames 1990, Van Boekel 2001).

Properties of ghee residue samples as affected by clarification temperature

Ghee residue is obtained from heating the cream or butter at temperatures above 100°C and several thermo-chemical reactions are controlled by temperature. Thus, the temperature used for clarification may also affect various ghee and ghee residue properties. With this background, the effect of two clarification temperatures (110°C and 120°C) was evaluated on the properties of GR samples. Moisture, lactose, ash, browning index and HMF content of ghee residue were significantly affected ($p<0.05$) by temperature of clarification while proteins, fats, phospholipids and, antioxidant activity were non-significantly affected ($p>0.05$) (Table 3). Ghee residue from the ghee processed at 120°C had lower moisture content ($11.79 \pm 0.44\%$) than the ghee residue obtained at 110°C ($13.15 \pm 0.45\%$) which may be due to losses of moisture due to evaporation which occurs at higher temperature of clarification. Similar trend with respect to decreasing moisture content with increasing clarification temperature was also reported by Santha and Narayanan (1979). Also, at 120°C, the ash content ($5.52 \pm 0.13\%$) of ghee residue samples were higher due to the minerals concentration resulting from moisture loss at higher clarification temperature.

The HMF content increased significantly ($p<0.05$) with the increase in temperature of clarification (Table 3). This is due to the positive correlation between temperature and the formation of browning pigments, resulting from condensation of lactose and maillard reaction between the sugar moiety and free amine groups. This also led to the changes in colour of the samples from light brown (at110°C) to darker brown (at 120°C) due to condensation of lactose, reaction between sugar and free amine groups and decomposition of sugar (Kiriyaga et al. 1971). Due to this, browning index is also affected. These findings related to HMF content are also supported by the reports of Sripad (1988), where, the amount of HMF in GR was 113.93 µg/g at 110°C clarification temperature and 460.64 µg/g at 120°C, indicating an increase in HMF content with increasing temperature. McGookin (1991) reported that lactose degradation also contributes to higher HMF content. Thus, the results obtained for HMF content and browning index of the ghee residue support the foundation of increasing maillard reaction products upon increasing the clarification temperature used for ghee manufacture.

Conclusion

It could be observed from the present study that ghee residue composition was affected by ghee preparation method. The cow's breed had significant ($p<0.05$) effect on the protein, fat and the browning index of the ghee residue, with higher protein for the ghee residue obtained from processing of Deoni cow's milk and higher fat for HF crossbred cows. The method of ghee preparation significantly ($p<0.05$) affected the protein, fat, lactose, phospholipids content and antioxidant activity with higher protein, phospholipids content and antioxidant activity; while lower fat and lactose content were found for the ghee residue obtained from creamery butter method. The moisture and lactose content of ghee residue decreased significantly ($p<0.05$) while; hydroxy methyl furfural (HMF) content and ash content increased significantly ($p<0.05$) with increasing clarification temperature for ghee preparation. Thus, the present study gave good insights for the factors affecting ghee residue composition.

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RESEARCH ARTICLE

Comparative study of aspartame and neotame stability in Ice cream and Cake

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Abstract: The suitability of artificial sweeteners aspartame and neotame for the incorporation in products like ice cream and cake was studied. These sweeteners were compared for their stability during processing and storage and the effect of addition on different physico-chemical properties were also considered. The recovery of the HPLC method used for the estimation of aspartame and neotame from ice cream and cake was 96.22-98.57% and 96.08-98.62%, respectively. During pasteurization of ice cream mix (68°C/30 min), 25% of aspartame was degraded, however, no loss of neotame was observed. Aspartame and neotame content decreased significantly from 74.55 to 62.97% and 99.42 to 89.93%, respectively during storage (-18°C/90 days) of ice cream. During baking (180°C/20 min), the degradation of aspartame and neotame was about 50 and 13%, respectively. However, aspartame and neotame content decreased significantly from 49.90 to 24.61% and 87.20 to 62.40%, respectively during storage (25°C/20 days) of cake.

Introduction

Neotame (Dimethyl butyl-aspartyl phenylalanine-methyl ester) (NTM), a derivative of aspartame (Aspartyl phenylalanine-methyl ester) (APM) is gaining popularity in the food industry due to its greater sweetness potency (30-60 time sweeter than APM), higher stability at neutral pH with no adverse health effects as compared to APM (Kumari et al. 2016b). Besides the approval from FDA as food additives, APM consumption always remains controversial among the consumers, as some of the published reports suggested APM's ill health effects and regenerated controversy among consumers (Landrigan and Straif, 2021; Soffritti et al. 2007, Soffritti et al. 2010; Belpoggi et al. 2006; Andreatta et al. 2008; Tibaldi et al. 2020). However, more than 100 studies claimed its safety and negate any association with adverse health effects (EFSA, 2006; EFSA, 2013; Butchko et al. 2002). In 1996, FDA declared APM to be safe at a daily acceptable level of 50 mg/kg/day, except for phenylketonuric person. Phenylalanine (Phe), a degradation product of APM is toxic for phenylketonuric persons as it results in mental retardation (Harper, 1984). However, in NTM, the presence of dimethyl butyl (DMB) group restricts the production of Phe and makes it safer for consumption. Additionally, DMB also provides remarkable sweetness potency, flavour enhancing property, and stability during the baking process. Since 2002, NTM is permitted in the food industry as a general-purpose sweetener but still not extensively used in food products. The stability of the sweetener is greatly affected by pH, temperature, duration and water activity (Tsoubeli and Labuza, 1991). These sweeteners are most stable between pH 4 to 5 (Kumari et al. 2016b). At alkaline or neutral pH, APM can be degraded into different degradation products like aspartyl-phenylalanine (ASP-Phe), which may be further hydrolyzed into aspartic acid or Phe or may cyclize into diketopiperazine (DKP) etc. At acidic pH, α-APM can be isomerized into β-APM, which can be further degraded into aspartyl-phenylalanine (Prodolliet and Bruuelhart, 1993). NTM when used at a much higher concentration (200 ppm) in a beverage (pH 3.2, stored at 20°C/8 weeks) only de-esterified NTM was the principal degradation product (7% of original NTM), however, three minor degradation products less than 1% concentration was obtained by cyclization, beta arrangement or hydrolysis. At a lower initial concentration (15 ppm) these products were not detected (JECFA 52, 2004). Very few published reports are available regarding the stability of NTM in the food system. APM when used in products like yoghurt (heating temperature 85°C/30 min and pH 4.5) and flavoured milk (temperature 90°C/20 min, pH 6.6) about 40-50% of APM was degraded into ASP-Phe, however only 3 to 7% of NTM was degraded at same condition (Kumari et al. 2016b, 2018).

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APM was completely degraded into ASP-Phe and Phe during the preparation of in-bottle sterilized milk, however, 50% NTM was degraded (Kumari et al. 2016b). The stability of APM was also studied in chocolate milk (Keller et al. 1991b), *burfi* (Arora et al. 2010), whey lemon beverage (Arora et al. 2013), orange flavoured soft drinks by HPLC (Yakici and Arici, 2013). APM sweetened cake was evaluated by several workers for its sensory properties (Wetzel et al. 1997; Baeva 2000; Nourmohammadi et al. 2011) and this sweetener was found to be a successful sweetener in frozen desserts also (Keller et al. 1991a; Guzeler et al. 2011, Rathod et al. 2013). Cake and ice cream are the most widely consumed products by all age groups and have potential industrial applications for sweeteners. But before using any artificial sweeteners in these products, their stability and breakdown products should be properly analyzed. The acceptability of APM in ice cream and cake in terms of sensory properties has been reported in the literature, however, the relevant information is still lacking on its stability in ice cream and cake during processing and storage. Data also lacks on comparative stability study of APM and NTM as affected by different processing parameters in these products.

Hence, the present study was therefore designed to analyze the stability of APM and NTM by HPLC method in ice cream and cake during processing and storage. The effect of different processing parameters such as pH, temperature, water activity, and storage period was also studied.

Materials and Methods

Water and acetonitrile (HPLC grade), aspartyl-phenylalanine methyl ester (aspartame), L-phenylalanine were procured from Sigma-Aldrich (Layoffs, Missouri, USA). HPLC grade potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Qualigens fine chemicals (Mumbai, India). The degradation product L-Aspartyl-L-phenylalanine was procured from Sigma-Aldrich (Steinheim, Germany). Zinc sulphate and potassium ferrocyanide were procured from S.D. Fine Chem. Ltd. (Mumbai, India). SPE cartridge DSC-18LT Discovery (6 ml tube of 1 g capacity) and Vacuum assembly Visiprep™ DL were procured from Supelco (Bellefonte, PA, USA).

Milk sample was obtained from the dairy plant of National Dairy Research Institute (Karnal, India). APM and NTM were procured from NutraSweet Sweetener Company (Georgia, USA). Polydextrose was procured from Danisco India Private Limited (Gurgaon, India) and maltodextrin from M/S Sukhjit Starch and Chemicals limited (Punjab, India). The wheat flour was obtained from Victoria foods Private Limited (New Delhi, India) and shortening from Bunge India Private Limited (Mumbai, India). The cornflour and baking powder were obtained from Weikfield foods Private Limited (Maharastra, India). Whey protein concentrate-70 (WPC-70) was procured from Modern Dairies Limited (Haryana, India) and cake gel from AB Mauri India Private Limited (Maharashtra, India). The vanilla flavour was obtained from Ajanta Enterprises (Himachal Pradesh, India) and the mixture of stabilizer and emulsifier from Danisco India Private Limited (Gurgaon, India).

Preparation of Ice cream

Ice cream was prepared according to the method described by Kumari et al. (2016a). The sugar was completely replaced by APM and NTM in ice cream at an acceptable level of APM (1200 mg/kg ice cream mix) and NTM (30 mg/kg ice cream mix). As a bulking agent, WPC-70 (1.5%) and maltodextrin (9.9%) were used.

Preparation of Cake

The cake was prepared according to the method of Kumari et al. (2016a). For the preparation of APM and NTM sweetened cake, the sugar was completely replaced by the best acceptable level of APM (7000 mg/kg cake mix) and NTM (60 mg/kg cake mix), respectively.

Extraction of APM and NTM from Ice cream

For the extraction of APM, the APM sweetened ice cream sample was first degassed using on an ultrasonication bath for 20 min at 30°C. Ten grams of the degassed sample were taken in a 100 ml beaker. 50 ml aqueous methanol solution (20%) was added into it and sonicated for 20 min at 40°C. It was cooled to ambient temperature (30°C) and transferred to 100 ml volumetric flask. Three milliliters of each carrez I (3.6 g potassium ferrocyanide in 100 ml water) and carrez II (7.2 g zinc sulphate in 100 ml water) were added into it and kept undisturbed for 10 min. Diluted up to the mark by water (HPLC grade) and filtered through filter paper (Whatman no.1). The NTM extraction from ice cream was done as per the method of kumari et al. (2016a). The filtrate is further purified by passing through an SPE cartridge.

Extraction of APM and NTM from cake

For the extraction of APM, five grams of APM sweetened cake sample was added into 50 ml of aqueous methanol solution (20%). After vortex for 2 min, it was sonicated for 20 min at 40°C. It was cooled to ambient temperature (30°C) and centrifuged (Kubota Corporation 6800, Tokyo, Japan) for 10 min at 2000×g. The supernatant was transferred into a 50 ml volumetric flask and added into 2 ml carrez 1 and II each and shaken vigorously. It was kept undisturbed for 10 min at ambient temperature (30°C), diluted up to the mark with water (HPLC grade) and filtered through filter paper (Whatman no.1). Further purification was done by passing through the SPE cartridge. The extraction of NTM from cake was done as per the method described by Kumari et al. (2016a).

The solid-phase extraction of APM and NTM from sample filtrate was done as per the method described by Kumari et al. (2016b). The 20µl of extracts were filtered through a Hamilton microlitre

syringe filter (Hamilton Company, Nevada, USA) before being injected into the HPLC system.

HPLC conditions

Waters HPLC apparatus equipped with PDA detector (Waters 2998, Massachusetts, USA), Empower 2 software and manual injector with 20 µl sample loop were used for the analysis of sweeteners. Phenomenex C_{18} column (4.6×250 mm, pore size 100 Å, particle size 5 µm) maintained at 40°C was used for analysis of sweeteners. APM, NTM and its degradation products were quantified according to the method described by Kumari et al. (2016a and 2016b). APM and its degradation products (Phe and ASP-Phe) were analyzed under gradient conditions as described by Kumari et al. (2016b). The mobile phase A consisted of 0.02 M phosphate buffer, pH 5.0: acetonitrile (97:3) and mobile phase B consisted of 0.02 M phosphate buffer, pH 3.5: acetonitrile (80:20). The gradient condition is depicted in Table 1. The flow rate of the mobile phase was 1 ml/min and wavelength for analysis was 200 nm. NTM was analyzed under the isocratic condition as followed by Kumari et al. (2016a). The mobile phase consisted of a 60:40 mixture of acetonitrile and water (0.09%TFA) was used at 0.6 ml/min flow rate and 210 nm wavelength.

Method validation

Individual solutions of APM, ASP-Phe, Phe (5-100 mg/l) were prepared in mobile phase A and B (1:1) and NTM solution (5-100 mg/l) was prepared in methanol: water (20:100). These prepared solutions were used for the preparation of the standard curve, which was plotted by taking concentration against peak area.

The LOD and LOQ were determined by using the formula LOD= 3 (standard deviation of curve's response)/calibration curve slope and LOQ= 10 (standard deviation of curve's response)/calibration curve slope.

The precision and accuracy of the method were calculated according to the method mentioned by Kumari et al. (2016a).

The percentage recovery of the method was calculated by using the following formula:

$$\% \text{ Recovery} = \frac{X}{Y} \times 100$$

Where,

X = amount of sweetener recovered

Y = amount of sweetener added to the product

pH

The pH of ice cream and cake was determined by the method as described in IS: SP: 18, part XI (1981) and AOAC method (2005), respectively.

Water activity

The water activity was measured by water activity meter "Aqual Lab' (Model Series 3 TE) supplied by Decagon Devices, W. A., USA. (Plate-6).

Stability monitoring

Effect of pasteurization and baking

The ice cream mix sample was cooled to 4-7°C just after heating the ice cream mix (68°C/30 min) and was analyzed in triplicate for the sweeteners in ice cream mix

The cake was cooled to ambient temperature (30°C) after baking (180°C/20 min) and was analyzed in triplicate for the sweeteners in cake

Storage stability

Ice cream samples were stored at -18°C for 90 days and samples were analyzed weekly during storage for the sweetener content.

Cake samples were stored at 25°C for 20 days and samples were analyzed at 0, 4th, 8th, 12th, 16th and 20th days of storage the sweetener content.

Statistical analysis

Means, relative standard deviation (RSD), standard error mean (SEM), 95% confidence intervals and linear regression analysis were evaluated by Microsoft Excel (2007). A significant difference between mean values was tested using ANOVA.

Results and Discussion

Method validation

Table 2 represented the correlation coefficients and regression equation of the standard curve. The correlation coefficient for

Table 1 Gradient programming for detection of Aspartame and its degradation products

Time (min)	A concentration (%)	B concentration (%)
0.1	100	0
8	100	0
13	0	100
25	0	100
27	100	0
30	100	0

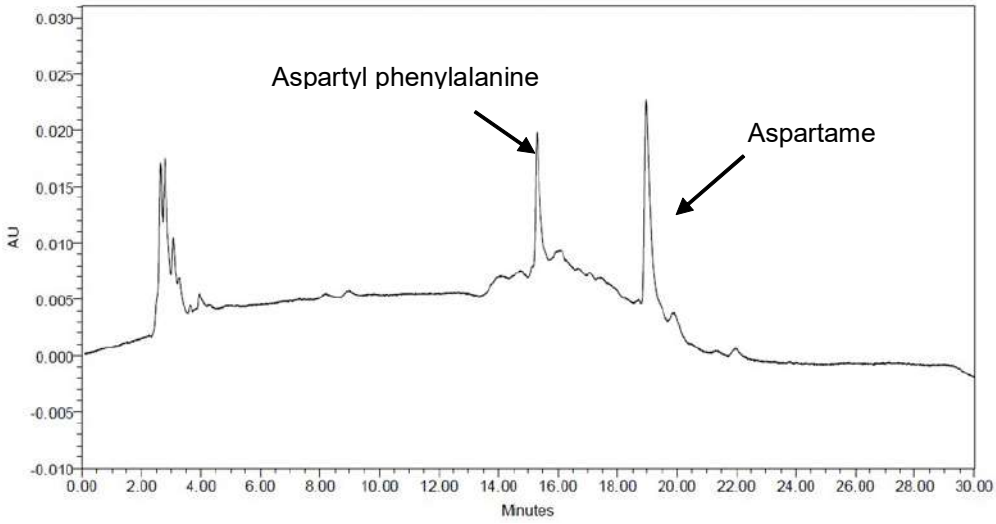

Fig. 1a Chromatogram of aspartame and its degradation product in ice cream

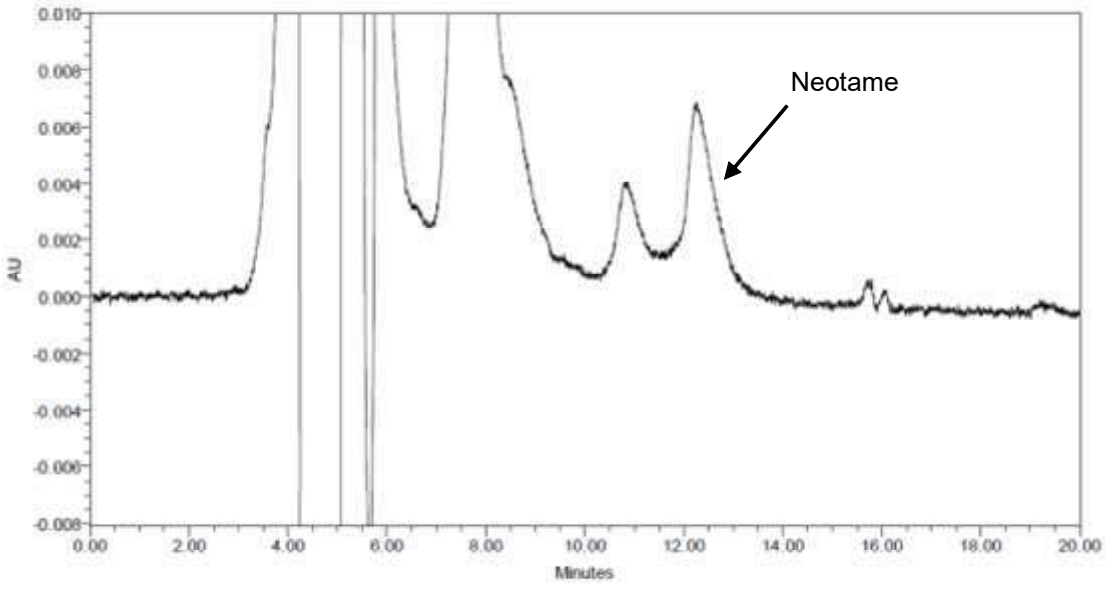

Fig. 1b Chromatogram of neotame in ice cream

Table 2 Sensitivity and Linearity of the method

Sweetener/Degradation product	Regression equation	R^2 Correlation Coefficient	LOD (mg/l)	LOQ (mg/l)
Aspartame	y = 45137x-4036.5	1.0	1.5	4.5
Aspartyl-phenylalanine	y = 8683.7x-21040	0.998	1.0	3.0
Phenylalanine	y = 30095x-14721	0.9989	1.0	3.0
Neotame	y = 54004x-32859	1.0	0.25	0.50

APM, NTM and its degradation product was greater than 0.99. The calibration curve was found to be linear from 5 to 100 mg/l concentration.

Table 2 represented the values of LOD and LOQ. A similar value of LOD for APM was reported by George et al. (2010), Lawrence and Charbonneau (1988) and Arora et al. (2008; 2010; 2013) by using the C_{18} column at wavelength 200 nm. Our results for NTM were in accordance with Yang and Chen (2009 and 2010). The chromatogram for APM, NTM and its degradation products in ice cream and cake samples were represented in Figures 1a, 1b, 2a and 2b.

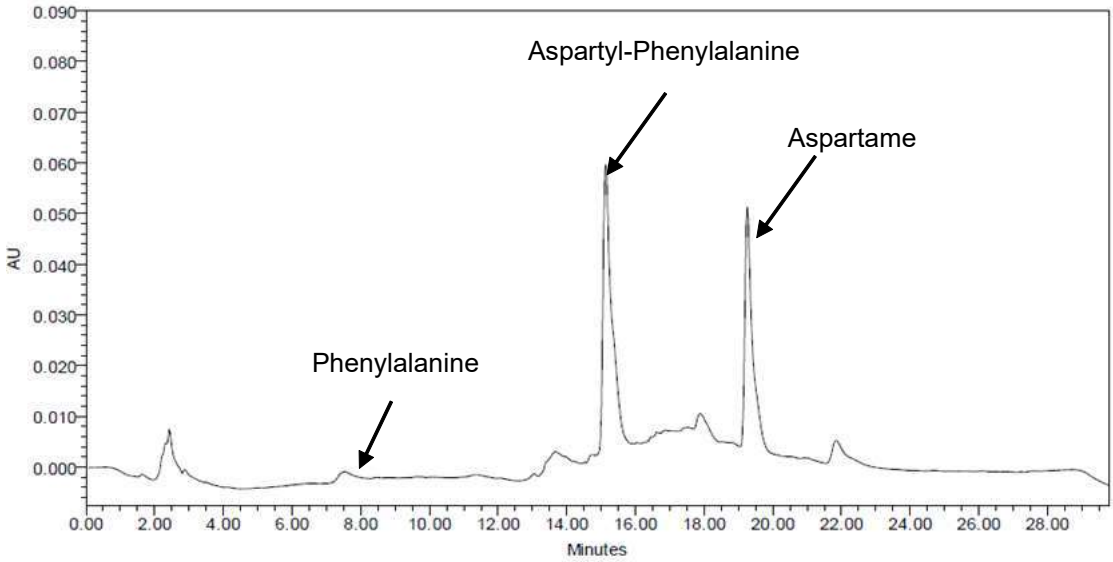

Fig. 2a Chromatogram of aspartame and its degradation products in cake

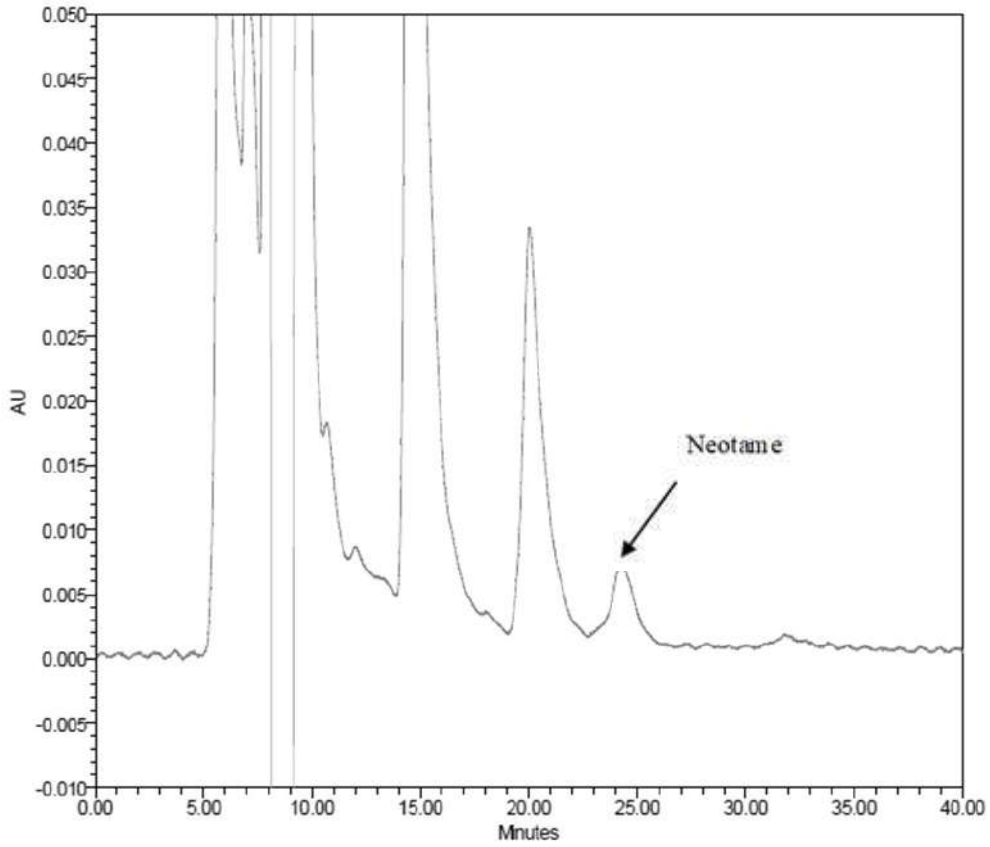

Fig. 2b Chromatogram of neotame in cake

The accuracy was represented as per cent recovery of a specific component in the sample, the recovery (mean±%RSD) of APM and NTM from ice cream and cake is depicted in Table 3 and 4, which shows the high accuracy of the method.

The values for interday and intraday precisions (% RSD) are depicted in Table 3 and 4. All these values were within the 5% limit, indicating repeatability of the method.

pH

The stability of sweeteners is greatly affected by the pH of the food (Kumari et al. 2016b). The pH of ice cream (6.5) and cake (6.9) is close to neutral pH. The activation energy of the sweetener degradation is closely related to the pH condition and it is lower at neutral pH (Ozol, 1986). The stability with respect to pH needs to be monitored during the storage period in the present study.

In the case of ice cream, no significant ($p>0.05$) difference among the sample of control, APM and NTM sweetened ice cream was observed. A significant decrease in the pH of control (6.57-6.52), APM sweetened (6.55-6.52) and NTM sweetened (6.56-6.52) ice cream was observed during the 0^{th} to 90^{th} day of storage at -18°C.

There is a slight decrease in the pH of control (7.05-6.97), APM (6.91-6.85) and NTM (6.93-6.86) sweetened cake during the storage period (25°C/25 days), however, the pH of control, APM and NTM cake did not differ significantly ($p>0.05$) both from each other and throughout the storage period.

Water activity

There is a combined effect of pH and water activity on the activation energy of sweeteners degradation (Bell and Labuza, 1991a). At pH 7 the activation energy of sweetener degradation declines on increasing water activity from 0.56 to 0.99 (Bell and Labuza 1991b). Due to these reasons, the present study monitors the water activity along with the sweetener stability during the storage period.

The water activity of the cake containing APM and NTM was significantly higher ($p<0.05$) than the control (cake with sucrose) (Table 5). The lower water activity of the control cake is due to the presence of sugar, which could not be compensated by the bulking agents (maltodextrin and polydextrose) used for APM and NTM sweetened cake (Wetzel et al.., 1997). The water activity of control, APM and NTM sweetened cake significantly ($p<0.05$) decreases during the storage (25°C/25 days).

Stability of APM and NTM during processing of ice cream and cake

In APM sweetened ice cream, pasteurization (68°C/30 min) of the ice cream mix resulted in partial degradation of APM into ASP-Phe. Results revealed that 74.55±0.08% of APM remained intact and the degradation of APM resulted in the formation of ASP-

Table 3 Precision and Accuracy of the method for analysis of APM (n=6)

Sample	Concentration added (mg/l)	Observed concentration	% Recovery	Intraday Precision (%RSD)	Interday Precision (%RSD)
Ice cream	Low level (1000)	985.7	98.57	0.61	0.86
	Medium level (1200)	1178.64	98.22	0.57	0.92
	High level (1400)	1378.44	98.46	0.85	0.99
Cake	Low level (1000)	962.2	96.22	1.25	1.25
	Medium level (5000)	4815.5	96.31	0.85	1.17
	High level (7000)	6759.9	96.57	0.89	1.21

Table 4 Precision and Accuracy of the method for analysis of NTM (n=6)

Sample	Concentration added (mg/l)	Observed concentration	% Recovery	Intraday Precision (%RSD)	Interday Precision (%RSD)
Ice cream	Low level (20)	19.71	98.54	1.43	1.17
	Medium level (40)	39.43	98.57	0.85	1.86
	High level (60)	59.17	98.62	0.92	1.52
Cake	Low level (25)	24.04	97.98	1.86	2.85
	Medium level (60)	58.78	96.17	1.34	2.70
	High level (100)	96.08	96.08	1.86	2.15

Table 5 Water activity of cake during storage

Storage period (days)	Control	APM Sweetened cake	NTM Sweetened cake
0	0.83 ± 0.01^{aD}	0.91 ± 0.01^{bD}	0.92 ± 0.01^{bD}
4	0.79 ± 0.01^{aC}	0.87 ± 0.01^{bC}	0.87 ± 0.01^{bC}
8	0.76 ± 0.02^{aB}	0.83 ± 0.01^{bB}	0.84 ± 0.01^{bB}
12	0.76 ± 0.01^{aB}	0.82 ± 0.03^{bB}	0.83 ± 0.01^{bB}
16	0.76 ± 0.01^{aB}	0.83 ± 0.01^{bB}	0.84 ± 0.01^{bB}
20	0.74 ± 0.01^{aA}	0.80 ± 0.01^{bA}	0.79 ± 0.01^{bA}

Values are expressed as means±SEM (n=3)

$^{a-b}$Means within rows with dissimilar lower case superscript differ significantly ($p<0.05$)

$^{A-D}$Means within the column with dissimilar uppercase superscript differ significantly ($p<0.05$)

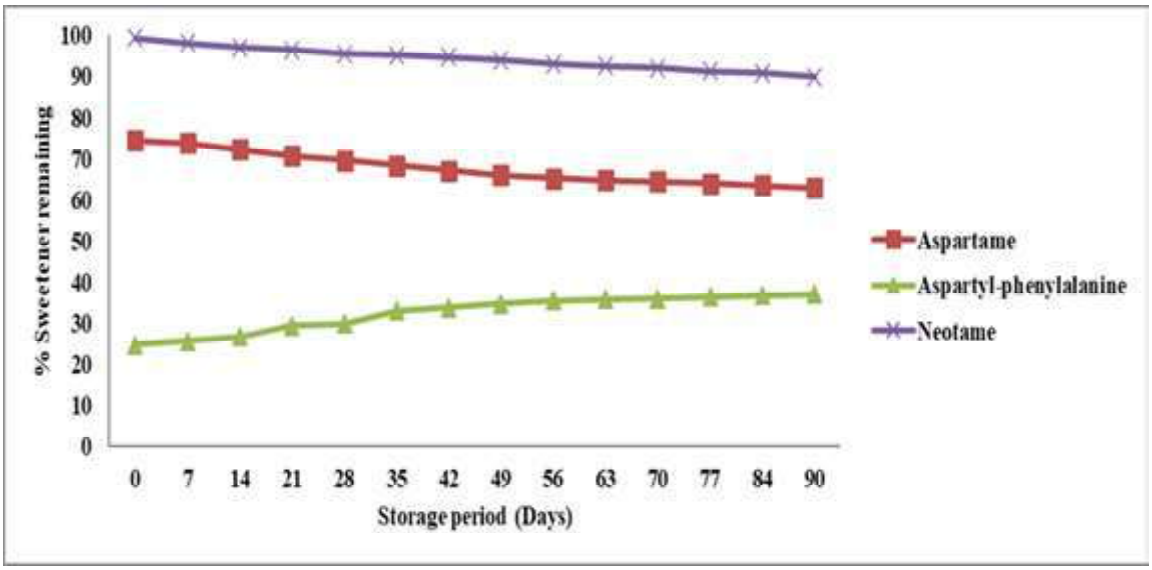

Fig. 3 Levels of sweeteners and degradation products in ice cream during storage (-18°C/90 days)

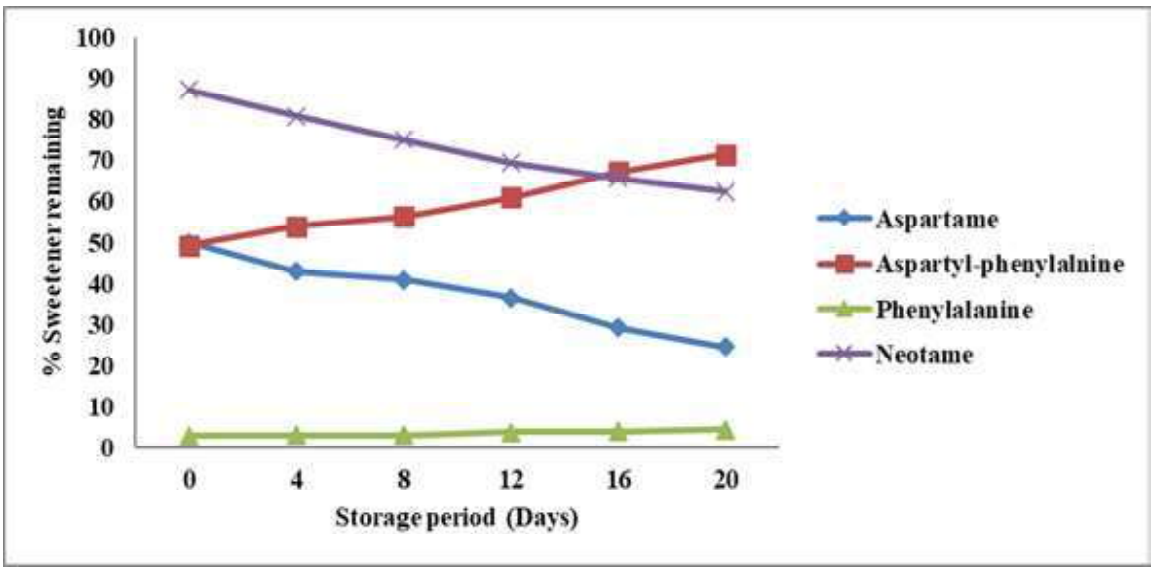

Figure 4. Levels of sweeteners and degradation products in cake during storage (25°C/20 days)

Phe (24.81±0.12%). However, in NTM sweetened ice cream, after pasteurization 99.27 ± 0.21% of the NTM remained intact.

During the preparation of APM sweetened cake, baking (180°C/ 20 min) resulted in degradation of APM into ASP-Phe and Phe. During baking, 49.30±0.20% of APM remained intact and 49.72±0.09% ASP-Phe and 2.85±0.01% Phe were formed. However, in the case of NTM sweetened cake, during baking 87.29 ± 0.19% of NTM remained intact.

Our results were in accordance with Wetzel and Bell (1998) and Prodolliet and Bruuelhart (1993), who reported that APM is sensitive to degradation when heated for a longer duration at pH >6 and can be hydrolyzed into ASP-Phe or Phe at this pH. Heating (63°C/30 min) of APM containing phosphate buffer (0.1 M, pH 7.0) resulted in 83% loss of APM (Tsoubeli & Labuza, 1991). In pasteurized flavoured milk (90°C/20 min), about 40% of APM was degraded into ASP-Phe (Kumari et al. 2016b). However, in in-bottle sterilized flavoured milk (121°C/15 min), APM was completely degraded into ASP-Phe and Phe. At temperature 136°C/15 sec and pH 6.0, 42% APM loss was observed by O'Donnell (2012). In cake, 33-34% encapsulated APM was recovered after baking, while non-encapsulated APM recovery was 22% (Wetzel and Bell, 1998).

Stability of APM and NTM during storage

In APM sweetened ice cream, the amount of APM significantly decreased ($p<0.05$) in the range of ~75 % to 63% during the storage period (-18°C/90 days). However, the amount of ASP-Phe increased significantly ($p<0.05$) in the range of ~25% to 37% during this period (Figure 3). During storage of NTM sweetened ice cream, the amount of NTM reduced significantly ($p<0.05$) from ~99% to 90% (Figure 3). Although the ice cream

pH (6.5) is not optimal for sweeteners stability, the rate of degradation was slower due to the frozen temperature at storage conditions. Our results were in agreement with Gloria (2003) who reported that in frozen dairy dessert (pH 6.5-7.0), due to frozen state the degradation rate decreased and the maximum stability of sweetener was detected in the lower temperature range used for frozen and refrigerated storage. Besides, due to the lower amount of free moisture, APM stability is more than expected (Abegaz et al. 2012). Our results were in accordance with Nofre and Tinti (2000) who stated that the degradation rate of NTM is lower at a lower temperature. During storage of in-bottle sterilized milk (30°C/60 days) and pasteurized milk (4-7°C/7days), the amount of NTM declined significantly from 50.36 to 8.67% and 91.78 to 87.18%, respectively (Kumari et al. 2016b).

During storage of APM sweetened cake (25°C/20 days), the APM level decreased significantly ($p<0.05$) from ~50% to 25%, however, a significant increase in the level of ASP-Phe (from ~49% to 71%) and Phe (from ~3% to 4%) was observed (Figure 4). The pH of the cake was about 6.9 during storage which is favourable for APM degradation (Homler, 1984; Ozol, 1986; Tsoubeli & Labuza, 1991; Bell & Labuza, 1991b and Gloria, 2003). The amount of NTM declined significantly ($p<0.05$) from ~87% to 62% during the storage of NTM sweetened cake (pH 6.9) (Figure 4). Our results were in accordance with Nofre and Tinti (2000) who found 85% recovery of NTM after baking and on storage at room temperature for 5 days, 81% NTM was retained. Alkaline pH, storage temperature and duration all combinedly affect the sweetener degradation.

Conclusions

In ice cream, pasteurization of ice cream mix resulted in 25.35% loss of APM and the APM amount was decreased to about 12% during storage. However, no loss of NTM was found during pasteurization but NTM amount was decreased to 9% during storage. The extent of decrease of both the sweeteners was comparatively slower due to frozen conditions. In case of cake, 50.7% of APM was lost by baking and about 25% of the loss was observed during storage period. However, only 13% of NTM was lost during baking and about 24% during storage. Heating at a higher temperature for a longer duration resulted in faster degradation of sweeteners. APM was found to be more sensitive towards heat treatment as compared to NTM. Thus NTM was found to be more stable as compared to APM during processing and storage conditions of ice cream and cake. These features allow NTM's application in high heated products.

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RESEARCH ARTICLE

ACE- inhibitory and antioxidant activities in probiotic Cheddar cheese incorporated with Inulin and Whey Protein Concentrate

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Abstract: The aim of this study was to compare angiotensin-converting enzyme (ACE) - inhibitory and antioxidant activities of Cheddar and probiotic Cheddar cheese using *Lactiplantibacillus plantarum* DSM 20174 with inulin and whey protein concentrate (WPC). Water soluble extracts (WSE) of probiotic Cheddar cheese exhibited higher ACE-inhibitory activity than their Cheddar cheese counterparts. A similar trend was observed for antioxidant activity. The highest antioxidant activity (2183.55 µM of Trolox) was obtained when cheese supplemented with WPC and probiotic (PCW) after 6 months of ripening among all the samples followed by probiotic cheese with inulin (PCI). Similarly, PCW had highest ACE- inhibitory activity (88.51%) after 6 months of ripening. The electrophoretic and RP-HPLC profiles indicated that the rate of degradation of proteins resulted in formation of smaller peptides were highest in PCW followed by PCI among all the samples. The results suggest the potential use of WPC in presence of *L. plantarum* DSM 20174 for production of Cheddar cheese enhanced ACE-inhibitory and antioxidant properties.

Keywords:ACE- inhibition, Antioxidant, Probiotic Cheddar Cheese, Inulin, WPC

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Introduction

Many chemical, biochemical changes occur during cheese ripening and these improve the taste, flavour and nutrimental properties of the cheese. Among the milk constituents, milk proteins and its hydrolysis/ hydrolysate received a renewed interest related to their physiological role in humans and are the main source of biologically active peptides (Pihlanto 2006). Most of these peptides are encrypted within the primary structure of the native protein and released from milk protein sequences by digestive proteases, microbial or plant enzymes or by fermentation using dairy starter cultures with proteolytic activities. Such peptides posses various biological properties including antimicrobial (Meira et al. 2012 ; Mohanty et al. 2014), cholesterol-lowering (Hartmann & Meisel 2007), mineral-binding (Vegarud,et al. 2000), immunomodulatory, opioid, antioxidative and antihypertensive (Korhonen 2009; Moslehishad et al. 2013; Perna et al. 2015).

Angiotensin-I-converting enzyme (ACE) increases blood pressure by converting angiotensin- I to angiotensin-II, a potent vasoconstrictor. ACE also inactivates the vasodilating peptide bradykinin (nonapeptide) and endogenous opioid peptide Met-enkephalin. ACE inhibition results in an antihypertensive effect and may also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity. These peptides have already been isolated from a variety of fermented dairy products including cheese (Hartmann and Meisel 2007; Lu et al. 2016), yoghurt (Donkor 2007) and fermented bovine milk (Qian et al. 2011).

The role of free radicals and active oxygen species in various diseases, including aging, cancer, inflammation, neurodegenerative disorder, cardiovascular disease, cataract and the toxicity of numerous compounds, has been well documented. The body has its own dependent system against reactive oxygen species (ROS) on the basis of antioxidant enzymes (i.e. superoxide dismutase, catalase and glutathione peroxidase) and endogenous antioxidant (i.e. glutathione). Oxidative stress occurs when ROS overload the body's antioxidant defense or when the antioxidant defenses system loses its capacity for response (e.g. elderly people) and can lead to damage of vital cellular components. To

enhance antioxidant defense, the body must be provided with a constant supply of antioxidants through proper diet (Herna´ndez-Ledesma et al. 2005). The search for natural antioxidants as alternatives to synthetic ones is a subject of great interest nowadays. In recent years, some of the milk protein-derived bioactive peptides have been considered as a novel class of antioxidants (Pihlanto 2006). Health-Promoting role of *Lactobacillus plantarum* recently denominated as *Lactiplantibacillus* (Lpb.) *plantarum* isolated from fermented foods has been reviewed by Garcia-Gonzalez et al. (2021). Food-associated *Lpb. plantarum* showed a good adaptation and adhesion ability in the gastro-intestinal tract and the potential to affect host health through various beneficial activities, e.g., antimicrobial, antioxidative, antigen toxic, anti-inflammatory and immunomodulatory, in several in vitro and in vivo studies.

The benefits of the fermented dairy products in the diet are well accepted. The central role of microorganisms in fermentation, especially Lactic Acid Bacteria (LAB) is now widely acknowledged, and it is accepted that these microorganisms can exert beneficial effects through two mechanisms: direct effects or indirect effects during fermentation where these microbes act as cell factories for the generation of secondary metabolites with health promoting properties. Among the latter the most important components in fermented milk are bioactive peptides released from milk proteins by members of the genera *Lactobacillus*, *Bifidobacterium* and other LAB. There are few reports on the bioactive peptides release in cheeses during ripening.

Consumers are becoming more aware that foods directly contribute to their health improvement. Therefore, they are in search of a food which would facilitate to improve their health, i.e. functional food which contributes to reduce the risk of disease occurrence and helps in promoting health apart from satisfying hunger. Probiotic and prebiotic are combined to form a synbiotic food. *Lpb. plantarum* is versatile probiotic organism with various health benefits and it is very compatible with cheese matrix (Milesi et al. 2008). Enzymes produced by this microorganism hydrolyze cheese proteins and generate large and intermediate sized peptides which may be further hydrolyzed into smaller peptides and free fatty acids (Lynch et al. 2014) .More proteolytic activity of *Lactobacillus plantarum* (new *Lpb. plantarum*) stains in cheddar cheese as adjunct cultures has been observed by Duan et al. (2019). Inulin is a widely used prebiotic. It is a soluble dietary fibre forming a subset of nutraceutical ingredients that are increasingly used in food products. WPC is considered as functional ingredient. It serves as a source of nitrogen and amino acids. Incorporation of probiotic enhances the proteolysis and accelerates cheese ripening which improves functional properties. It is presumed that inulin & WPC act as growth promoters for probiotic bacteria, they may enhance functional properties and helps to reduce ripening time.

In this study, it is hypothesized that development of Cheddar cheese incorporated with functional ingredients (inulin & WPC) in presence of probiotic (*Lactobacillus plantarum*) would increase the rate of proteolysis and lipolysis. Higher proteolysis means formation of more number of low molecular weight bioactive peptides. These peptides have been proved to have beneficial health effects like being antihypertensive (with ACE-inhibitory property), opioid, antimicrobial, immunomodulatory and mineral binding activities. Thus, this study was aimed to enrich probiotic cheddar cheese with inulin & WPC and to assess their impact on rate of proteolysis and functional properties like antihypertensive & antioxidant activities.

Materials and Methods

The pooled cow milk, used for the manufacture of cheddar cheese, was obtained from Experimental Dairy Plant of National Dairy Research Institute, Bengaluru. Mesophilic mixed starter consisting of *Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis* was obtained from dairy bacteriology unit of the Institute. The probiotic culture of *Lpb.* DSM 20174 was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Two additives were used to prepare different types of Cheddar cheese: Inulin as prebiotic from M/s. DKSH India Pvt. Ltd. and Whey protein concentrates (WPC 70 % protein) as growth promoter from M/s Mahan proteins India Ltd. were procured for this investigation.

Cheddar cheese manufacture

Cheeses were manufactured according to the standard procedures (Kosikowski 1977). Whole milk was standardized with skim milk to adjust casein to fat ratio of 0.68- 0.70 and then pasteurized at 63 ºC for 30 min. The milk was then cooled to 30-31 ºC before inoculation of the culture. Cheddar cheese starter culture was added at 0.5% (v/v) to prepare control Cheddar cheese (CC), Cheddar cheese with inulin (CI) and Cheddar cheese with WPC (CW). Additional 1% probiotic bacteria was added along with 0.5% cheese starter culture for control probiotic Cheddar cheese (PCC), probiotic Cheddar cheese with inulin (PCI) and probiotic Cheddar cheese with WPC (PCW). The milk was kept for ripening for 30 min before the addition of rennet @ 3 g per100 L of milk. The milk coagulated after 45 min and the resulted curd was cut with cheese knives. The curd was cooked to 38 -39 ºC with slow agitation. Whey was drained and curd was cheddared at 39 ºC and turned every 15 min until the acidity increased to 0.45-0.50 % lactic acid. The cheddared curd was milled. Milled curd of 5 kg lots was mixed with 2% additive and 1% salt on the curd basis individually. The curd was hooped in cheese moulds and pressed overnight. The cheese blocks were turned next day and pressed further for 24 h. After pressing, cheese blocks were removed from press and kept in ripening room for 2 days for surface drying and then paraffined. The paraffined blocks of cheese were stored

at 7± 2 ºC for ripening. Three replicates of experimental (CI, CW, PCI & PCW) and control cheddar cheese (CC & PCC) were made and analysed for the ACE- inhibitory and antioxidant activity at zero day, 6 months and 10 months.

Preparation of Water soluble extract

Water soluble extracts of all Cheddar cheese were prepared using the method developed by Kuchroo and Fox (1982), with some modification. Grated Cheddar cheese of 20 g was mixed with 60 mL of distilled water in small mixer. The mixtures were centrifuged at 4250 g for 30 min at 4 ºC (cooling centrifuge, REMI, Mumbai). The fat layers were removed and the water extracts were filtered through Whatman No.1. The pH of the extracts was adjusted to 4.6 using 1N HCl. The precipitated proteins were removed by filtering through Whatman No.1. To further remove any impurities, the water soluble extracts were filtered through 0.22 µm pore size filter (Millipore, Billerica, MA, USA). The water soluble extract was kept in deep freeze and used for HPLC, ACE and antioxidant activity.

Analysis by Urea-PAGE

The Cheddar cheese samples were analysed by alkaline urea-polyacrylamide gel electrophoresis using the method of Creamer (1991). Samples were prepared by taking 0.3 g of each cheese into 15 mL of sample buffer (0.092 g EDTA, 1.08 g Tris base, 0.55 g boric acid, 36 g urea made up in 100 mL and adjusted to pH 8.4). Each sample was centrifuged at 3000 rpm for 30 min. 1 mL of middle portion was taken and were mixed with 3% each of 0.1% (w/v) bromophenol blue solution and mercaptoethanol. The resolving gel solution was made using 8 mL of a 30% solution of " acrylamide/ BIS 37.55:1.0" and 11.9 mL of resolving gel buffer(9.2g Tris, 54 g urea, 100 mL water, adjusted to pH 8.8 and made to 200 mL). The gel solution was mixed with 10 µL of TEMED and 100 µL of 10% (w/v) of freshly made ammonium persulfate solution. The gel solution was poured into the Biorad mini-protean apparatus and overlaid with about 0.5 mL of water. After the gel had set, the water was removed. The stacking gel as made from 10 mL of a gelling solution (1.08g Tris, 36 g urea, 0.55 g boric acid, 0.092g EDTA and 3.0 g "acrylamide/ BIS 37.55:1.0 made up to 100 mL and adjusted the pH to 8.4 with HCl) was mixed with 10 µL of TEMED and 50 µL of ammonium persulfate. The solution was poured and the comb was inserted. After gel formation, comb was removed and 30 µL of 2% cheese solutions was carefully put into each well. Stock chamber buffer (0.925g, EDTA, 10.79 g Tris and 5.5 g boric acid made upto 1L and adjusted to pH 8.4). was diluted 1:4 with water and then put into the apparatus. The electrophoresis was started at a 60 V till the sample crossed the stacking gel. Thereafter, the voltage was increased to 90V and it was run for about 3 h till the dye front was close to the bottom of the gel slab. The gel was stained with staining solution (1 g Comassie brilliant blue R, 500 mL isopropanol and 200 mL glacial acetic acid made up to 2L) for 1- 2 h and destained for 1-2 h with several changes of destaining solution (200 mL isopropanol and 200 mL glacial acetic acid made up to 1L) until a transparent gel background was obtained. The gel was then scanned for record.

RP-HPLC

RP-HPLC of water soluble extract of cheese samples were carried as per the procedure of Prithard et al. (2010). Extracts of the cheese samples were separated by a Sun Fire ™ C18 Column using RP-HPLC (Waters, Milliford, MA, USA). Solvent A was 0.1% Trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile. For each sample, 50 µL was injected and run with a linear gradient 0.2–60% of solvent B (0.1% TFA in acetonitrile) to 50 min followed by 0.2% of solvent B to 55 min at a flow rate of 1 mL/min at room temperature. The separated peptides were monitored at 215 nm using UV detector.

ACE- inhibitory activity

Angiotensin converting enzyme (ACE) inhibitory activity was measured using the method Herna´ndez-Ledesma et al. (2003). The method is based on the liberation of hippuric acid from Hippuryl-l-histidyl-l-leucine (HHL) catalyzed by ACE. To 110 µl of substrate (5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) 20 µL of sample was added. The reaction was initiated by the addition of 20 µL (4 mU) of ACE solution and incubated at 37^0 C for 30 min. The reaction was terminated by addition of 250 µL of 1 N HCl. Subsequently, the hippuric acid formed in the enzymatic process was extracted with 1.5 mL ethyl acetate by centrifugation at 3000 g for 10 min. An aliquot of 1 mL of the upper organic layer was collected and dried out completely by heating at 95 ^{0}C for 20 min. The dried material was re dissolved in 1 mL of double distilled water and the absorbance was measured at 228 nm. The positive control of the reaction was carried out by adding only substrate, ACE and water (no sample). Results are the mean values of three triplicates.

The extent of inhibition was calculated as follows:% Inhibition of ACE (% ACE) = $\frac{[(A-B)-(C-D)] \times 100}{(A-B)}$

where,

A = the absorbance in the presence of ACE (substrate + ACE)

B = the absorbance of the reaction blank (substrate alone)

C = the absorbance in the presence of ACE and the sample/ inhibitor

(substrate + ACE + sample)

D = the absorbance of the sample blank (substrate + sample)

The inhibitory activity of the sample was expressed as percentage of ACE inhibition.

Antioxidant activity

Antioxidant activity was measured using the method of Herna´ndez-Ledesma et al. (2005). The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7mM ABTS stock solution with 2.45mM potassium persulfate (final concentration in 10 mL of water) and keeping the mixture in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted in 5mM phosphate-buffered saline (PBS, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734nm in a 1cm cuvette at 30°C. After addition of 1mL of diluted $ABTS^{\bullet+}$ solution to 10 µL of samples or Trolox standard (0 to 2500 µM) in PBS, the absorbance was recorded every min for 10 min at 30 °C. Appropriate solvent blanks were run in each assay. Results are the mean values of three triplicates. The percent inhibition of absorbance at 734nm was calculated as

$$\% \text{ inhibition} = ((A734\ nm_{blank} - A734\ nm_{sample}) / A734\ nm_{blank}) \times 100$$

To calculate the Trolox equivalent antioxidant capacity (TEAC), the gradient of the plot of the percentage inhibition of absorbance versus sample concentration was divided by the gradient of the plot for Trolox. This gives the TEAC at the specific time.

Statistical Analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA, 2007).

Results and Discussion

Urea-PAGE

The alkaline Urea- PAGE of all Cheddar and probiotic Cheddar cheeses was performed to see the extent of proteolysis with ripening time and electrophoretic patterns are presented in Fig. 1-3. Protein bands were identified by comparison with standards of Sodium caseinate where, β- Casein (β-CN) and α- Casein (α-CN) and peptide positions are shown (plate1a). Cheese samples of different ages were analysed to compare the degradation pattern of these two caseins during ripening. αs_1-CN and αs_2-CN could not be separated on 12% acrylamide gel because the molecular weights of these two fractions are very similar. As the ripening progressed, the concentrations of α-CN & β-CN decreased and the breakdown products were formed. These breakdown products which appeared below α-CN, are low molecular weight peptides. The intensity of these peptides gradually increased till 6 months of ripening thereafter started decreasing till 10 months for all the cheeses. These peptides formation are due to hydrolysis of α-CN & β-CN by the action of microbial proteinase and peptidase leading to further formation of smaller peptides and amino acids. The degradation of β-casein by plasmin in all the cheeses **(Fig.1-3)** is clearly indicated by the decrease in the intensity of β-casein. However, during ripening β-CN did not undergo as much degradation as α-CN (Brandsma et al. 1994; Mistry and Kasperson 1998). This shows that the bacterial peptidases hydrolyse α-CN more readily than β–CN. This may be due to lower activity of natural enzyme of plasmin in cheddar cheese as it is neither cooked at high temperature nor any plasminogen activator was added (Fox et al. 2000).

The differences in the degradation and development of peptides at 6 months ripening of the Cheddar & probiotic Cheddar cheeses are shown in Fig. 4a &1b. The α-CN degradation was more than β -CN in all the cheese samples as the intensity of α-CN tended to be lower than β -CN among the six samples. Cheese made with Inulin and WPC showed higher hydrolysis of both the caseins as compared to control indicating a faster proteolysis in these cheeses.

Comparison between the electrophoretic pattern of 0 day and 10 months ripened cheese is shown in Fig. 2 & 3. There was not much peptides formation in fresh samples. Therefore, there was not much difference in the intensity of the peptides observed. At 10 months of ripening, hydrolysis of α-CN was higher in probiotic cheese (Fig. 3) as compared to Cheddar cheese (Fig. 2) irrespective incorporation of additives as visible by disappearance of α-CN band. All factors, which have influenced the rate of hydrolysis of both caseins, were kept constant during production & ripening, except probiotic addition. This faster α- CN degradation in probiotic was due to the action of probiotic peptidases (Ong et al. 2007) and more proteolytic activity of *Lpb plantarum* (Duan, 2019). In Cheddar cheese, WPC added cheese showed highest proteolysis among the five samples (Fig. 2, lane 7**)**. Similarly, WPC added cheese showed highest proteolysis among the five probiotic Cheddar cheese samples followed by inulin added cheese (Fig. 3, lane7). The increased rates of proteolysis in these two cheeses were mainly due to higher growth and survival of bacteria in presence Inulin and WPC, which might have resulted in high bacterial peptidase activity and may be due to higher growth and more proteolytic activity of *Lpb planatrum* (Duan et al. 2019).The results were also reflected in the amount of soluble amino nitrogen content (proteolysis) in probiotic cheeses.

RP-HPLC

The RP- HPLC chromatogram of standard α- lactalbumin (12 kDa) was run and eluted out at 35.806 min (Fig. 4). It was inferred that all peptides which eluted before 35 min are less than 12 kDa. Assuming this phenomenon, water soluble extracts (WSE) of all cheddar cheeses were analysed for RP-HPLC chromatogram restricting to 35 min run. Comparison between the RP-HPLC chromatograms of 6 months old, Cheddar cheese (data not shown) and probiotic cheddar cheese, with additives showed that total

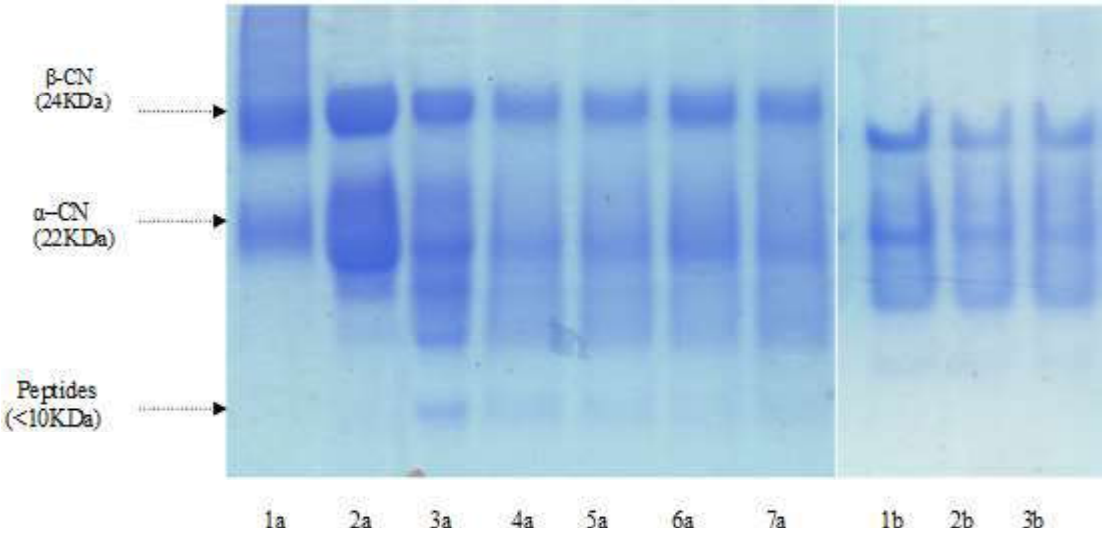


Fig. 1 Electrophoretic pattern of Urea-PAGE of Cheddar and Probiotic Cheddar cheese with additives at 6 months. Lane 1a- Standard (Na Casienate); lane 2a – 0 day; lane 3a- 2 months old; lane 4a- 6 months old Cheddar cheese (CC); lane 5a- 6 months old Cheddar cheese with inulin (CI); lane 6a- 6 months old Cheddar cheese with inulin -lactose; lane 7a- 6 months old Cheddar cheese with WPC (CW); lane 1b- 6 months old probiotic Cheddar cheese (PCC); lane 2b- 6 months old probiotic Cheddar cheese with inulin (PCI); lane 3b- 6 months old probiotic Cheddar cheese with WPC (PCW).

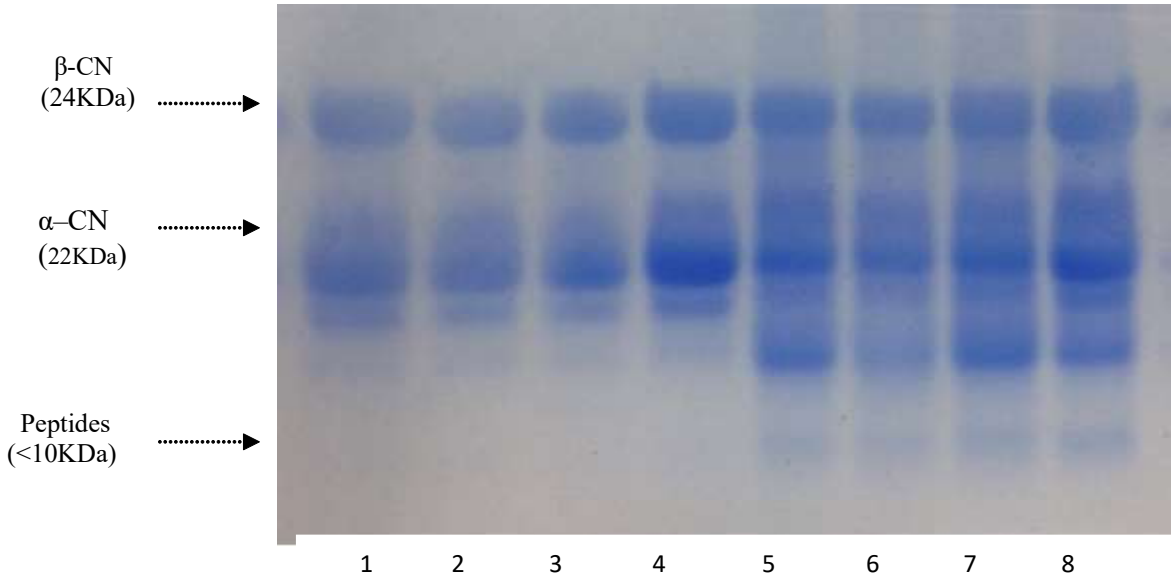


Fig. 2 Electrophoretic pattern of Urea-PAGE of Cheddar cheese with additives at 0 day & 10 months. Lane 1- 0 day ; lane 2 – 0 day with inulin; lane 3- 0 day with WPC; lane 4- 0 day with lactose; lane 5- 10 months old; lane 6- 10 months old with inulin; lane 7- 10 months old with WPC; lane 8- 10 months old with lactose.

number of peaks and their intensity were more in probiotic cheddar cheese as compared to corresponding Cheddar cheese. It was observed that more number of main peaks (15peaks) in probiotic cheddar cheese (PCC) than the control cheese (13 peaks). When peaks and intensity of CW & PCW were compared, it was found that PCW had 18 main peaks whereas CW had 15 main peaks and peak intensity was also higher in PCW. Since probiotic Cheddar cheese samples had higher number of peaks and higher intensity as compared with their corresponding Cheddar cheese samples, it may be inferred that probiotic addition would enhance the rate of proteolysis. The soluble amino nitrogen and electrophorograms also indicated that the rate of proteolysis was more in inulin and WPC added cheese.

A comparative study of peptide profile between PCC, PCW and PCI was done for 0 day, 6 and 10 months (Fig. 5). It can be inferred that as the age of cheese increased, new peaks appeared while existing peaks at the initial stages of ripening either increased or

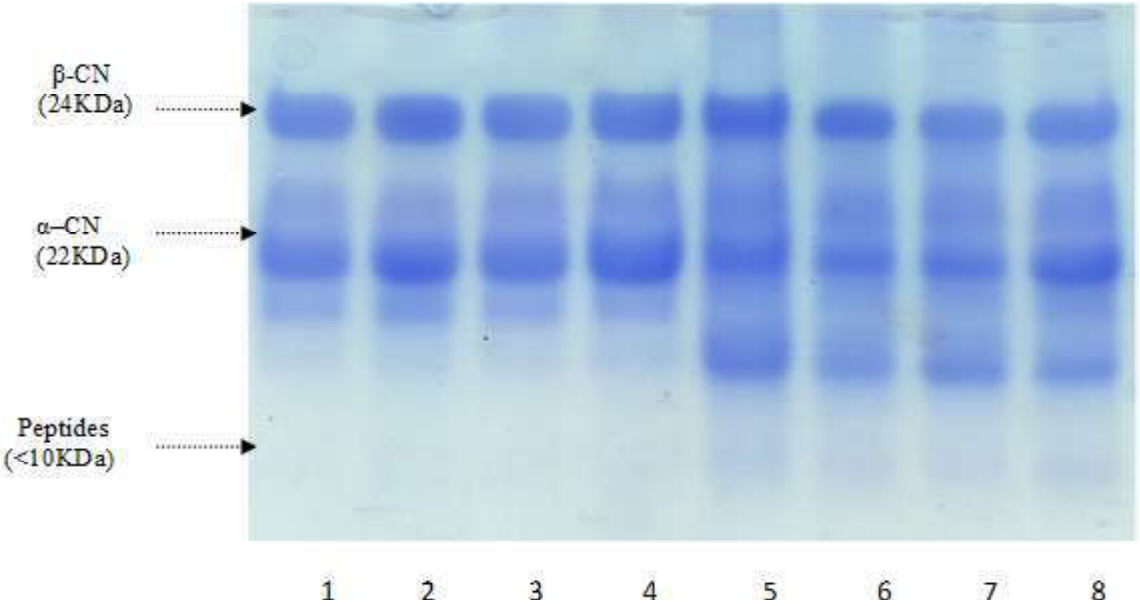


Fig. 3 Electrophoretic pattern of Urea-PAGE of probiotic Cheddar cheese with additives at 0 day & 10 months. Lane 1- 0 day ; lane 2 – 0 day with inulin; lane 3- 0 day with WPC; lane 4- 0 day with lactose; lane 5- 10 months old; lane 6- 10 months old with inulin; lane 7- 10 months old with WPC; lane 8- 10 months old with lactose.

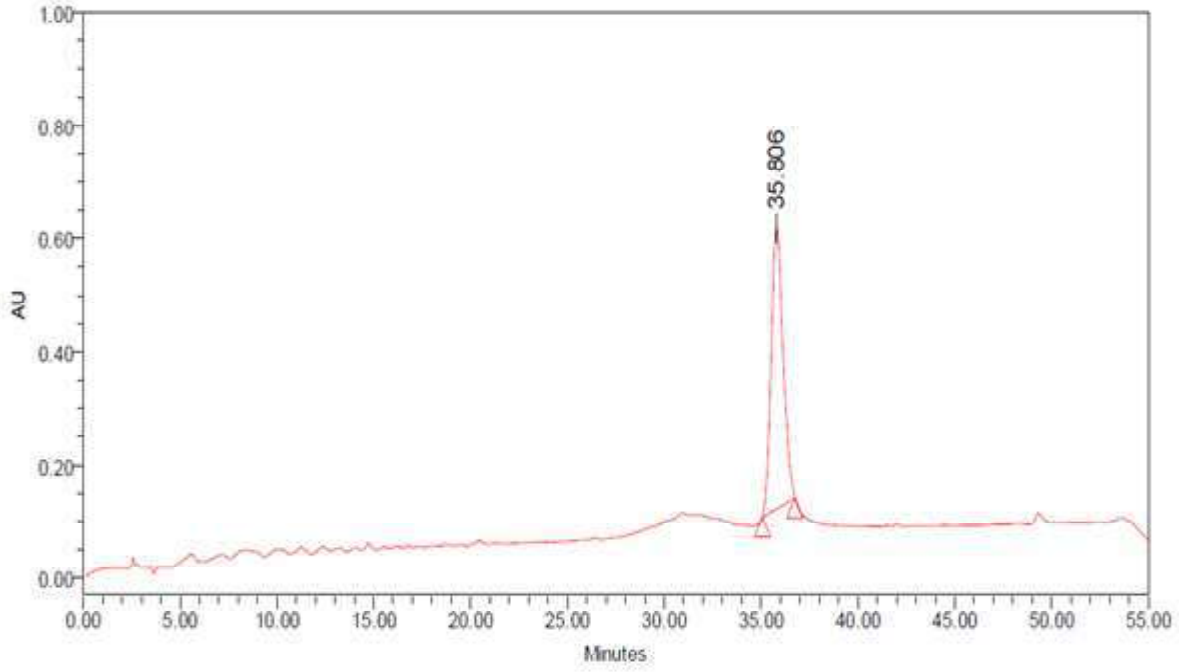
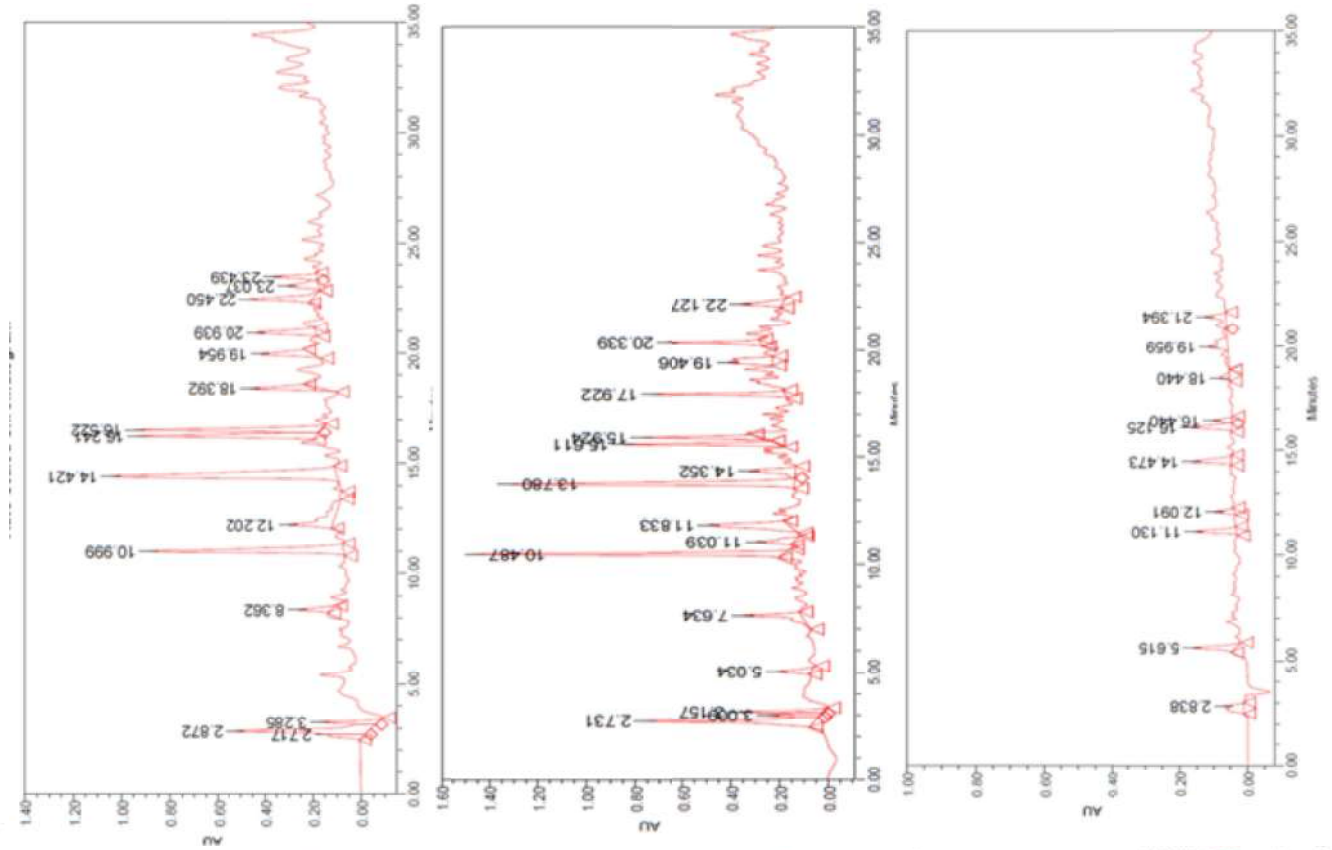
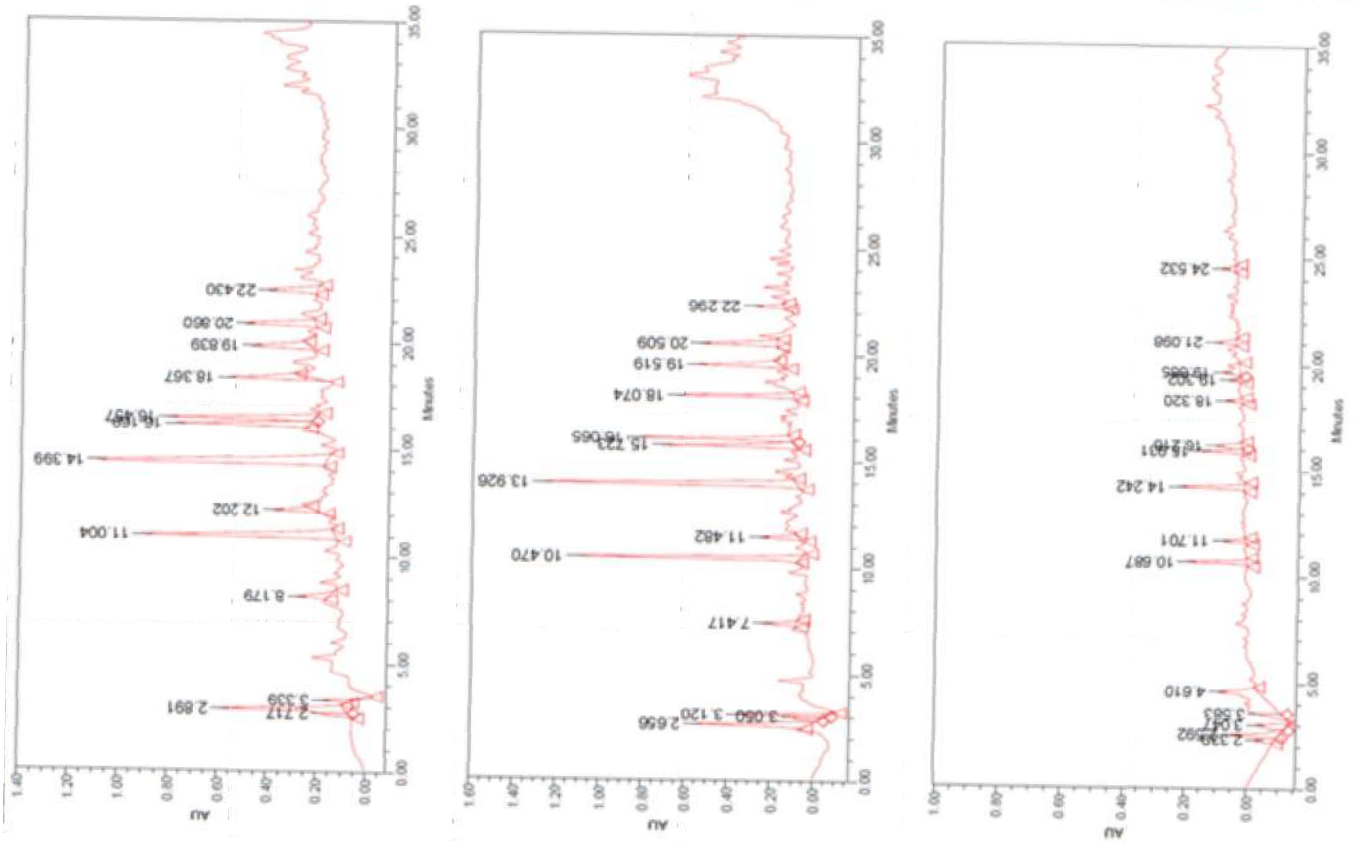


Fig. 4 RP-HPLC chromatograms of α lactalbumin standard

decreased. Peptide profile of cheeses at zero day showed that the number of peaks and their intensity were very less; however it did not differ among the samples. As the ripening increased the rate of formation of peptides in the cheeses made with WPC and inulin were higher as compared to control cheese. PCW had 18 main peaks, whereas 16 main peaks were observed in PCI and 13 main peaks in PCC. It was noted that the elution times of peptides with higher retention times of peptides were often affected by the average hydrophobicity on the reverse phase column (Champion and Starley 1982). Higher absorbance values of peaks indicate higher concentration of peptide contents in WSEs of Cheddar cheeses. With the increase in the ripening time from 6 to 10 months the electrophoretic pattern and elution profile changed. The number of peaks and their intensity was higher in cheese with WPC even after 10 months of ripening. However, there was decrease in number of peaks in RP- HPLC elution profile. This



5 b) PCI



5 a) PCC

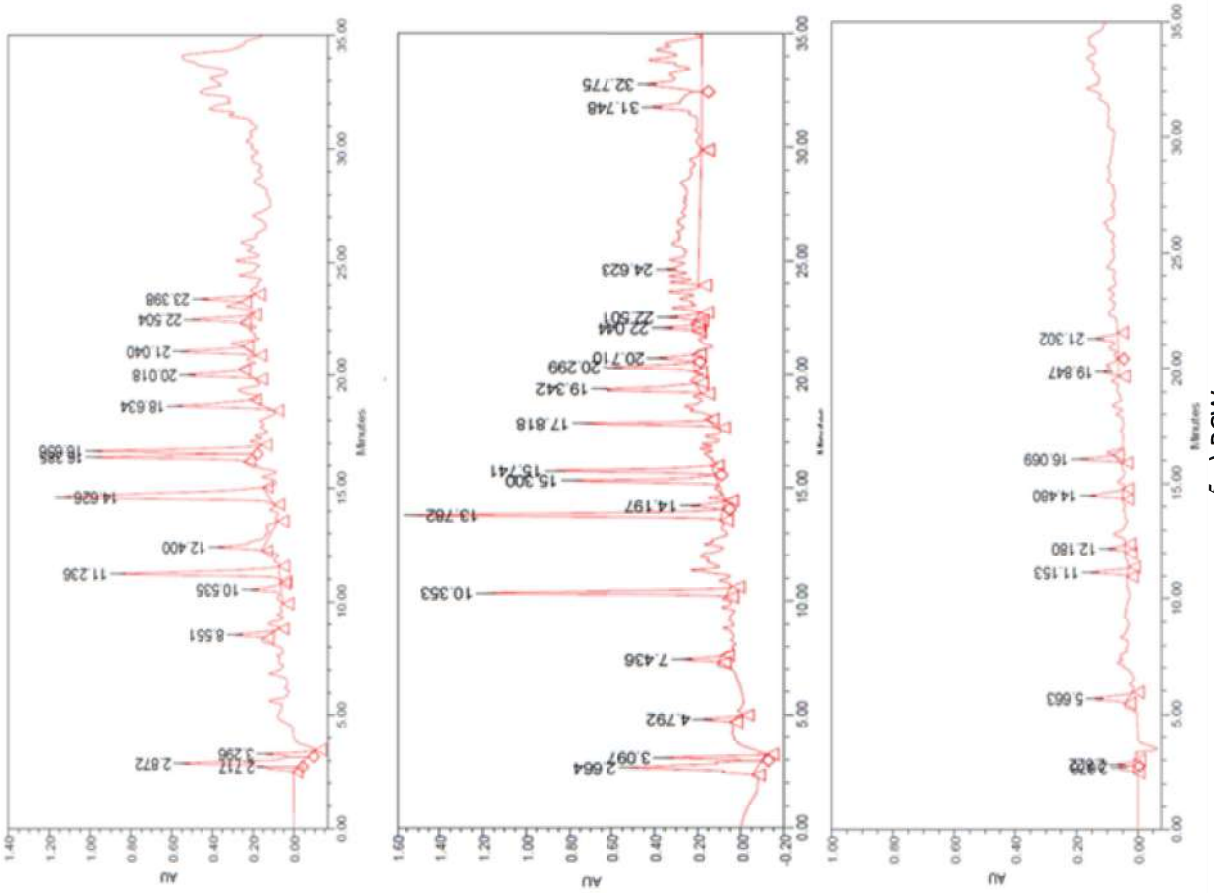


Fig. 5 Peptide profile of water-soluble extract (WSE) of (a) control probiotic Cheddar cheese (PCC), (b) probiotic Cheddar cheeses with inulin (PCI) and (c) probiotic Cheddar cheese with WPC (PCW) at 0 day, 6 mon & 10 mon. Eluent A was 0.01% TFA in water . Eluent B was 0.01% TFA in acetonitrile. Gradient: 0 to 50min, 0.2 to 60% eluent B; 50 to 55 min, 60 to 0.02% eluent B.. Detection was at 215 nm

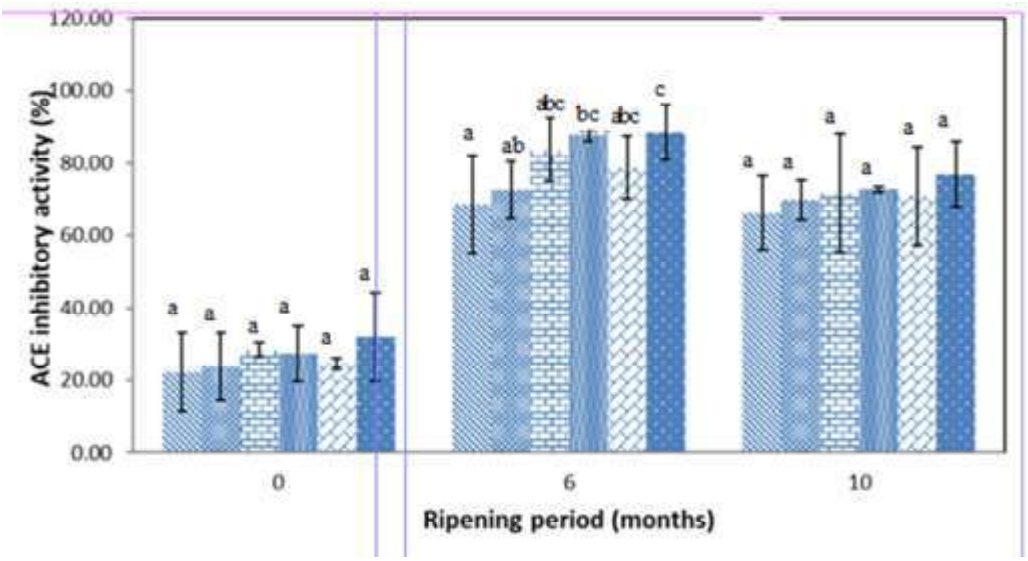


Fig. 6 ACE- inhibitory activity of WSE of CC ( ), PCC ( ), CI ( ), PCI( ), CW( ) and PCW( ). The data measured as Trolox equivalent antioxidant capacity (µM Trolox). Error bars show standard error of the mean of duplicate measurements of three independent experiments (batches).

may be due to further breakdown of peptides into smaller peptides and amino acids. As the ripening increased, more peptides were released into the cheeses and chromatogram became more complex which are similar to the observations for Cheddar cheese as reported earlier (Duan et al. 2019, Gupta et al. 2013, Ong et al. 2007 ; De Wit et al. 2005). It can be concluded that

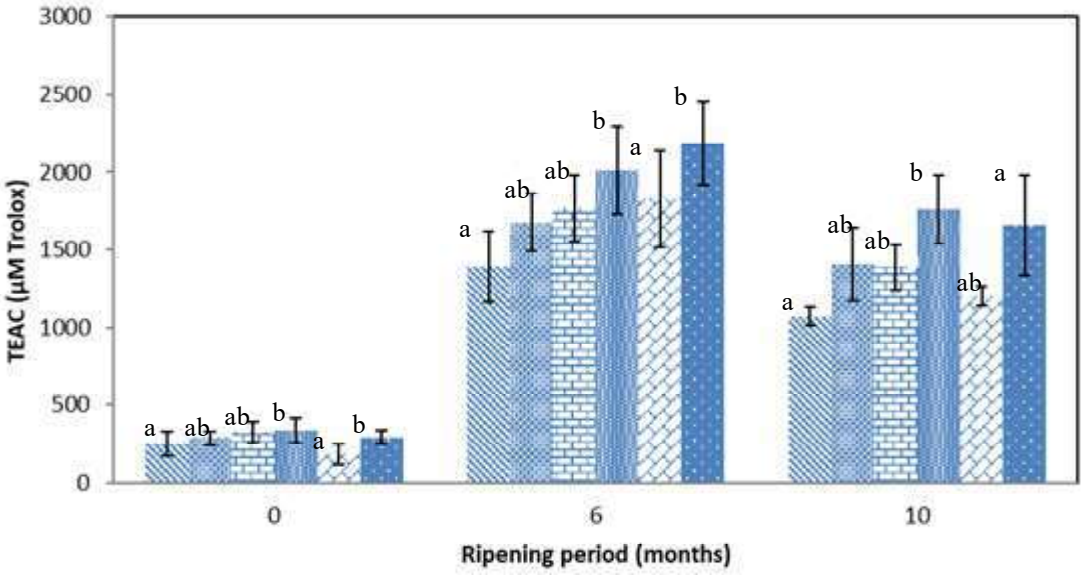


Fig. 7 Antioxidant activity of WSE of CC( ), PCC ( ), CI ( ), PCI( ), CW( ) and PCW( ). The data measured as Trolox equivalent antioxidant capacity (µM Trolox). Error bars show standard error of the mean of duplicate measurements of three independent experiments (batches). Mean values with different small letters for the same ripening period are significantly different ($P < 0.05$).

addition of WPC in presence of probiotic resulted in highest proteolysis.

ACE- inhibitory activity

The ACE- inhibitory activity of WSE of cheese samples collected at 0 day, 6 months and 10 months was compared by determining % ACE- inhibitory activity. Initial ACE- inhibitory activity of six cheeses with additives ranged between 22.21 to 32.00% (Fig. 6). The ACE- inhibitory activity among the cheeses at zero day of ripening did not show any significant difference ($p>0.05$).

All six cheese samples showed a significant increase in ACE-inhibitory activity after 6 months of ripening. ACE- inhibitory activity of PCW (88.51%) was highest among all the samples. It was significantly higher ($p<0.05$) than both of CC (68.68%) and PCC (72.50%) after 6 months followed by PCI (87.82%), CI (83.62%) & CW (78.80%). ACE- inhibitory activities of probiotic cheeses were more than that of Cheddar cheeses irrespective of additives.

ACE- inhibitory activity did not show any significant ($p>0.05$) difference among the samples after 10 months of ripening. ACE-inhibitory activity increases till 6 months and showed decease after 10 months of ripening. Our study suggests that ACE-inhibitory activity did not increase continuously till 10 months ripening unlike proteolysis but reached to a maximum value within 6 months and thereafter started decreasing. This may be due to some peptides produced at the early or intermediate stage of hydrolysis, getting degraded and subsequently forming new peptides which had less inhibition activity or may be further degraded and formed amino acid upon longer period of ripening.

Higher ACE inhibitory activity of probiotic Cheddar cheese compared to Cheddar cheese suggests that ACE- inhibitory activity is related to the bacterial strains used in the cheese ripening process. Similar trends were previously reported by Gupta et al. (2013) and Ong et al. (2007). ACE- inhibitory activity of probiotic cheese with WPC was highest among all the samples, may be due to extensive proteolysis in presence of WPC. Proteolysis was also highest for this sample as observed earlier. Many investigations showed that ACE- inhibitory activity is dependent on the degree of proteolysis and peptide formation. The activity increases until it reached maximum due some peptide formation, decreasing thereafter as a result of extensive proteolytic degradation of ACE- inhibitory peptides (Ong et al. 2007). (In contrast, Moslehishad et al. (2013) found that the ACE- inhibition did not follow any specific trend during 21 days of storage at 5 ºC in fermented milk.

Antioxidant activity

Antioxidant activities of all cheeses were evaluated by scavenging activity of 2, 2'- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical and expressed in Trolox equivalent antioxidant activity (TEAC) values. A comparative analysis of antioxidant activity between cheddar and probiotic cheddar cheeses with two additives (Inulin and WPC) including their control were studied at 0, 6 & 10 months of ripening (Fig. 7).

The TEAC values of Cheddar cheese with additives initially were low for all six samples and ranged from 183.09 to 334.19 µM of Trolox. The ABTS scavenging activities of both PCI & PCW samples were significantly higher than control samples at 0 day of ripening. There was a significant increase in ABTS scavenging activity till 6 months and reached to maximum value irrespective of the treatments. Highest TEAC value (1828.20 µM of Trolox) in Cheddar cheese was observed in the cheese with WPC followed by the cheese of CI and CC after 6 months of ripening. Similarly, in probiotic Cheddar cheese, ABTS scavenging activity was maximum in cheese with WPC (2183.55 µM of Trolox). When the antioxidant activity assays of all six samples was compared, it was observed that radical scavenging activity of cheeses at 6 months of ripening was significantly highest ($p<0.05$) in PCW as compared to CC but not significantly ($p>0.05$) different from PCC, followed by PCI. However, overall ABTS scavenging activity of the probiotic Cheddar cheeses was higher than Cheddar cheeses. The ABTS scavenging activity decreased after 6 months till 10 months of ripening irrespective of the treatment. TEAC value of PCI was highest among the six samples after 10 months of ripening. However, TEAC value (1385.87 µM of Trolox) was more in cheese with inulin as compared to the cheese with WPC (1200.26 µM of Trolox) after 10 months of ripening. Simliar trend was observed in probiotic Cheddar cheese.

ABTS scavenging activity was maximum at 6 months of ripening which may be due to hydrolysis of protein to smaller peptides having antioxidant activity. Reduction in ABTS scavenging activity after 6 months may be due to further hydrolysis of these peptides to smaller peptides and amino acids which may not have antioxidant activity as reported by Moslehishad et al. (2013). According to them, the 5-10 kDa peptide fractions exhibited the highest radical scavenging activities compared with lower molecular masses of peptides (3-5 kDa) in fermented milk.

ABTS scavenging activity of the probiotic Cheddar cheeses was higher than Cheddar cheeses may be due to the more hydrolysis of both α and β-CN by proteolytic/ peptidolytic enzymes of *Lpb.* In electrophoretic study (plate 1), more hydrolysis of α and β – CN was observed in probiotic cheeses. Gjorgievski et al. (2014) reported that the antioxidative capacity of fermented milk product (yogurt) was strain dependent. The lowest value of 45.17% indicates that milk was fermented with symbiosis of *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophiles* in the product. A few studies related to the production of antioxidant peptides in fermented milk with lactic acid bacteria indicated that the development of radical scavenging activity was a strain-specific characteristic and radical scavenging was related to proteolysis (Hernandez et al. 2005; Virtanen et al. 2007).

When the extent of proteolysis and peptide formation was compared with antioxidant activity, it was observed that changes in antioxidant activity were very similar to the change in the rate of formation of soluble peptides (proteolysis) in all the cheese samples till 6 months of ripening. As shown earlier, the soluble amino nitrogen was reported to be more in cheese made with WPC than in control cheese. Therefore, ABTS radical scavenging activity of probiotic cheese with WPC was highest. Perna et al. (2015) investigated antioxidant activity in Caciocavolla cheese by ABTS method and reported antioxidant activity varied depending on the rate of formation of soluble peptides during ripening. In Cheddar cheese and probiotic cheddar cheese, ABTS scavenging activity was highest in WPC added cheese after 6 months of ripening.

Conclusions

From the results, it can be concluded that addition of WPC increased the proteolysis more than inulin in cheddar cheese. Our results further indicate that proteolytic enzymes of probiotic culture, play an important role in increasing the production of peptides (detected via urea PAGE) which contributed towards ACE– inhibitory and antioxidant activity. Lower activity observed after 10 ripening may be due to further degradation and formation of amino acid upon longer period of ripening and also due to higher activity of *Lpb. plantarum* which was enhanced in the presence of WPC and thereby shown more ACE- inhibitory and antioxidant activity after 6 months of ripening.

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RESEARCH ARTICLE

Process optimization for the development of grape pulp enriched Low- calorie ice cream made with sucralose and sorbitol

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Abstract: Grape pulp is a rich source of antioxidants whereas, ice cream is a poor source of these antioxidants; therefore, the present study was carried out on physico-chemical, nutritional quality and sensory evaluation of low-calorie ice cream fortified with different levels of grape pulp. The grape pulp was added to the low-calorie ice cream to improve the sensory and nutritional quality like antioxidant activity of the product. The low-calorie ice cream was prepared with sucralose 300 ppm along with 3% sorbitol was kept as control. Low-calorie ice cream is enriched with grape pulp by adding at three different levels i.e. 8%, 10% and 12%. Based on the physico-chemical and sensory evaluation, 10% grape pulp added low-calorie ice cream was found to be acceptable. The resulted low-calorie ice cream enriched with grape pulp had enhanced functional and nutritional attributes.The results of current study demonstrated that the addition of fruits to the Yogurt significantly improved the quality of Yogurt.

Keywords: Ice cream, Low-calorie, Grape pulp, Physico-chemical quality, Antioxidant activity

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Introduction

The ice cream market in India reached a value of Rs. 165.2 billions in 2021 and exhibiting at a CAGR of 17.69 per cent during 2022-2027. Factors such as the rising demand for innovative flavors, types, and the rising demand for impulse ice creams such as cones, sandwiches, and pops in developing countries are expected to drive the market growth. The increasing health consciousness among consumers is also expected to fuel the demand for premium ice creams in the upcoming years. New varieties of ice cream are coming out targeting the health conscious consumers, and also new manufacturing processes giving more value for money spent by consumers (Sasikala et al. 2020). At present focus on nutritional enrichment has shifted from the provision of nutrient deficiency to the pursuit of optimal health and dietary intake. The consumers are now more interested in healthy foods and looking for foods that have added beneficial compounds such as antioxidants, phenolics and phytosterols. Thus producers have to add functional ingredients to food products to attract the attention of health conscious consumers (Shaviklo et al. 2011). Increasing preference of consumers towards natural ingredients has tempted the ice cream manufactures to search for new innovations in components having favourable health effects. Sugar has many roles in foods and sugar not only makes dairy products more palatable but also a acts as bulking agent, adds viscosity, enhances flavor, provides texture, adds color, is a preservative, and inhibits protein coagulation (Davis, 1995; Silcock, 2017). When sugar is replaced or reduced, another bulking agent, such as insoluble fiber or polydextrose system, often must take its place (Silcock, 2017). However, when sucrose is replaced with a bulking agent, the bulking agents also contribute calories to the product and may negate the original purpose of removing the sugar (Cardoso and Bolini, 2008). Sugar can also add viscosity to dairy products and so, when it is removed, viscosity is reduced (Kappes et al. 2006; Saint-Eve et al. 2010; Cadena et al. 2012; Leksrisompong et al. 2012). Sugar reduces the water activity of diary products and beverages, which makes water unavailable for bacterial and fungal growth. Thus, when sugar is removed, it has to be replaced with another preservative, which is often less appealing to consumers. Sugar acts as an anticoagulant agent, in that it delays a liquid

from changing into a solid or semi-solid state (Mizukoshi et al. 1979).

Grapes contain a variety of phytochemicals, like phenolic acids, stilbenes, anthocyanins, and proanthocyanidins, all of which act as strong antioxidants (Yang et al. 2009). From a health perspective, grape phytochemicals has been shown active against HIV by inhibiting virus expression and replication (Nair et al. 2002), anticarcinogenic (Roy et al. 2002) and cardio-protective agent (Shafiee et al. 2003). Clinical data has shown that the antioxidant potential of grape phytochemicals is twenty and fifty fold greater than vitamins E and C respectively (Shi et al. 2003) which is arising from increased levels of polyphenol proanthocyanidins and oligomers of flavan-3-ol units, especially catechin and epicatechin present in GSE (Yilmaz and Toledo, 2004). Mechanism of antioxidant action of grape phytochemicals includes oxygen radical scavenging activity (Bagchi et al. 2000), stimulation of the enzymatic production of nitric oxide and inhibition of nitrositive stress (Roychowdhury et al. 2001). Thus, the present study was undertaken to develop a grape pulp enriched low-calorie ice cream made with sucralose and sorbitol.

Materials and Methods

Fresh chilled raw cow milk and cream was procured from Experimental Section, Department of Dairy Technology, College of Dairy Technology, Tirupati, Andhra Pradesh. Sucralose was purchased from Shandong Kanbosweet Biochemical Technology Co., Ltd. China, whereas, sorbitol used as bulking agent was procured from Panchamrut chemicals, Mumbai. Other ingredients such as skim milk powder, sugar, vanilla essence were purchased from the local market.

Preparation of low-calorie ice cream

In the present study, low-calorie ice cream (control) was prepared using 10% fat, 11% MSNF, 300 ppm sucralose, 3% sorbitol, 0.3% stabilizer and emulsifier and 0.2% vanilla flavour used. Liquid ingredients (milk and cream) were mixed and heated to 49°C. Thereafter, dry ingredients (skim milk powder, sorbitol and stabilizer) were added. The ice cream mix was then pasteurized at 68°C for 30 minutes, homogenized. The mixture was cooled to 30°C. Calculated quantity of sucralose was first dissolved in small quantity water and mixed with the mixture properly. This mixture was kept for ageing at 0 to 4°C for 4 hrs. After addition of vanilla essence, the mix was subjected to freezing at -4 to -5°C, filled in polystyrene cups of 100 ml capacity and kept for hardening at -23°C.

Preparation of grape pulp

Good quality well ripened black grapes were purchased from local market of Tirupati, Andhra Pradesh, India. The grapes were washed thoroughly and rinsed in tap water followed by with distilled water. The whole fruits were pureed well using a fruit Pulper and then filtered through a wire mesh to obtain pure pulp. The pulp was pasteurized (80 to 90°C about 30 minutes) later concentrated to maintain the total soluble solids 15°brix and then cooled at 4°C for ice cream preparation.

Preparation of grape pulp enriched low-calorie ice cream

Grape pulp enriched low-calorie ice cream preparation by incorporating grape pulp is presented in Fig 1. The mixes were homogenized at 150 kg/cm^2 and ice cream mix was kept for ageing at 4°C for 4 hours and for freezing at -4°C. After packing of the ice cream was kept for hardening and storage at -23°C.

Analysis of ice cream

The ice cream was evaluated for compositional, physico-chemical and sensory characteristics. The fat content of the ice cream was determined by the standard method as suggested in ISI Hand Book (1989) for ice cream mixes using 5g ice cream mix sample. The total nitrogen in the sample was determined by Macro-Kjeldahl method (AOAC, 2000). Ash content of ice cream samples was determined by procedure described in IS: 1547-1985. Total solids content of the ice cream mix was determined by gravimetric method (IS: 2802-1964). The total carbohydrate content in the

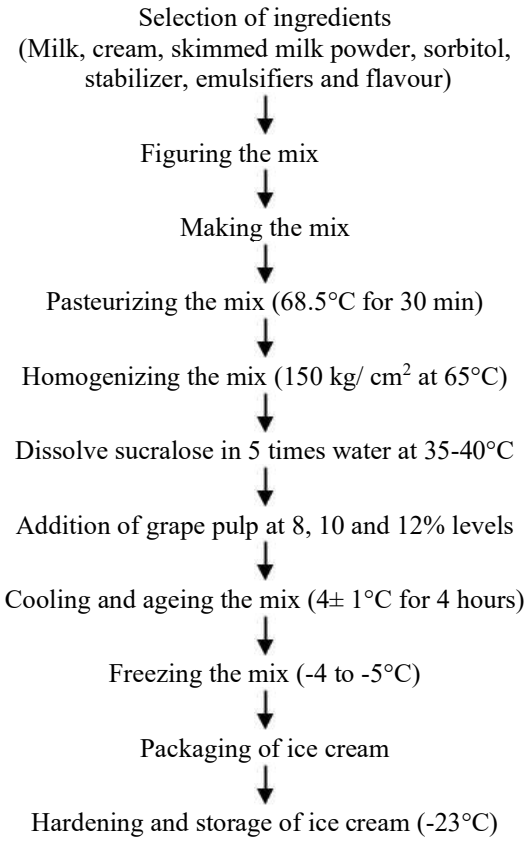

Fig 1. Flow chart for preparation of grape pulp enriched low-calorie ice cream

samples was determined by difference i.e. the sum of moisture, protein, fat and total ash percent was subtracted from 100. The titratable acidity of the ice cream was determined by the standard method suggested in ISI Hand Book (1989). The pH of ice cream mix was determined after ageing using a digital pH meter (Elico Pvt. Ltd., Hyderabad) (AOAC, 2000).

The viscosity of ice cream mix was determined by the method of Loweenstein and Haddad (1972) using a Brookfield Viscometer, Model LTD2T, (Brookfield Engineering Laboratories, Chennai). The overrun in ice cream was determined as per the method of Marshall et al. (2003). The penetration value of the hardened frozen product was measured using cone penetrometer. The melting rate was determined as per the procedure given below by Specter and Setser, (1994).

Sensory evaluation

The acceptability of ice cream by substituting with low-calorie sweetener was added at different levels were studied by conducting sensory evaluation with the help of 5 member panel of trained judges were assessed by using a 9- point hedonic scale.

DPPH radical scavenging activity

The ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by ice cream enriched with grape pulp was determined by the method of Singh et al. (2002). 100 µl ice cream enriched with grape pulp extracts were diluted with 0.1 M Tris–HCl buffer (pH 7.4) and mixed with 1 ml of DPPH (250 µM) with vigorous shaking. The reaction mixture was stored in the dark at room temperature for 20 min and then absorbance was measured at 517 nm using a UV–VIS spectrophotometer (UV-VIS spectrophotometer; Model: UV-1700 PharmaSpec, SHIMADZU, Japan). The scavenging activity was calculated by the following equation:

Scavenging activity % = (Absorbance of Blank –Absorbance of Sample/Absorbance of Blank) X 100

Statistical analysis

The results obtained during the course of investigation were subjected to statistical analysis using the software OPSTAT, as proposed by Sheoran et al. 1998.

Results and Discussion

Utilization of fruits in milk products for value addition is great challenge to dairy processing industry. Now a day's consumers prefer value-added milk products. There is a large scope in dairy processing industry for conversion of milk into innovative fruit based milk products. Ice cream is rich in macronutrients i.e. carbohydrates, fats, proteins, and some micronutrients i.e. vitamin A, E and calcium. However, commercially available ice creams are generally poor in natural antioxidants like vitamin C, colours and phenols.

Optimization of different levels of grape pulp for low-calorie ice cream prepared with sucralose and sorbitol

Grape is a rich source of antioxidants including phenolic, flavonoid, and anthocyanin. To improve the functional and nutritional attributes of low-calorie ice cream a trail has been conducted by addition of grape pulp levels (G_4) 8, (G_5) 10 and (G_6) 12 percent level with control low-calorie ice cream without grape pulp (Cs).

Compositional and physico-chemical analysis for low-calorie ice cream enriched with different levels of grape pulp

On perusal of the data presented in Table 1 pertaining to the compositional and physico-chemical analysis reveals that the mean values of fat, protein, carbohydrate, ash, total solids, pH and acidity for different levels of grape pulp enriched low-calorie ice cream and Cs cream are discussed below.

Fat

The mean fat percentage in ice cream for Cs, G_4, G_5 and G_6 were 9.64, 9.51, 9.43 and 9.36 respectively, which was significantly higher (Pd 0.05) in Cs than in other treatments. The present findings illustrated that control sample (Cs), had the highest (9.64%) fat than remaining three ice cream mixes added with grape

Table 1 Average compositional and physico–chemical analysis for low-calorie ice cream enriched with different levels of grape pulp

Treatments	Fat (%)	Protein (%)	CHO* (%)	Ash (%)	Total solids (%)	pH	Acidity (% LA)	Antioxidant activity (% inhibition of DPPH)
Cs	$9.64^{a}\pm0.01$	$3.73^{a}\pm0.07$	$12.46^{a}\pm0.03$	$0.85^{a}\pm0.01$	$26.69^{a}\pm0.36$	6.582 ± 0.02	$0.21^{c}\pm0.01$	$29.36^{d}\pm0.05$
G_4	$9.51^{b}\pm0.01$	$3.59^{ab}\pm0.07$	$12.18^{a}\pm0.05$	$0.80^{b}\pm0.01$	$26.09^{b}\pm0.03$	$6.39^{b}\pm0.03$	$0.31^{b}\pm0.01$	$34.79^{c}\pm0.04$
G_5	$9.43^{c}\pm0.01$	$3.50^{b}\pm0.04$	$11.93^{ab}\pm0.09$	$0.76^{c}\pm0.01$	$25.64^{b}\pm0.03$	$6.34^{bc}\pm0.06$	$0.34^{a}\pm0.01$	$41.93^{b}\pm0.05$
G_6	$9.36^{d}\pm0.01$	$3.45^{b}\pm0.03$	$11.51^{b}\pm0.02$	$0.701^{d}\pm0.01$	$25.03^{c}\pm0.03$	$6.20^{c}\pm0.05$	$0.36^{a}\pm0.01$	$54.07^{a}\pm0.03$
CD (P≤0.05)	0.03	0.17	0.57	0.04	0.57	0.14	0.04	0.63

CHO*-Carbohydrate**;** Values mentioned above are mean ± SE; (n=5);
abcd: Means in the same column with different superscripts differ significantly (P≤0.05)

pulp. Irrespective of treatments grape pulp ice cream had low-fat percent. The decrease in the fat content of ice cream with increasing levels of grape pulp is ascribed due to low-fat content of grape pulp. Similar observations were recorded by Shelke et al. (2020) who reported a gradual decrease in fat content with increase in the level of jamun pomance in ice cream.

Protein

The mean protein percentage of ice cream prepared using grape pulp for different treatments was ranged from 3.73 to 3.45. The protein content of grape pulp ice cream in all treatments grape pulp enriched was significantly lower than Cs (3.73 percent). The observations revealed that as the pulp level in the ice cream increased, the protein content of ice cream decreased. The observations revealed that as the pulp level in the ice cream increased, the protein content was decreased; the reason might be due to low protein content of grape pulp. Similarly, Bajwa et al. (2003) also reported similar findings in ice cream incorporated with 0, 10, 15, 20 and 25% strawberry pulp and Murtaza et al. (2004) observed significant effect on protein content in ice cream manufactured with guar gum and xanthan gum and distilled monoglyceride.

Carbohydrate

The mean carbohydrate percentage of ice cream were ranged from 12.46 to 11.51 in treatments Cs to G_6 respectively, which was significantly higher in Cs than in other treatments. It was observed that the decrease in the carbohydrate content of ice cream with increase levels of grape pulp. The results corroborates the earlier observations made by Bajwa et al. (2003) and Murtaza et al. (2004), who also observed a decreasing trend of carbohydrate content with increasing addition of strawberry pulp and fig pulp respectively into the ice cream.

Ash

The mean ash percentage of grape pulp ice cream in all treatments with Cs was higher than G_4, G_5 and G_6. Addition of grape pulp significantly affected the ash percentage of ice cream. It was observed that the decrease in the ash content of ice cream with increasing levels of grape pulp due to the high moisture content of grape pulp. The results in accordance with Goraya and Bajwa, (2015) and Shelke et al. (2020) who observed a decreasing trend of total ash content with increasing addition of jamun pomace into the ice cream

Total solids

The mean total solids percentage of ice cream was range from 26.69 and 25.03. The total solids percentage of ice cream in all treatments with Cs was higher than other treatments. However, the variation in total solids content due to different treatments was significant, although decrease in total solids content with an increase in grape pulp was noticed. Similar observations were recorded by Shelke et al. (2020) who reported that the decrease in total solids content of ice cream with an increase in level of jamun pomace, orange and pineapple juice, respectively.

Acidity

The acidity of Cs ice cream was found to be lower and pH to be higher than ice cream prepared with grape pulp. The pH decreased from 6.58 to 6.20 with addition of grape pulp from 8 to 12 percent, while acidity of ice cream increased from 0.21 to 0.36 percent. Grape pulp addition at increased levels caused a significant increment in acidity and decrease in the pH of ice cream samples, this was due to higher acidity of grape pulp than Cs sample and pulp had good content of acidic phenolic substances which increased the acidity. The results are in accordance with Pinto et al. (2004) who observed that acidity increased with addition of ginger juice. The results are in correlation with Poul et al. (2009) reported that decrease in pH values in custard apple pulp ice cream and custard apple milk shake.

Antioxidant activity

The antioxidant activity ranged from 29.36 percent (Cs) to 54.07 percent (G_6). The antioxidant activity increased significantly ($P \leq 0.05$) and was greater at 12 percent level than 8 percent level. However, it is observed increasing level of grape pulp an increasing in antioxidant activity. Low- calorie ice cream samples prepared with different levels of grape pulp were found to have higher amount of antioxidant activity than control low-calorie ice cream (Cs). This remarkable increase in antioxidant activity was

Table 2 Physical properties of different levels of grape pulp for low-calorie ice cream prepared with sucralose and sorbitol

Treatments	Viscosity (before ageing at 37°C)	Viscosity (after ageing at 4°C)	Over run (%)	Penetration value (mm/5s)
Cs	132.80a± 2.93	280.40a± 11.69	79.34d± 0.25	70.46d± 1.09
G4	127.20ab± 2.76	234.38b± 11.91	82.44c± 0.50	76.62c± 0.99
G5	123.40bc± 3.05	223.56b± 3.77	86.14b±0.17	81.60b± 1.33
G6	116.80c± 2.63	211.04b± 4.00	88.10a±0.25	87.14a± 1.13
CD (P≤0.05)	8.55	36.33	0.96	3.43

Values mentioned above is mean ± SE, (n=5), abcd: means in the same column with different superscripts differ significantly (P≤0.05)

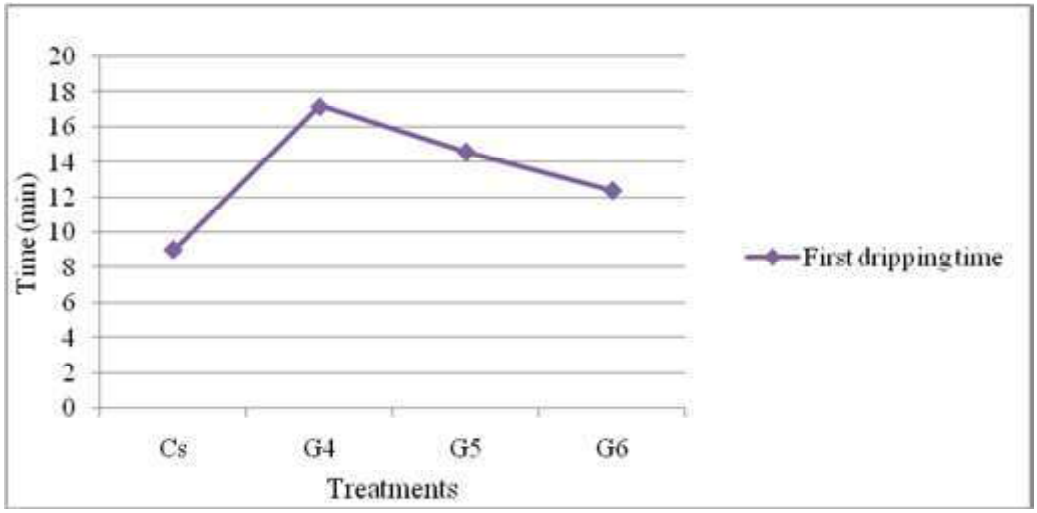

Fig 2. Effect of different levels grape pulp on first dripping for low-calorie ice cream prepared with sucralose and sorbitol

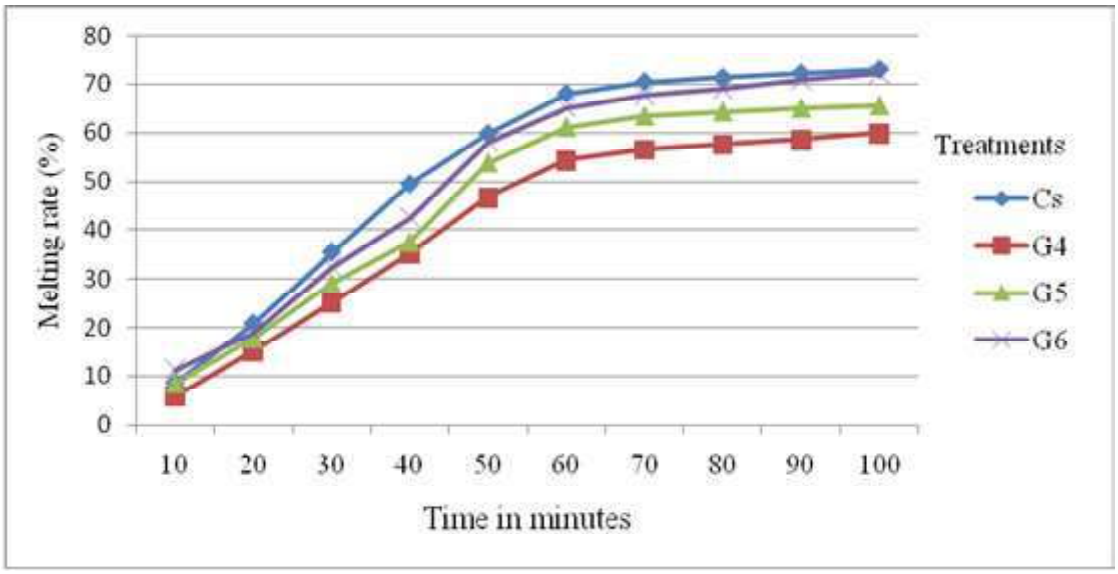

Fig 3. Effect of different levels of grape pulp addition in low-calorie ice cream prepared with sucralose and sorbitol on the first dripping time and melting rate

due to more phenols and tannins infusing from the grape pulp into the ice cream matrix. The results are similar with (Goraya and Bajwa, 2015) reported that the processed amla (Indian gooseberry) incorporated ice cream samples were also found to have higher antioxidant activity, total phenols and tannins than control due to more total phenols and tannins infusing from the amla into the ice cream matrix.

Physical properties of different levels of grape pulp for low-calorie ice cream prepared with sucralose and sorbitol

On perusal of the data presented in Table 2 pertaining to the physical properties reveal that the mean values of viscosity, overrun and penetration value for low-calorie ice cream (Cs) and ice cream prepared with different levels of grape pulp are discussed below.

Viscosity

The mean viscosity (Cp) of mix before ageing at 37°C the values are 132.80, 127.20, 123.40, 116.80 and after ageing the mix at 4°C the values are 280.40, 234.38, 223.56, 211.04 in treatments Cs to G_6 respectively. All the treatments are significantly different ($P \leq 0.05$) from each other. The above observations indicated that, increasing level grape pulp addition decreased the viscosity of ice-cream mix, this might be due to high moisture content of grape pulp contributing a low level of viscosity in low-calorie ice cream. These results are in agreement with Sasikala et al. (2020) in viscosity of various ice cream mixtures.

Overrun

It is evident from Table 2 the overrun of Cs was 79.34 percent. The overrun of G_6 was higher than Cs and that of G_4 and G_5 lower than G_6. Addition of grape pulp significantly affects the overrun of the ice cream. The reason might be due to a decrease in viscosity

with increasing level of grape pulp as contributing to the increment of overrun. Sasikala et al. (2020) reported that overrun of artificially sweetened frozen dessert increased when maltodextrin and sorbitol were used in combination.

Penetration value

The mean penetration value (mm/5s) indicated that there was increment in penetrometer reading revealing a decreasing hardness of sample with increasing levels of grape pulp (8 to 12% i.e. G_4 to G_6) respectively, which was significantly different (Pd"0.05) among the treatments. The penetration values were in the range 70.46 in Cm to 87.14 in G_6. However, the low-calorie containing 10% grape pulp was found to give a softer frozen dessert compared to Cm as well as G_1 and G_2, though very slightly. However, the hardness could depend on the overall structure of the product. It is observed a slight increase in penetrometer reading (that is decrease in hardness) by increased addition of grape pulp. Pawar et al. (2012) noted that use of ingredients added in ice cream affects the penetration value of ice cream.

Effect of different levels of grape pulp on the first dripping time and melting rate for low- calorie ice cream prepared with sucralose and sorbitol

The values given in the Table 3 (Fig 2 and 3) indicates that there was significantly lower in G_6 than in other treatments for first dripping time, in case of melting rate showed that there was significantly lower in G_4 than in other treatments. It was observed from the table that the first dripping time decreased and melting rate increased with increasing level of grape pulp because it contains high moisture content which caused decrease in viscosity and thus enhanced the melting resistance.

Sensory characteristics for selection of grape pulp level for low-calorie ice cream prepared with sucralose and sorbitol

Flavour

From the results presented in Table 4 (Fig.4) revealed that the mean flavour score for Cs had significantly lower score (7.280)

Table 3 Effect of different levels of grape pulp on the first dripping time and melting rate for low-calorie ice cream made with sucralose and sorbitol

Treatments	Melting rate % (Time in minutes)										
	FDT	10	20	30	40	50	60	70	80	90	100
Cs	9.00^{c}± 0.447	8.58^{b}± 0.33	20.79 ^{a}±0.38	35.42^{a} ± 0.56	49.48^{a} ± 0.29	59.90^{a} ± 0.81	68.13^{a} ± 0.55	70.55^{a} ± 0.52	71.43^{a} ± 0.46	72.32^{a} ± 0.33	73.11^{a} ± 0.20
G_4	17.20^{a}±0.9 69	5.79^{c}± 0.56	15.00^{c} ± 0.88	25.04^{d} ± 1.46	35.10^{d} ± 2.03	46.59^{c} ± 1.61	54.30^{d} ± 1.09	56.61^{d} ± 0.69	57.5^{c}± 0.80	58.58^{c} ± 1.17	59.92^{c} ± 1.34
G_5	14.60^{b}± 0.678	8.48^{b}± 0.37	17.72^{b} ± 0.44	29.08^{c} ± 0.55	37.47^{c} ± 0.61	53.76^{b} ± 0.65	61.10^{c} ± 0.47	63.38^{c} ± 0.73	64.29^{b} ± 0.85	64.97^{b} ± 0.97	65.52^{b} ± 1.15
G_6	12.40^{b}± 0.812	11.24^{a} ± 0.54	18.83^{b} ± 0.56	32.39^{b} ± 0.68	42.50^{b} ± 0.85	58.05^{a} ± 0.47	65.10^{b} ± 0.98	67.66^{b} ± 1.37	68.93^{a} ± 1.70	70.74^{a} ± 1.62	72.19^{a} ± 1.79
CD (P=0.05)	2.254	1.31	1.80	2.70	3.46	2.97	2.45	2.67	3.41	3.38	3.78

Values mentioned above are mean ± SE; (n=5);
abcd: Means in the same column with different superscripts differ significantly (P≤0.05)

Table 4 Average sensory scores for selection of grape pulp level of low-calorie ice cream prepared with sucralose and sorbitol

Treatments	Flavour	Body and texture	Colour and appearance	Sweetness	Mouth feel	Overall acceptability	Remarks
Cs	7.28^{c} ± 0.02	8.01^{b} ± 0.05	7.90^{d} ± 0.05	8.38^{b} ± 0.01	8.03^{c} ± 0.03	7.92^{b} ± 0.17	Acceptable sweetness
G_4	8.36^{a} ± 0.01	7.88^{b} ± 0.05	8.12^{c} ± 0.05	7.87^{c} ± 0.04	8.23^{b} ± 0.01	8.09^{b} ± 0.09	Low level of sour taste
G_5	8.54^{a} ± 0.02	8.45^{a} ± 0.02	8.66^{a} ± 0.03	8.53^{a} ± 0.02	8.64^{a} ± 0.01	8.56^{a} ± 0.03	Acceptable level of sour taste
G_6	7.62^{b} ± 0.13	7.71^{c} ± 0.05	8.50^{b} ± 0.01	7.15^{d} ± 0.05	7.54^{d} ± 0.01	7.70^{b} ± 0.21	High level of sour taste
CD (P=0.05)	0.21	0.15	0.12	0.11	0.06	0.45	-

Values mentioned above are mean ± SE; (n=5);
abcd: Means in the same column with different superscripts differ significantly (P≤0.05)

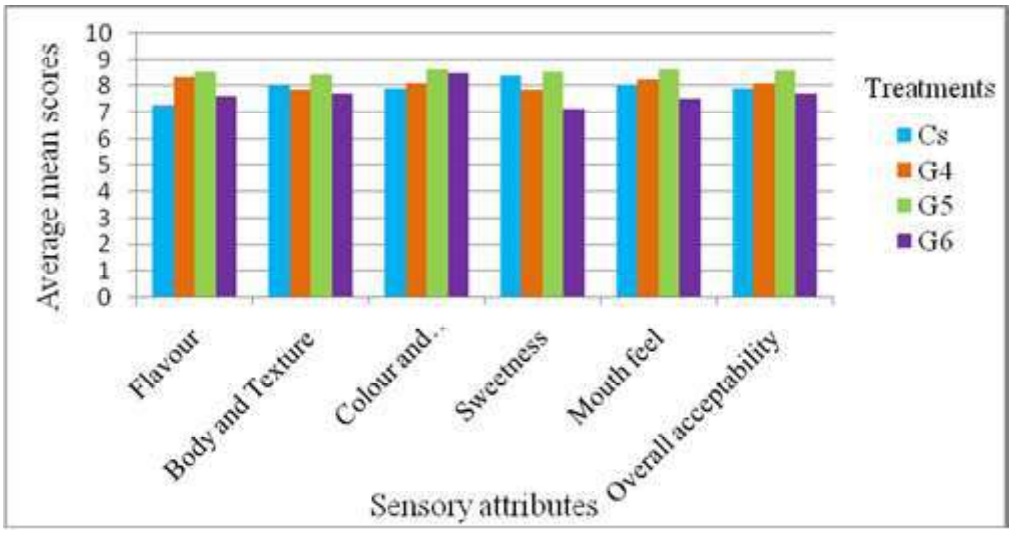

Fig 4. Sensory scores for selection of grape pulp level for low-calorie ice cream

than low-calorie ice cream made with grape pulp. Among the treated sample G_5 had significantly higher score (8.548) than G_4 and G_6 (8.362 and 7.628). A significant decrease in flavour scores observed as grape pulp level increases in ice-cream. Control had significant ($P<0.05$) lower flavor scores than grape pulp added ice cream samples. Addition of grape pulp increase flavour score up to 10 per cent level and thereon a significant ($P<0.05$) reduction of flavor scores of 12 % grape pulp added ice cream was recorded.

Body and texture

The mean body and texture scores of ice cream were 8.012, 7.884, 8.456 and 7.712 in treatments Cs to G_6 respectively, which was significantly higher (Pd"0.05) in G_5 than in other treatments. It was observed from above finding that 10% grape pulp ice cream developed a superior body and texture whereas the lowest noticed for ice cream prepared with 12 percent grape pulp. Similar findings were noticed by Morley and Ashton (1982) suggested that 10.8% sorbitol is optimum to produce softness in soft scoop ice cream. On the other hand, Finney and Dea (1978) obtained the soft scoop ice cream with 3% sorbitol.

Colour and appearance

Colour and appearance score observed for sample Cs was significantly lower score (7.960) than low-calorie ice cream added with grape pulp. Among the treated sample G_5 had significantly higher score (8.662) than G_4 (8.120) and G_6 (8.500) respectively. It is observed that the colour has been changed from white to light purple colour as the level of grape pulp was increased and colour and appearance score decreased. From the results, it was concluded that increased level of grape pulp beyond 12 per cent decreased colour and appearance score of product.

Sweetness

The mean sweetness scores of ice cream were 8.384, 7.874, 8.530 and 7.156 respectively, which was significantly higher (Pd"0.05) in G_5 than in other treatments. A significant decrease in sweetness scores observed as grape pulp level increases in ice-cream.

Mouth feel

The mean mouth feel scores were 8.034, 8.236, 8.644 and 7.542 respectively, which was significantly (Pd"0.05) higher in G_5 than in other treatments. Ice cream containing 12% grape pulp obtained lower acceptance. This was because of sourness of samples at higher levels.

Overall acceptability

The overall acceptability scores were significantly (Pd"0.05) higher for ice cream prepared with 10 percent grape pulp than all others and similar findings were noticed by Goraya and Bajwa, (2015) that 10 percent amla candy was optimal for incorporation in ice cream. The overall acceptability scores for 10 percent grape pulp are significantly higher than the other levels of grape pulp.

Based on the sensory attributes of the above study confirming that addition 10% grape pulp enriched ice cream was better acceptable since it had optimum flavour and mouth feel as compared to other treatments. Therefore, 10% grape pulp enriched low-calorie ice cream was selected for storage studies.

Conclusions

The addition of grape pulp to low-calorie ice cream improved the appearance and flavour of low-calorie ice cream, giving it a good natural colour and flavour. To improve the functional property of low-calorie ice cream (Cs) added at three different levels of grape pulp (8, 10 and 12 percent) compared with the control low-calorie ice cream. The composition, physico-chemical and sensory

attributes of the product have been studied. The overall acceptability scores were highest score (8.568) for 10 percent grape pulp with 300 ppm sucralose and 3 percent sorbitol added low-calorie ice cream (G_5). So, low-calorie ice cream can be prepared by addition of grape pulp with improved colour, flavour and enriched with antioxidants.

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RESEARCH ARTICLE

Spray-dried Probiotic adjunct with *in vitro* acid and bile salt tolerance

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Abstract: Probiotic *Lactobacillus acidophilus* LA1 was encapsulated using spray drying with concentrated skim milk with total solids (17%), and maltodextrin (25%), and gum acacia (15%) as carriers. The encapsulated probiotics were assessed for viability following drying at an inlet temperature of 170°C ± 3°C and an outlet temperature of 80°C ± 3°C. The viable count enumerated after drying was 10^8 log cfu/g. The prepared probiotic adjunct was further assessed for viability through *in vitro* acid and bile salt exposure assay. The results indicated that the cell count decreased at lower pH (1, 2) and high incubation periods (1 hr and 2 hr). A similar trend was observed with bile. However, they showed better survivability for different incubation periods at low pH and in high bile salt concentrations. The spray-dried probiotic adjunct was kept for storage at three different temperatures i.e., 8 °C, 25 °C, and 37 °C for eight weeks, and assessed weekly for cell viability. An inverse relationship between cell survival and storage temperature was observed during storage.

Keywords: Bile; Encapsulation; Gum acacia; Maltodextrin; *Lactobacillus acidophilus*; pH; Skim milk

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Introduction

Probiotics are functional ingredients incorporated into foods to enhance their nutritional value. With the growing acceptance of probiotic products, customers usually desire that the health benefits of probiotic strains be kept in the foods they purchase up until the point of consumption. The use of probiotics as an active food ingredient is not easy because, to be beneficial, they must contain at least 10^{6-7} CFU of live microbes per gram or milliliter of food consumed (Huang et al. 2017). However, the FSSAI recommends 10^8 CFU/g of live organisms in food products containing probiotic ingredients also if they are in a lower number than the recommended levels, it must be supported by proven research studies for health benefits as approved by the food authority (FSSAI 2016). Maintenance of probiotic viability throughout product shelf life until consumed is an important consideration. High processing temperatures, low pH, and bile salt sensitivity during gastrointestinal (GI) transit are the most severe threats to survival. A microencapsulation is a promising approach that has appeared recently as a probiotic protective barrier. Numerous microencapsulants and techniques are available to enhance probiotic viability; alginate encapsulation is the most popular among them. However, it suffers from the disadvantage of being expensive and difficult to scale up. Since product cost is an important criterion to survive in the competitive market, some alternatives must be tried.

Spray drying is a well-known technology in the food industry and may serve as an alternative low-cost encapsulating technique to produce significant quantities of dehydrated cells.

However, microorganisms may face survival difficulties due to high temperatures and dehydration during spray drying. Thus, microbial survival is a critical parameter to control here. On the contrary, this technology can elevate survival rates by selectively choosing processing conditions and carriers (Arslan et al. 2015; Gul 2017). It also maintains good viability and functionality of cultures when subjected to simulated gastrointestinal conditions (De Castro-Cislaghi et al. 2012) and increases survival at room temperature (Ananta et al. 2005).

Various approaches to improve the performance parameters of the spray-drying technique have been tried. One method of approach is to use protectants. For instance, adding growth-promoting ingredients like prebiotics and thermoprotectants like trehalose, non-fat milk solids, and adonitol has increased culture viability throughout drying, storage, and gastro-intestinal transit. Regarding entrapment matrix, skim milk and carbohydrates like trehalose and maltodextrin were among the most commonly used and showed significantly improved cell viability during drying (Fu, & Chen 2011).

Gum Arabic, or Gum Acacia (G.A.), is a polysaccharide and glycoprotein polymer. It has good solubility, low viscosity, and surface activity and is widely used as an entrapment agent in spray drying because of its excellent emulsifying properties. Higher survival rates have been reported with gum acacia (Gul 2017; Tao et al. 2019). G.A. also inhibits complete water loss of cell components and helps to stabilize microbial cells throughout drying and storage (Liu et al. 2016).

Due to its nontoxicity, affordability, quick rate of dissolving, low viscosity even at high solid content, and ease of availability, maltodextrin (M.D.) is a commonly used coating material for encapsulation. (Silva et al. 2014).

An attempt was made to study the survival rate of *L acidophilus* LA1 spray dried in a combination matrix of three carrier matrices viz skim milk, maltodextrin, and gum acacia. The present study has been undertaken to assess the efficacy of spray-dried microcapsules in enhancing the survival rate of LA during the storage of formulation at different storage temperatures and simulated GI tract conditions.

Materials and Methods

Probiotic organism and culture environment

Lactobacillus acidophilus LA1 was obtained as a pure freeze-dried culture from the National Dairy Research Institute's NCDC National Collection of Dairy Cultures (Karnal, India). The freeze-dried culture was activated in Chalk Litmus milk for 24 hours at 37 °C before being grown in 100 ml MRS Broth at 37 °C for 24-48 h. Before cell encapsulation, the culture was transferred 2-3 times. The reactivated cultures were centrifuged twice in distilled water at 2500 g for 10 minutes at 4 °C using a refrigerated bench-top centrifuge (Hermle Z 382 K, Maschinenfabrik Berhold Hermle A G, Gosheim, Germany).

Growth and maintenance of culture

The culture was subcultured in MRS broth before use and harvested during the stationary phase to avoid cell injury during the spray drying process. The cells were collected and washed twice with saline after being centrifuged at 8000 rpm for 10 minutes at 4°C. Thereafter the cells were kept under refrigeration until used.

Carriers

Whole cow milk procured from the institute's cattle yard was separated in the Dairy Technology division's, Experimental Dairy Plant, and the resulting skim milk was used in the present study. Maltodextrin was procured from Goodrich carbohydrates, Karnal. The gum acacia was acquired from Central Drug House in New Delhi.

Microencapsulation procedure for spray-dried microcapsules

Microencapsulated adjunct containing concentrated skim milk with total solids (17%), maltodextrin (25%), and gum acacia (15%), as well as *L. acidophilus* LA1, was dried in a spray dryer (SSP Pvt Ltd Faridabad) with incoming air at 170°C ± 3°C and an outlet temperature of 80°C ± 3°C. After cooling to room temperature, the microencapsulated powder was vacuum-packed in laminate sachets.

Encapsulation efficiency

The probiotic bacteria's survival rate during spray drying was measured as the encapsulation efficiency (E.E.), which was computed as follows:

$$EE = 100 \times N / N^0$$

Where E.E. is the encapsulation efficiency (%), N^0 is the number of bacteria before drying (log Cfu/g), and N is the number of bacteria after the drying process (log Cfu/g) (Rajam,& Anandharamakrishnan 2015).

Enumeration of encapsulated cells

Eleven gram of powder was dissolved in 99 ml of maximum recovery diluents. After this, about 1ml of the solution was taken, serially diluted, and appropriate dilutions were spread plated with MRS agar. The viable cell count was determined after 48-72 hours of incubation at 37°C. For every sample, three different dilutions were enumerated and averaged. The results were expressed on dry basis.

Stability under simulated GI conditions

Effect of Low pH

The Lee and Heo (2000) approach were used to investigate the impact of pH on the survival of probiotic microbes. As recommended by Rao et al. (1989), simulated gastric solutions containing 0.2% NaCl at pH 1.0, 1.5, and 2.0 (pH adjusted with 0.1 N HCl) were prepared. A simulated stomach solution with a different pH was combined with one g of the microencapsulated culture, and the mixture was then incubated at 37°C for 1, 2, and

3 hours. As a control, free cell culture without encapsulants was kept through the same processes as powder. After being incubated for 48 hours at 37 °C, colony-forming units were counted and noted.

Effect of high bile salt concentration

Lee and Heo (2000) technique was used to examine impact of bile salts. One g of microcapsules was kept in test tubes with 10 ml each of 1.0%, 1.5%, and 2.0% bile salt solution. After incubating at 37 °C for 1, 2, and 3 hours, the cells were counted from each concentration level and compared to a control.

Statistical analysis

The data obtained in the present study were subjected to a one-way analysis of variance (ANOVA) using SPSS v.16.0 for Windows 246 software (SPSS South Asia (P) Limited, Bangalore, India). The mean values and the standard error were calculated from the data obtained with triplicate trials.

Results and Discussion

Encapsulation efficiency and viability of spray-dried microencapsulated bacteria

Table 1 reveals that following drying, the number of survivors decreased from an initial log count of 9.32 to 8.11 log counts for spray-dried microencapsulated *L. acidophilus* LA1. Further, the overall log count reduction was observed to be 1.21 log counts. Therefore, the encapsulation efficiency calculated in the present study was 87%. The high encapsulation efficiency of matrix materials, more than 80 % for encapsulation of observed organisms in this study, agreed with previous research (Ying et al. 2012). Microcapsules containing gum arabic demonstrated the highest entrapment efficiency of 97.1% (highest viability 7.78 log CFU/g) (Tao et al. 2019). Pereira et al. (2014) reported a higher encapsulation yield of 77 – 82% using maltodextrin and gum arabic. Maltodextrin and gum acacia has long been used as wall materials to encapsulate probiotics and are effective as protectants for culture drying (Gul 2017).

The bar diagram in Fig 1 shows that the percent survival of the microencapsulated probiotic of live cells survived (87.07%) the spray drying process. The overall percent reduction was observed to be 12.93 %. It has been observed that during spray drying there is reduction in the bacterial cells due to heat stress and dehydration effects which may lead to injury at cellular levels. Exposure to higher outlet temperatures also may result in higher viability loss (Ananta et al. 2005). Approaches like use of stationary phase cultures and use of thermoprotectants can be of benefit. In the present study we have employed both approaches that might have resulted in lesser decline in cell from an initial value. According to Lian et al. (2002), the most effective carriers of probiotic *bifidobacteria* for improved survival after spray-drying were skim milk (80% survival) and gum acacia (25 to 60% survival).

Table 1 Log cfu/g of live cells before and after drying

Live cells	Log cfu/g
Before drying	9.32±0.32
After drying	8.11±0.42
Log reduction	1.2±0.31
Encapsulation efficiency	87.07%

Values are Mean±SE (n=3)

Several authors suggest that the effectiveness of dairy carriers in protecting cell viability during drying is related to the presence of lactose, milk proteins (García 2011), and prebiotics (Salaria et al. 2013). As a result of the good viable count obtained after spray drying, the use of gum acacia, maltodextrin, and skim milk as carrier materials is justified in the current experiment.

Stability of *L. Acidophilus* LA1 at simulated pH

The stability of free without encapsulation and microencapsulated *L. acidophilus* LA1 at simulated pH for different time intervals is presented in Table 2.

When free cells were exposed at pH 1.0 for 1 hour, the initial concentration decreased by 4.65 log count, while no colony-forming units could be found after 2-hour incubation at pH 1.0. In contrast, enhanced acid tolerance was observed at pH 2. A 2.77 log and 5.65 log reduction were observed for microencapsulated cells following 1 and 2-hour incubation, respectively.

At pH 2.0 approximately 2.88 log and 4.76 log cycles reduction were observed in free cells after 1 and 2 h of incubation, whereas in the case of microencapsulated cells, the decline was only 0.61 and 1.54 log, respectively. Thus, microencapsulated cells also declined in numbers during incubation at very low pH 1.0. Nonetheless, microcapsules were found to be significantly more acid tolerant than free cells, with > 10^2 cfu/g remaining after 2 hours at pH 1.0.

Milk proteins operate as buffering agents *in vivo*, protecting ingested strains of bacteria throughout upper GI transit, according to Charteris et al. (1998). The current findings agree with Fritzen-Freire et al. (2013), who found that after three hours of incubation at pH 2.0, the number of encapsulated cells decreased by about 2 log. Ilha et al. (2015) also found that encapsulated cells survived well, losing an average of only 1 log and 0.3 log after exposure to pH 2.0 and 3.0, respectively, as opposed to free cells, which lost an average of 4.25 log. It was also reported that in acidic circumstances, *L. casei Shirota* microencapsulated with RSM: GA experienced a modest decline in viability (Gul 2017). An appropriate spray-drying media can shield probiotics from stress

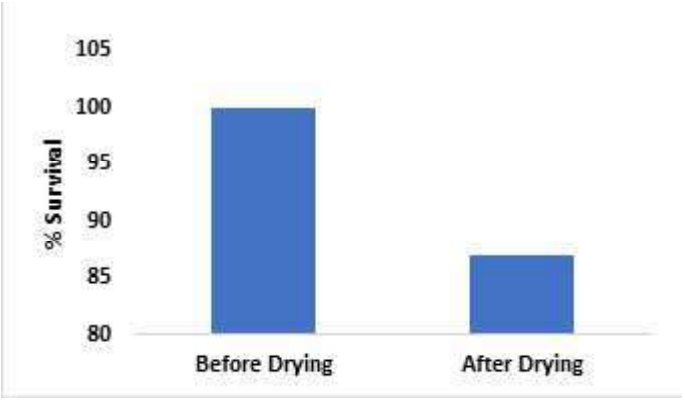

Fig. 1 Percent (%) survival in *L. acidophilus* LA1 following spray drying

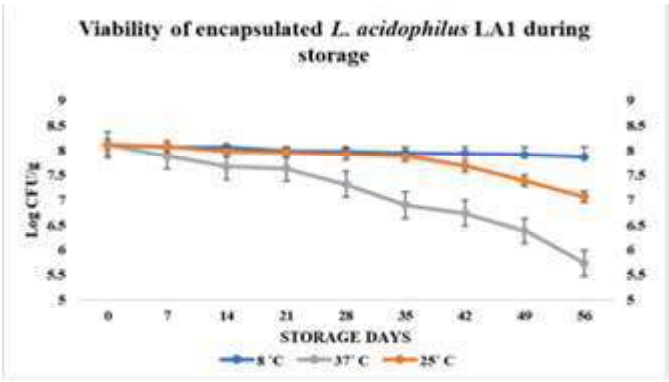

Fig.2 Effect of temperature on the viability of encapsulated *L. acidophilus* LA1 during storage

Table 2 Effect of simulated gastric pH on the viable count of *L. acidophilus* LA1 (Log cfu/g)

pH	Time(h)	Initial count	Free cells	Initial count	Protected cells	t stat
1.0	1	9.32	4.59±0.09	8.11	5.34±0.16	4.14*
	2	9.32	0.00±0.00	8.11	2.46±0.23	10.57**
2.0	1	9.32	6.36±0.18	8.11	7.50±0.24	3.86**
	2	9.32	4.48±0.20	8.11	6.57±0.25	6.49**

Values are Mean±SE (n=3), ** highly significant, * significant

Table 3 Effect of bile salt concentration on the viable count of microcapsules containing free and encapsulated *L. acidophilus* LA1 (Log cfu/g)

Bile %	Time(h)	Free cells	Protected cells	t stat
1%	1	7.32±0.17	7.91±0.06	3.27*
	2	6.80±0.08	7.74±0.06	9.63**
	3	6.46±0.26	7.57±0.11	3.93**
1.5%	1	7.14±0.09	7.73±0.12	3.86**
	2	6.52±0.19	7.41±0.24	11.92**
	3	6.05±0.12	6.78±0.07	5.32**
2%	1	6.87±0.03	7.29±0.23	1.81^{NS}
	2	5.48±0.08	6.79±0.08	11.92**
	3	4.57±0.11	6.24±0.23	6.61**

Values are Mean±SE (n=3), ** highly significant, * significant, NS=Not Significant

during digestion, according to *in vitro* research (Arslan et al. 2015).

Stability of *L. acidophilus* LA1 to simulated bile concentrations

L. acidophilus LA1 containing spray-dried adjunct was exposed to varying concentrations of bile salts (1%, 1.5%, and 2%). It is clear from the results (Table 3) that a similar trend was followed as with low pH. The cell numbers steadily declined with increasing bile levels and incubation time. The reduction rate was more for free cells. Statistically, the difference in the reduction of the free cells (F.C.) and encapsulated cells (E.C.) of *L. acidophilus* LA1 was significant at all bile levels ($p<0.01$) (Table 3). These findings agree with several earlier reports. Milk proteins and gum acacia may be responsible for the protective effects against bile (Gul 2017).

Viability of encapsulated *L. acidophilus* LA1 during storage

The results of the survival of microencapsulated *L. acidophilus* LA1 kept at different storage temperatures is presented in Fig. 2. It is evident from the figure that the numbers declined steadily as the temperature and storage period increased. The rate of decrease was more significant ($p<0.01$) at higher temperatures. The results may also be observed that there was no significant reduction at 8°C and 25 °C temperatures for the entire storage period. However, at 37°C, there was a gradual and significant ($p>0.05$) decline from an initial 8.11 log count value to 5.73 and was more rapid. At the end of the storage period, the 7.86 log

survival at 8 °C, 7.07 at 25°C, and 5.73 at 37 °C was reported. Thus, it can be inferred from the results that temperature has a marked effect on probiotic survival. The results indicated that eight weeks of storage of microencapsulated *L. acidophilus* LA1 at 8°C and 25°C corresponds to the advised therapeutic minimum dose of 10^{6-7} Cfu/g.

Statistical analysis also showed a highly significant ($p>0.01$) effect of temperatures on the survival and decline dynamics of probiotic *L. acidophilus* LA1. There has been a significant effect of temperature and interval interaction. The log count declined significantly at 37°C from an initial value and followed a non-linear trend, whereas, at refrigeration storage, it followed a linear pattern with no significant loss in viability until the end of the storage period. At ambient temperature during the initial part, there has been a linear trend for up to 5 weeks; afterward, a sharp decline can be observed. The statistical comparison showed significant differences ($p>0.05$) in survival kinetics during storage interval and temperature.

Low storage temperatures boost microbiological survival rates while reducing the stability in terms of cell survival of spray-dried samples (Kearney et al. 2009; Dimitrellou et al. 2008; Behboudi-Jobbehdar et al. 2013). Significant losses in viability were observed during storage at 37°C. Other researchers reported similar results (Ranadheera et al. 2015; Simpson et al. 2005). At higher storage temperatures, such as 37°C, a more significant loss of cell viability has been observed (Kearney et al. 2009; Strasser et al. 2009).

Our results agree with other studies that evaluated *L. acidophilus* viability using different encapsulating agents during refrigerated and higher storage temperatures.

Conclusions

In this work, a probiotic microorganism (*L. acidophilus* LA1) was spray-dried to protect the probiotic and check its efficacy against *in vitro* acid and bile salt conditions. Skim milk, maltodextrin, and gum acacia gave satisfactory effects as protectants. The viability of the probiotic strain was enhanced significantly by adding protectants during spray drying and storage. On the other hand, storage temperature was a crucial factor impacting strain viability. As was predicted, higher storage temperature led to a higher inactivation rate.

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RESEARCH ARTICLE

Validation of methods for pesticide residue analysis in milk and milk products as per FSSAI regulation

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Abstract: Accurate analysis of 55 pesticides as per the regulatory requirement of FSSAI for milk and milk products is a challenging task as it requires performing six different types of extractions and detection techniques. Further, before putting to use methods has to be thoroughly validated or checked for its fitness of purpose as per the National or International guidelines to ensure the accuracy of test results. The objective of this study was to develop a validated test method for the determination of 55 pesticides in milk and milk products as per FSSAI regulation. QuEChERS based simultaneous extraction of multiclass pesticides involves different steps i.e. mixing of reagents, separation of phases, extraction of analytes, clean up followed by reconstitution in suitable solvents. For analysis of few pesticides of different chemical nature, certain analytical steps which are saponification, derivatisation, digestion of analyte to release CS_2, extractions using specific solvents, purification using specific cartridges at specific pH, temperature and flow conditions are required to be performed. For detection of pesticide residues, mass spectrometry either with gas or liquid chromatography is one of the best approach to meet method performance and validation criteria. In this study, the optimised extraction protocols were investigated for parameters like Linearity, Matrix Effect (ME), Limit of Quantification (LOQ), Specificity, Trueness, Precision, Ion Ratio and Retention Time (RT) to validate the fitness for purpose of the methods. The calibration curves for all the pesticides were linear over the tested range as the concentration of every analyte at each calibration level fall within the residual limit of ±20 %. The LOQ for most of pesticides is established at 5 µg/kg, whereas it is 10 µg/kg for Bifenthrin, Cypermethrin, Dichlorvos, Etofenprox, Phorate, Glufosinate Ammonium, and 25 µg/kg for Dithiocarbamtes as CS_2. The Trueness and Precision of the methods were evaluated by analysing control samples spiked at LOQ and 2 to 10x the RL/LOQ in 6 replicates as per SANTE/11321/2021 guideline. Results for Trueness and Precision meet validation criteria of recovery 70 to 120 % and % RSDr < 20 % respectively for all targeted pesticides. The methods fulfilled all other the requirements of SANTE/11321/2021 guidelines and can be extended for routine analysis of pesticide residues in milk and milk products.

Keywords: FSSAI, GC-MS/MS, Milk, Milk Products, LC-MS/MS, Pesticides, SANTE/11321/2021, Validation

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Introduction

The substances intended for preventing, destroying, and repelling any 'pest' are known as pesticides. Pesticides play an important role in sustainable agriculture by protecting crops and commodities from pests and diseases (Tripathy et al. 2019). The agro produce used as food and feed sources treated with pesticides may retain some amount of these residues (Muppalla et al. 2019). These pesticide residues get into the human body through the food chain and cause health and safety problems (Johansen and Muir 2004). Therefore, it is necessary to ascertain the levels of pesticides of food matrices, so that it remains within the limit *i.e.* Maximum Residue Limits (MRL) set by national and international regulatory body.

When animals are fed feed containing pesticide residues, it may lead to excretion of residues of pesticides in animal products including milk. FSSAI has set regulatory limits for various pesticides in milk and milk products. Therefore, to ascertain the compliance in milk and milk products, the use of accurate methodology of testing of pesticides residues is of paramount importance. FSSAI list of regulated pesticides has wide chemical nature due to which laboratory has to perform different protocols for extractions, chromatographic separations and mass spectrometric detections. Use of mass spectrometry with gas or liquid chromatography offers high selectivity and sensitivity at very low levels of MRL.

Milk is a complex biological matrix, its components (Lipids and proteins) interfere with extraction and quantification (Tripathy et al. 2019). Hence for optimum simultaneous extraction of multiclass pesticide residues, milk and milk products are required to be optimally extracted using solvents and salts followed by clean up with sorbents like C-18, PSA, and dehydrating agents like Na_2SO_4 and $MgSO_4$ before analysis. QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique has emerged as a method of choice for simple, fast, efficient and economical extraction of pesticides residues from food produce (Anastassiades et al. 2003; Wilkowska et al. 2011) and milk products (Singh et al. 2012; Golge et al. 2018). Few chemically different classes of pesticides requires to perform methods with different principles for their extraction which involves steps like saponification, derivatisation, digestion of analyte to release CS_2 and extraction using a specific solvents or purification or clean-up through specific cartridge at specific pH, temperature and flow conditions. All these methods need to be thoroughly validated at and around MRLs set by legislative bodies for analytical parameters like Linearity, Matrix Effect (ME), Limit of Quantification (LOQ), Specificity, Trueness, Precision, Ion Ratio and Retention Time (RT) for use in laboratory to meet regulatory requirement. In this context main objective of the present study is to provide a robust single laboratory validated methods meeting requirement of SANTE/11321/2021 for adoption to analyse milk and milk products for residues of pesticides as per FSSAI requirement in the country.

Materials and Methods

The method setup, development and validation work was conducted at CALF, NDDB, Anand which is an analytical laboratory, accredited by NABL as per ISO 17025:2017, recognised by Export Inspection Council (EIC), BIS and is a National Reference Laboratory (NRL) of FSSAI for milk and milk products.

Standards, Reagents, and Chemicals

Pesticides standards, acetonitrile, toluene, hexane, ethyl acetate, methanol, ammonium formate, formic acid, anhydrous $MgSO_4$, PSA, C18 and all other chemicals and reagents were purchased from authorised distributors of Sigma Aldrich, Dr. Ehrenstofer and Indian companies. All chemicals, reagents, and solvents used were of pesticide residue analysis or HPLC or MS grade and are supplied by major Indian chemical suppliers.

Milk samples

Milk samples collected from the local market were analysed to investigate the presence of pesticides for the selection of blank control sample to prepare calibration curve using the standard addition approach and other quality control checks.

Standard Preparation

Stock standards of pesticides were prepared from analytical grade Certified Reference Material (CRM) of purity more than 95 %. An amount of about 10 mg of pure CRM standards were accurately weighed (Sartorius CP225D) and were dissolved and diluted to 10 ml with a suitable solvent (toluene, methanol, acetonitrile, acetone, etc.) for better solubility. The standards were immediately labelled for a minimum requirement of identification (name, date of preparation, date of expiry, concentration) and stored at -20 ºC (Thermo Fischer Scientific Model 7320 U) in the amber coloured bottles and care was taken to prevent loss of solvent and entry of water. Stock standards need to be checked for solubility, there shall not be any precipitates if required, standards were sonicated (Cole Parmer 8894) for dissolution. The concentration of stock standard was calculated considering its purity and salts. Intermediate standards of 10 mg/litre in a group were prepared by pipetting (Eppendorf) required volume of individual stock standard and dissolving in a 10 ml volumetric flask. A mixture of working standards of 1 mg/litre from a mixture of all intermediate standards was prepared by dissolving the required volume of all intermediate standards in volumetric flask of 10 ml. All intermediate and working standards were labelled indelibly and stored at -20 ºC. In case, stability is an issue working standards needs to be prepared fresh. For preparation of standard for analysis of Dithiocarbamates as CS_2, the conversion factor (liberation of CS_2 from Thiram) of 0.633 was used.

Regulatory requirement of Pesticides for Milk and Milk Products as per FSSAI

The list of pesticides to be tested in milk and milk products as per FSSAI is given in Table No. 1. The table also contains information about the instrumental technique used for analysis of each pesticide. For a complete analysis of FSSAI listed pesticides for milk and milk products laboratory requires performing six different extraction protocols due to the different chemical and physicochemical nature of these pesticides.

Methods for analysis of Pesticides residues as per FSSAI for Milk and Milk products

Multiresidue (MR-1) analysis of multiclass pesticides (GC-MS/MS and LC-MS/MS)

Weigh 10.00 ± 0.01 g of homogenized sample for products containing approximately less than 30 % total solids (liquid milk, dahi, and buttermilk), 5 ±.0.01 g of homogenized sample for products containing approximately more than 30 % total solids (ice-cream, milk powder, paneer, cheese, khoa, and other traditional Indian dairy products) and 2 ±.0.01 g of homogenized sample for high fat products (cream, butter and ghee) in a 50 mL polypropylene centrifuge tube. Make slurry of milk products with more than 30% total solids by adding 5 ml water after weighing in a tube. Add 10 ml of Acetonitrile containing 1 % glacial acetic acid to weighed samples in 50 ml centrifuge tube and shake tubes

Table 1 Pesticide in Milk and Milk Products as per FSSR 2011(V-19.08.2020)

Sr. No.	Name of pesticides	MRL mg/kg	Techniques of analysis
1	Bifenthrin	0.20	GC-MS/MS MR1
2	Chlorothalonil	0.07	
3	Chlorpyriphos	0.02	
4	Cypermethrin (sum of isomers) (Fat soluble residue)	0.05	
5	Deltamethrin (Decamethrin)	0.05	
6	Dichlorvos (DDVP) (content of D.C.A. to be reported where possible)	0.01	
7	Ethofenprox (Etofenprox)	0.02	
8	Fenpropathrin	0.1	
9	Fenvalerate (Fat soluble residue)	0.01	
10	Fipronil	0.02	
11	Pirimiphos-methyl	0.05	
12	Phorate	0.05	
13	Acephate (expressed as mixture of Methamidophos and acephate).	0.02	LC-MS/MS MR1
14	Acetamiprid	0.02	
15	Azoxystrobin	0.01	
16	Sum of benomyl and carbendazim expressed as carbendazim	0.1	
17	Bitertanol	0.05	
18	Buprofezin	0.01	
19	Carbaryl	0.05	
20	Carbendazim	0.1	
21	Carbofuran (sum of carbofuran and 3-hydroxy carbofuran)	0.05	
22	Chlorantraniliprole	0.05	
23	Chlothianidin (Chlothianidin and its metabolites)	0.02	
24	Difenoconazole	0.02	
25	Dimethoate	0.05	
26	Dinotefuran	0.1	
27	Edifenphos	0.01	
28	Emamectin Benzoate	0.01	
29	Ethion (Residues to be determined as ethion and its oxygen analogue)	0.5	
30	Flubendiamide	0.1	
31	Flusilazole	0.05	
32	Imidacloprid	0.1	
33	Indoxacarb	0.1	
34	Kresoxim Methyl	0.01	
35	Methomyl	0.02	
36	Metolachlor	0.01	
37	Monocrotophos	0.02	
38	Oxydemeton-Methyl	0.01	
39	Penconazole	0.01	
40	Phenthoate	0.01	
41	Oxygen analogues of Phorate *i.e.* Phorate sulphoxides and Phorate sulphones, expressed as phorate)		0.05
42	Propiconazole	0.01	
43	Pyraclostrobin	0.03	
44	Tebuconazole	0.01	
45	Thiacloprid	0.05	
46	Thiamethoxam	0.05	
47	Thiophanate-Methyl	0.05	
48	Trichlorfon	0.05	
49	Triadimefon	0.01	
50	2,4-Dichlorophenoxy Acetic Acid (2, 4 D)	0.05	LC-MS/MS MR2
51	Methyl Chlorophenoxy Acetic Acid (MCPA)	0.04	
52	Glufosinate Ammonium	0.02	LC-MS/MS SR1
53	Paraquat dichloride (Determined as Paraquat cations)	0.01	LC-MS/MS SR2
54	Triacontanol	0.01	GC-MS/MS SR3
55	Dithiocarbamates (Mancozeb and metiram as CS2)	0.05	GC-MS SR4
	SR-Single Residue method, MR-Multi-Residue method, LC-Liquid Chromatography, GC-Gas Chromatography, MS-Mass Spectrometry, MRL-Maximum Residue Limit as per FSSAI		

vigorously for a minute and keep tubes aside in ice-cold water or a freezer at 4 °C for 15 minutes before extraction. Add 4 gm MgSO4 and 1.5 gm sodium acetate, shake tubes for 10 minutes (Tarsons Rotaspin tube shaker) and centrifuge (Thermo Scientific Sorvall Legend XTR) at 4000 RPM for 10 minutes, the supernatant would be used for further analysis.

For GC-MS/MS amenable pesticides (Table No. 1) take 2 ml of supernatant into 20 ml glass tube, dry the sample by using a nitrogen evaporator (Caliper Life Sciences TerboVap LV) at 40 °C. Reconstitute with 2 ml of ethyl acetate and vortex for 30 seconds and for cream, butter, and ghee samples reconstitute with 1 ml ethyl acetate. Transfer extract into a clean-up tube containing 150 mg $MgSO_4$, 50 mg PSA and 50 mg C18. Vortex (Abdos SWIRLEX) the clean-up tubes for 2 minutes and centrifuge at 4000 RPM for 10 minutes. Transfer the cleaned extract into a 2 ml auto-sampler GC vial through a 0.2 µm syringe filter (Nylon Agilent part No. 5190-5271 or equivalent).

For LC-MS/MS amenable pesticides (Table No. 1) transfer 2 ml of supernatant into a 5 ml clean-up tube containing 150 mg $MgSO_4$, 50 mg PSA and 50 mg C18. Vortex clean up tubes for 2 minutes and centrifuge at 4000 RPM for 10 minutes. Take 1 ml of supernatant into a 20 ml glass tube, dry the sample by using a nitrogen evaporator at 40 °C and reconstitute it with mobile phase A: B (80:20) as in Table No. 2. Transfer the cleaned extract into a 2 ml auto-sampler vial through a 0.2 µm Nylon syringe filter (Golge et al. 2018). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Multiresidue (MR-2) Analysis of 2, 4 D & MCPA - PGRs (LC-MS/MS)

Weigh 10.00 ± 0.01 g of homogenized sample for products containing approximately less than 30 % total solids (liquid milk, dahi, and buttermilk), 5 ±.0.01 g of homogenized sample for products containing approximately more than 30 % total solids (ice-cream, milk powder, paneer, cheese, khoa, and other traditional Indian dairy products) and 2 ±.0.01 g of homogenized sample for high fat products (cream, butter and ghee) in a 50 mL polypropylene centrifuge tube. Make slurry of milk products with more than 30% total solids by adding 5 ml water after weighing in a tube. Add 10 ml of Acetonitrile to weighed samples in 50 ml centrifuge tube and shake tubes vigorously for a minute and keep tubes aside for 15 minutes. Add 4 gm $MgSO_4$ and 1 gm NaCl shake tubes for a minute and centrifuge at 4000 RPM for 10 minutes. Transfer 2 ml supernatant into 5 ml centrifuge tube containing 500 mg $MgSO_4$, 50 mg PSA and 100 mg C18. Vortex tubes for a minute and centrifuge at 4000 rpm for 10 minutes. Take 1 ml supernatant and filter through Nylon syringe filter into LC-MS/MS vial (Chris S. 2015). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Single Residue (SR-1) Analysis of Glufosinate Ammonium (LC-MS/MS)

Weigh 1 gm of milk and milk products in 15 ml centrifuge tubes, add 3 ml extraction solvent containing 50mM acetic acid and 10mM Sodium EDTA (0.287 ml of acetic acid + 0.336 gm of sodium EDTA and make up the volume to 100 ml with water). Vortex for half a minute followed by centrifugation at 4000 RPM for 10 minutes at 4 °C. Take 2 ml supernatant into a 5 ml centrifuge tube containing 25 mg C18 for clean-up. Vortex the content and centrifuge at 4000 rpm for 5 minutes. Filter the supernatant through 0.2 µm Nylon syringe filter into vial for LC-MS/MS injection (Narong C. 2015). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Note: Glufosinate Ammonium tends to interact with glass surfaces. Such interactions are stronger in presence of aprotic solvents (e.g. acetonitrile). Therefore, use only plastic tubes and vials during this analysis. Always prepare fresh working standards from intermediate.

Single Residue (SR-2) Analysis of Paraquat Dichloride (LC-MS/MS)

Weigh 10.00 ± 0.01 g of homogenized sample for products containing approximately less than 30 % total solids (liquid milk, dahi, and buttermilk), 5 ±.0.01 g of homogenized sample for products containing approximately more than 30 % total solids (ice-cream, milk powder, paneer, cheese, khoa, and other traditional Indian dairy products) and 2 ±.0.01 g of homogenized sample for high fat products (cream, butter and ghee) in a 50 mL polypropylene centrifuge tube. Make slurry of milk products with more than 30 % total solids by adding 5 ml water after weighing in a tube. Add 10 ml methanol containing 1 % formic acid to weighed samples into a 50 ml centrifuge tube. Vortex the content for a minute and shake the tubes using a tube shaker for 15 minutes. Centrifuge the samples tubes at 4000 rpm for 10 minutes at 4 °C and collect the supernatant in another centrifuge tube and adjust the pH to 6-7 using disodium dihydrogen phosphate buffer (Prepare 400 mM buffer and adjust its pH to 7 using orthophosphoric acid). Condition the cartridge (Waters Oasis WCX 3 cc Vac Cartridge, 60 mg sorbent per cartridge, 30 µm or equivalent) with 3ml methanol containing 1 % formic acid followed by 3 ml water and 3 ml methanol containing 1 % formic acid. Pass the 9 ml of supernatant through cartridges at a flow rate of approximately 1 ml/minute. Allow air to pass through the cartridge for few minutes. Elute the analyte in 3 ml methanol containing 10 % formic acid. Evaporate the content to dryness under nitrogen at 50 °C and reconstitute the sample in 1 ml water containing 10 % acetonitrile and 0.1 % formic acid**.** Filter through a 0.2 µm Nylon syringe filter into vial (Ionara R. 2016). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Note: Paraquat Dichloride tends to interact with glass surfaces. Such interactions are stronger in presence of aprotic solvents (e.g. acetonitrile). Therefore, use only plastic tubes and vials during this analysis. Always prepare fresh working standards from intermediate.

Single Residue (SR-3) Analysis of Triacontanol (GC-MS/MS)

Weigh 2 gm of homogenized liquid milk and 1 gm of milk products in a 20 ml glass test tube. Make slurry of milk products with more than 30% total solids by adding 5 ml water after weighing in a tube. Add 10 ml of ethanolic NaOH (1N NaOH dissolved in 20 parts water/80 parts ethanol. Vortex the mixture for a minute and subject content to saponification at 80 ºC for 60 minutes. After saponification add 2 ml of 5N HCL and keep samples in oven at 70 ºC for 15 minutes. Extract the samples thrice with 3 ml of heptane and collect organic phase in another glass tubes after every extraction. Wash the heptane extract with 6 ml of ultrapure water and transfer solvent layer to another glass tube. Evaporate the organic solvent to dryness under stream of nitrogen at 40 °C. Reconstitute the residues in 500 ul of derivatizing agent N, O-Bis-trimethylsilyl-trifluoroacetamide (BSTFA product no. 15238 Merck make or equivalent), incubate tubes at 80 °C for 20 minutes in oven. Evaporate the derivatizing agent BSTFA at 40 °C under a stream of nitrogen to dryness and reconstitute with 1 ml of heptane. Filter it through a 0.2 um Nylon syringe filter and collect it in GC vial. Inject 1 µl of solution in GC-MS/MS. Prepare calibration standard using standard addition technique by spiking working standard to control samples (Daniela et al. 2009) Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Note: For complete dissolution of triacontanol prepare stock standard of around 100 ppm in 100 ml heptane and further prepare intermediate standard of 10 ppm in 10 ml of heptane. Analyst may observe the loss of response after few injections which may be because of acidic BSTFA affecting source or filament assembly leading to carbonization of source or oxidation of filament. It is suggested to completely dry derivatized samples before reconstitution with heptane. Further, keeping solvent delay of 6 minutes would save heavy mist of heptane and residual BSTFA ionizing in source and affecting the source conditions. Upon continual injection of samples, it is suggested to clean the MS source part after every 20 injections. For continual performance of method on wide variety of milk products laboratory would require fine-tuning of instrument method and saponification process.

Single Residue (SR-4) Analysis of Dithiocarbamates as CS_2 (GC-MSMS)

Weigh 25.00 ± 0.10 g milk and milk products in 250 ml of stoppered conical bottle, add 75 ml of the reaction mixture (take one litre water in glass bottle of capacity of 2.5 to 3.0 litres, gradually add solution of Tin (II) Chloride (30 g of 98 % purity) dissolved in one litre of concentrated HCl with continual stirring to obtain clear solution). Add 25 ml of isooctane and immediately stopper the bottle with a screw cap. Place the bottle in a water bath at 80 (±5) °C. Mix the content of the bottle by inversion after approximately every 20 minutes. After the total reaction time of 60 minutes, remove the bottle from the water bath and mix the contents of the bottle. Transfer the bottle in ice water bath to cool down the temperature quickly. After cooling the reaction mixture to about 10-20 °C, transfer 1.8 ml of isooctane layer in a 2 ml centrifuge tube and centrifuge at 4000 rpm for 10 min at 10 °C. Transfer the supernatant (1 ml) to an auto-sampler vial (Sumaiyya et al. 2014). Instrumental parameters and MRM method are summarised in Tables 2 and 5. (See supplementary file online)

Note: Analyse the samples immediately on GC-MSMS, avoid storage of the prepared sample vials. For quantification prepare calibration standards by spiking respective/representative sample commodity using standard addition approach.

Results and Discussion

Validation of methods

Validation of method is required to be done to demonstrate that a method is fit-for-intended purpose. A validated test method ensures accurate, reliable, and consistent results and validation of in-house test methods is a mandatory requirement of accreditation as per ISO17025:2017. Parameters and criteria of validation for analysis of pesticides residues as per SANTE/11321/2021 are indicated in Table 3. These parameters were evaluated and results obtained are discussed as per criteria of SANTE/11321/2021.

Linearity

Linearity can be tested by examination of a plot of residuals produced by linear regression of the responses verses concentrations. In general, the use of weighted-linear regression is recommended for low part per billion (µg/kg) concentrations. Ideally, the value of the intercept should be close to zero to reduce errors in the calculation of concentrations at lower levels, at the same time calibration curve should not be forced through the origin/zero without justification. Formula for linear equation is as given below,

$y = mx \pm c$

Where, y is the instrument response (plotted on Y axis)

m represents the slope (sensitivity),

c is a constant that describes the background (intercept on Y axis)

x is the analyte concentration (plotted on X axis) of unknown samples,

Linearity of a test method was studied by injecting standards at five concentration levels as showed in Table 4 and was considered

Table 2 Instrumental parameters

Multiclass: MR-1 Multiresidue method GC-MS/MS	Multiclass: MR-1 Multiresidue method LC-MS/MS	2, 4 D and MCPA: MR-2 (PGR) Multiresidue method
Instrument Conditions (Agilent 7010B)	Instrument conditions (Waters Xevo TQS)	LC-MS/MS
GC Oven conditions	Instrument Settings	Instrument conditions (Waters Xevo TQS)
Oven Temperature Program	Mobile Phase A: 0.1 % formic acid and 5	Instrument Settings
60 ºC for 1 minutes,	mM ammonium formate in water:	Mobile Phase A: 0.1 % acetic acid in water
40 ºC per minute to 170 ºC,	methanol (90:10)	Mobile Phase B: 0.1 % acetic acid in
10 ºC per minutes to 310 ºC,	Mobile Phase B: 0.1 % formic acid and 5	acetonitrile
3 minutes hold	mM ammonium formate in methanol:	Flow rate: 0.4 ml/min
Run time- 20.75 minutes	water (90:10)	BEHC18 1.7µm, 2.1 X 100mm Column
GC injection conditions	Flow rate: 0.4 ml/min	Temperature: 40 ºC
Inlet Type: Multi-Mode Inlet (MMI)	Column Temperature: 40 ºC	Injection Volume: 5 µL
Liner: 2 mm id Agilent's part no. 5190-2293	Injection Volume: 5 µL	Run Time: 11 minutes
Injection Volume: 1 µl (Syringe 10 µl)	Column:	UPLC Gradient:
Injection Mode: Split less	BEHC18 1.7µm, 2.1 X 100mm	Time / Mobile Phase (%)
Inlet Temperature (ºC): 280	Run Time: 22 minutes	Min. / A / B
Septum Purge (ml/min): 3	UPLC Gradient:	0.0 / 90 / 10
GC Column Flow Conditions	Time / Mobile Phase (%)	0.5 / 90 / 10
Carrier Gas: Helium	Min. / A / B	4.0 / 10 / 90
Column 1 and 2 connected through union	0.0 / 98 / 2	4.5 / 10 / 90
DB5MS 15m x 250µm x0.25µm	0.5 / 98 / 2	5.0 / 90 / 10
Column 1 flow: 1.197 ml /min	15 / 2 / 98	8.0 / 90 / 10
Column 2 flow: 1.397 ml/min	17 / 2 / 98	MS conditions
MS conditions	17.5 / 98 / 2	Mode: ESI (Negative mode)
MS Source (eV): 70	22 / 98 / 2	Capillary (kV): 2
Source temperature (^{0}C): 280	MS conditions	Cone (V): 20
Quadruple Temp (^{0}C).: 150 ºC	Mode: ESI (Positive mode)	Source offset (V): 80
Transfer Line Temp.: 280	Capillary (kV): 1.00	Source temperature (^{0}C): 150
Helium Quench (ml/min): 2.25	Source offset (V): 80.0	Desolvation Temp. (^{0}C): 550
N2 Collision (ml/min): 1.5	Source temperature (^{0}C): 150	Cone Gas Flow (L/Hr): 150
	Desolvation Temp. (^{0}C): 500	Desolvation Gas flow (ml/Min): 1000
	Cone Gas Flow (L/Hr): 150	Collision Gas (ml/Min): 0.15
	Desolvation Gas flow (ml/Min): 1000	
	Collision Gas (Bar): 0.15	

Glufosinate Ammonium: SR-1 Single Residue method LC-MS/MS
Instrument Settings
Mobile Phase A: 50 mM ammonium formate in water (pH adjusted to 2.9 using formic acid)
Mobile Phase B: Acetonitrile containing 0.5% formic acid
Flow rate: 0.5 ml/min
Column Temperature: 40 ºC
Injection Volume: 10 µL
Column: Torus DEA 1.7 µm, 2.1 X 100mm
Run Time: 10 minutes
UPLC Gradient

Time (Min.)	Mobile Phase (%) A	B
0.0	10	90
0.2	10	90
4.5	60	40
5	10	90
10	10	90

MS conditions
Mode: ESI (Negative mode)
Capillary (kV): 1.00
Source offset (V): 80.0
Source temperature (^{0}C): 150
Desolvation Temperature (^{0}C): 550
Cone Gas Flow (L/Hr): 150
Desolvation Gas flow (ml/Min): 1100
Collision Gas Flow (Bar): 0.15

Paraquat Dichloride: SR-2 Single Residue method LC-MS/MS
Instrument Settings
Mobile Phase A : 50 mM ammonium formate in water (pH adjusted to 2.9 using formic acid)
Mobile Phase B: Acetonitrile containing 0.5% formic acid
Flow rate: 0.6 ml/min
Column Temperature: 40 ºC
Injection Volume: 5 µL
Column: X-Bridge HILIC 2.5 µm, 2.1 X 100mm
Run Time: 10 minutes
UPLC Gradient

Time (Min.)	Mobile Phase (%) A	B
0.0	00	100
0.5	00	100
3.5	60	40
4.5	60	40
5.0	00	100
10	00	100

MS conditions
Mode: ESI (Positive mode)
Capillary (kV): 1
Cone (V): 20
Source offset (V): 80
Source temperature (^{0}C): 150
Desolvation Temperature (^{0}C): 550
Cone Gas Flow (L/Hr): 150
DesolvationGasflow (ml/Min): 1100
Collision Gas Flow (ml/Min): 0.15

Triacontanol: SR-3 Single Residue method GC-MS/MS
Instrument conditions (Agilent 7010B)
GC Oven conditions: 200 ºC hold for 1 min.
8 ºC per minute to 270 ºC hold 2 minutes,
20 ºC per minutes to 310 ºC hold 8 minutes
Run time- 20.75 minutes,
GC injection conditions
Inlet Type: Multi-Mode (MMI)
Injection Volume: 1µl
Injection Mode: Split less
Inlet Temperature: 320 ºC
Septum Purge: 3 ml/min
GC Column Flow Conditions
Carrier Gas: Helium
Column 1 and 2 connected through multi union
DB5MS, 15 m x 250 µm x 0.25 µm back flush.
Column 1 flow: 1.063 ml /min
Column 2 flow: 1.263 ml/min
MS conditions
MS Source (eV): 70
Source temperature (ºC): 300
Quadruple Temperature (ºC): 150
Transfer Line Temperature (ºC): 320
Solvent Delay (min): 6
Helium Quench gas (ml/min): 2.25
N2 Collision Gas (ml/min): 1.5
Acquisition mode: MRM
MRM transitions: 494.9>75(Q), 97.1(q1),

DTC (Mancozeb and metiram as CS2): SR-4 Single Residue method
GC-MS/MS-Instrument conditions (Agilent 7010B)
GC Oven conditions: 40 ºC for 5 minutes,
40 ºC per minute to 200 ºC hold for 3 minutes
Run time 12 minutes
GC injection conditions
Inlet Type: Multi-Mode Inlet (MMI)
Liner: 2 mm id
Injection Volume: 1 µl
Injection Mode: Splitless
Inlet Temperature: 100 ºC
Septum Purge: 3 ml/min
GC Column Flow Conditions
Carrier Gas: Helium
Column 1 and 2 connected through multi union
DB5MS, 15 m x 250 µm x 0.25 µm
Column 1 flow: 1.063 ml /min
Column 2 flow: 1.264 ml/min
MS conditions
MS Source (eV): 70
Source temperature (ºC): 250
Quadruple Temperature (ºC): 150
Transfer Line Temperature (ºC): 280
Solvent Delay (min): 1
Helium Quench gas (ml/min): 2.25
N2 Collision Gas (ml/min): 1.5
Acquisition mode: SIM, Ions: 76 (Q) and 78 (q)

Table 3 Pesticides Method Validation Parameters and Criteria as per SANTE/11321/2021

Sr. No.	Parameter	Details of the study	Criterion
1	Linearity	Linearity check from five levels	Deviation of back calculated concentration =±20 %
2	Matrix Effect (ME)	Comparison of response from solvent standards and matrix-matched standards	< 20 % signal suppression or enhancement, if ME >20 %, use procedural calibration.
3	LOQ	Lowest spike level meeting criteria for trueness and precision	≦ MRL Mean Recovery 70 to 120% Mean Precision RSD <20%
4	Specificity	Response in reagent blank and blank control samples	≦30 % of RL/LOQ
5	Recovery (Trueness)	Average recovery at spike level tested	70 to 120%
6	Precision (RSDr)	RSDr for each spike level tested	RSD <20%
7	Precision (RSDwR)	RSDwR from on-going method validation / verification	RSD <20%
8	Robustness	Average recovery and RSDwR, derived from on-going method validation	Mean Recovery 70 to 120% Mean Precision RSD <20%
9	Ion ratio	Shall comply requirements for MS	Within ±30% (relative)
10	Retention time	Repeatability throughout the batch	±0.1 Minute

acceptable when residuals (Deviation or difference of back-calculated concentration from calibration curve verses actual concentration) were ≤±20 %. Standard addition approach was used for checking the linearity. As evident from the data in Table 4, calibration curves were linear over the tested range as the residuals/deviation from back calculations were ≤±20 % for all pesticides and regression coefficients (r^2) were higher than 0.99 except for Edifenphos (0.984).

Matrix Effect

Matrix effect is an influence of the one or more undetected matrix components from the sample on the measurement of the analyte

concentration. The matrix effect of milk constituents at a retention time of the analyte of interest is determined by comparing the response of an analyte with and without matrix component. Matrix effect occurs frequently in both gas and liquid chromatographic techniques and should be assessed at the initial method validation stage. Majorly matrix effect in GC analysis is attributed to shielding of active sites in GC liner and column by matrix components which reduce the interaction of the analytes on these active sites and lead to enhanced analytes response. Matrix effect in LC analysis is attributed to the ionisation behaviour of the analytes in source in presence and absence of the matrix components. Percentage matrix effect (ME) can be calculated using following equation,

$$\% \text{ ME} = 100 - (100 \times A_{\text{m extract}} / A_{\text{s standard}})$$

Table 4 Results for Linearity Study

Multiresidue (MR-1) analysis of multiclass pesticides (GC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5.0	10.0	20.0	40.0	80.0
1	Bifenthrin	0.9985	-5.7	0.7	4.3	3.0	-2.3
2	Chlorothalonil	0.9950	-9.5	4.9	1.0	7.8	-4.1
3	Chlorpyriphos	0.9997	-2.4	0.7	2.9	-1.0	-0.2
4	Cypermethrin (Total)	0.9984	0.0	0.0	-0.7	1.8	-1.2
5	Deltamethrin	0.9996	0.4	1.8	-3.5	1.4	-0.1
6	Dichlorvos	0.9985	0.3	-1.3	1.3	1.0	-1.3
7	Etofenprox	0.9970	-7.8	2.1	3.7	5.3	-3.4
8	Fenproprathrin	0.9990	-4.9	0.6	4.3	1.8	-1.7
9	Fenvalerate (Total)	0.9996	1.1	-2.7	0.4	2.0	-0.8
10	Fipronil	0.9987	1.4	-6.5	4.6	1.9	-1.4
11	Phorate	0.9985	0.2	-0.6	-0.4	2.0	-1.2
12	Pirimiphos Methyl	0.9992	-0.8	-2.8	2.3	2.9	-1.6
Multiresidue (MR-1) analysis of multiclass pesticides (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	35	50
13	Acephate	0.9957	-4.0	-3.8	1.0	5.6	-2.3
14	Acetamiprid	0.9986	0.0	-2.0	-0.5	-4.5	3.6
15	Azoxystrobin	0.9980	-2.0	2.0	2.5	-9.4	5.4
16	Bitertanol	0.9979	10.0	0.0	-0.5	0.6	-0.6
17	Buprofezin	0.9996	0.0	-3.0	-4.0	1.7	0.8
18	Carbaryl	0.9985	0.0	-7.0	-7.5	-14.3	13.6
19	Carbendazim	0.9975	-6.0	14.0	19.5	4.9	-12.2
20	Carbofuran	0.9996	-8.0	9.0	11.0	0.0	-5.0
21	Carbofuran Hydroxy	0.9992	0.0	-2.0	-1.0	-5.7	4.6
22	Chlorantraniliprole	0.9992	-4.0	-2.0	1.5	-12.9	8.6
23	Clothianidin	0.9961	0.0	-1.0	1.5	-8.3	5.2
24	Difenoconazole	0.9997	-4.0	-4.0	-2.0	1.1	0.8
25	Dimethoate	0.9993	-2.0	-3.0	-2.0	-7.7	6.6
26	Dinotefuran	0.9895	-2.0	-15.0	-6.5	-16.3	-4.8
27	Edifenphos	0.9843	-2.0	3.0	1.0	0.5	-1.2
28	Emamectin Benzoate	0.9991	-2.0	-5.0	-4.0	-2.6	4.0
29	Ethion	0.9982	0.0	3.0	5.0	3.7	-4.8
30	Flubendiamde	0.9991	-15.6	-10.4	-3.4	2.0	8.0
31	Flusilazole	0.9971	-2.0	1.0	-2.5	-1.4	2.0
32	Imidacloprid	0.9989	0.0	-2.0	-3.5	-8.0	7.0
33	Indoxacarb	0.9998	0.0	-1.0	-4.5	-3.1	4.0
34	Kresoxim Methyl	0.9964	-6.0	3.0	7.5	-1.7	-1.8
35	Methamidophos	0.9932	-1.0	0.6	1.3	4.9	-9.1
36	Methomyl	0.9987	2.0	-2.0	2.0	-0.3	-0.4
37	Metolachlor	0.9995	-2.0	-2.0	-3.5	0.6	1.4
38	Monocrotophos	0.9989	-2.0	-2.0	-1.0	-5.1	4.4
39	Oxydemeton methyl	0.9994	-2.0	-6.0	-5.0	-9.7	9.4
40	Penconazole	0.9992	-4.0	-2.0	-2.5	-1.7	2.6
41	Phenthoate	0.9871	-4.0	2.0	3.5	-0.6	-1.0
42	Phorate-sulfone	0.9936	-6.9	-5.8	3.0	1.0	2.6
43	Phorate-sulfoxide	0.9971	-12.4	-5.6	-2.6	4.0	6.5
44	Propiconazole	0.9992	-4.0	-4.0	1.0	-5.1	4.0
45	Pyraclostrobin	0.9998	-4.0	-4.0	-3.0	-0.3	2.2
46	Tebuconazole	0.9988	-2.0	1.0	-0.5	1.1	-0.4
47	Thiacloprid	0.9962	14.0	2.0	2.0	-2.9	-15.8
48	Thiamethoxam	0.9968	-2.0	1.0	1.5	-5.4	3.4
49	Thiophanate methyl	0.9994	-2.0	-10.0	-6.0	-4.6	6.8
50	Triademefon	0.9989	-2.0	-1.0	0.5	1.4	-1.0
51	Trichlorfon	0.9992	-2.0	-2.0	-2.0	-7.7	6.2

Multiresidue (MR-2) analysis of PGR pesticides (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	50	100
52	2, 4 D	0.9921	-3.6	-1.5	-1.5	-2.8	1.8
53	MCPA	0.9919	-0.3	0.9	-2.0	6.2	-4.7
Single Residue (SR-1) analysis of Glufosinate Ammonium (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	40	60
54	Glufosinate ammonium	0.9970	0.2	-0.8	0.9	-0.5	0.2
Single Residue (SR-2) analysis of paraquat dichloride (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	50	100
55	Paraquat	0.9960	-0.6	0.5	4.4	0.4	-4.4
Single Residue (SR-4) analysis of Dithiocarbamates as CS2 (GC-MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			25	50	100	200	400
56	Dithiocarbamates as CS2	0.9989	-1.8	-5.3	4.2	6.4	-3.5

Where, % ME is the Percentage Matrix Effect

$A_{m\ extract}$ is the peak area of analyte of interest with matrix

$A_{s\ standard}$ is the peak area of the analyte without matrix

ME value less than zero (i.e. negative value) indicates matrix suppression, while greater than zero (i.e. positive value) is a sign of matrix enhancement. SANTE/11321/2021 has set criteria of matrix effect <20 % signal suppression or enhancement. In the present study, both signal suppression and enhancement were observed for most of the pesticides. To compensate for the matrix effect SANTE/11321/2021 has also suggested an approach of standard addition technique or use of isotopically labelled internal standards for quantification. Therefore, in this work standards addition technique was adopted for plotting calibration curves and quantitation.

Limit of Quantification (LOQ)

LOQ is commonly defined as the minimum concentration of the analyte in sample that can be quantified with acceptable precision (repeatability) and accuracy. As per the theoretical definition by analytical chemists, the LOQ is the concentration at which the signal/noise ratio (S/N) equals 10 in the analysis. Due to the the latest instrumental developments and improved detection capabilities of the LC-MS/MS or GC-MS/MS, the significance of this theoretical definition of LOQ is limited. Thus, in case of pesticides residue analysis spiking at the target LOQ (must be well below target regulatory MRL and within the linear dynamic range of the instrument) is the more descriptive and practical approach. In essence, the point of the establishing a LOQ is not to determine how low the instrument can detect analytes of interest, but to demonstrate that the lowest reported concentration is meeting the requirement of the need for the analysis at and around or below the target regulatory MRL.

In the current study LOQ for all the compounds is determined by checking mean relative standards deviation (RSD) and percentage average recovery of six replicates at target LOQ. To meet accuracy criteria average % recovery at target LOQ shall be between 70 to 120 % and to meet precision criteria for mean relative standard deviation shall be less than 20 % of data from a minimum of 6 replicate injections. As showed in Table 5 and 6 the target LOQ for most of pesticides is established at 5 µg/kg, whereas it is 10 µg/kg for Bifenthrin, Cypermethrin, Dichlorvos, Etofenprox, Phorate and Glufosinate Ammonium and 25 µg/kg for Dithiocarbamtes as CS_2.

Specificity/Selectivity

Specificity/Selectivity is the extent to which a method can determine a particular analyte in a mixture or matrix without interferences from other components of similar properties. Ideally, specificity/selectivity should be evaluated to demonstrate that interferences are not significantly influencing the results. It is impractical to test the method against every potential interferant, but it is required that common interferences are checked by analyzing a reagent (process) blank for every batch of reagents. When reagents and solvents are changed between analytical batches, additional process blank evaluations must be performed. Background levels of plasticizers, bleed of septa, cleaning agents, impurities of reagent, laboratory contamination, last run carry-over, etc. tend to appear in process blank which must be recognized by the analyst when they occur. Formula for calculation of specificity/selectivity in terms of interference at retention time (RT) of analytes of interests is as given below,

$$\text{Specificity / selectivity}_{(\%\ \text{interference at RT of analyte})} = (A_c / A_{LOQ})*100$$

Where, A_c is area of control samples at RT of analyte of interest

A_{LOQ} is area of standards at reporting level or LOQ at RT of analyte of interest

The specificity/selectivity was evaluated for each of the MRM transitions by analyzing the control sample, process blank, and solvent blank. Chromatograms did not show any response of

interfering peaks at the analyte retention time for any of the pesticides investigated in this work. SANTE/11321/2021 has given criteria for specificity/selectivity response of interfering compounds at a retention time of analytes of interest shall be less than 30 % of reporting limit or LOQ.

Trueness (bias)/Accuracy

The closeness of agreement between test results and the accepted reference value of the property being measured is termed as trueness. Quantitatively trueness is stated in terms of bias, smaller the bias greater the trueness. Typically bias is tested by evaluating the response of the method to a CRM with an assigned known value. Trueness (bias) can also be determined by calculating the recovery percentage of spiked samples at different levels and comparing them with acceptance criteria of 70 to 120 %. Recovery of an analyte is termed as the amount of analyte determined in the final result compared with the amount added to a control sample before extraction. The trueness for multiresidue method was evaluated by extracting 6 replicates of blank samples spiked at LOQ and at tested levels as in Table 5 and 6. Results showed in Table 5 and 6 shows that trueness (bias) meets validation criteria for all pesticides at all tested levels. Minimum and maximum acceptable % recovery of all pesticides among all tested levels were credited to Triademefon (73.4±1.2 % @ 40 µg/kg) and Fenproprathrin (118.5±6.4 % @ 5 µg/kg) respectively.

Table 5 and 6

Precision (RSDr)

Precision is the closeness of agreement between the results of replicate tests obtained under stipulated conditions and it is usually specified in terms of standard deviation (SD) or relative standard deviation (RSD) or the coefficient of variation (CV). It is expressed as repeatability in terms of relative standard deviation (RSD %). SANTE/11321/2021 has given criteria for precision in terms of repeatability (RSDr) at each spike level tested, shall be less than equal to ±20 %. The precision was evaluated by calculating % RSDr upon analysis of 6 replicates of spiked control samples at LOQ and tested level as in Table 5 and 6. It is evident from Table 5 and 6 that results for precision meet validation criteria for all pesticides at all tested levels. Minimum and maximum acceptable precision expressed as % RSDr of all pesticides among all tested levels were attributed to Tebuconazole and 2, 4 D (1.0 % @ 5 and 50 µg/kg respectively) and Paraquate Dichloride (19.5 % @ 10 µg/kg) respectively.

Precession (RSDwr) and Robustness

Both parameters can be carried out during on-going method validation and can be performed during routine testing and are required to be evaluated statistically as evaluated in the above parameters. Precession (RSDwr) is a within lab reproducibility which can be evaluated by checking the precision of six replicates injection at LOQ, and at levels as in trueness or precision trials on three different days. Robustness (often synonymous with ruggedness) of an analytical method is the resistance to change in the results produced by the analytical method. The aspects of the method should be identified that are likely to affect results, and their influence on method performance evaluated by using ruggedness tests. Examples of the factors that a robustness test could address are small changes in the instrument, brand/lot of reagent or operator or analyst, the concentration of a reagent, pH of a solution, the temperature of a reaction, the time allowed for completion of a process, and/or other pertinent factors.

Ion ratio

It is the % relative abundance of qualifier (q) transition with the quantifier (Q) transition. Based on the relative abundance of two transitions, the ion ratio in samples, it should be within 30 % of the average reference value from the standard calibration curve. The average ion ratio obtained from the calibration curve was used as a reference ion ratio. The ion ratios for different concentrations of the standards for all pesticides were consistent and were within 30 % of the average reference value from the standard calibration curve. Calculation of Ion ratio with the example of Phorate is as given below,

% Ion Ratio= (Area of qualifier ion/Area of quantifier ion)*100

Example: When, Area of qualifier ion of phorate *i.e.,* 121.0 > 47.0 (q1) is 28003 at 5 µg/kg

Area of Quantifier ion of phorate *i.e. 260* > 75 (Q) is 93346 at 5 µg/kg

% Ion Ratio for phorate @ 5 µg/kg = (28003/93346)*100 = 29.99%

For positive confirmation of an analyte in the unknown sample average of ion ratio from the multi-level calibration curve should be taken as reference value to check ion ratio confirmation criteria of ±30 % for positive determination.

Retention Time (RT)

The retention time shift of the analyte in the extract and calibration standard is evaluated for acceptable tolerance of ±0.1 minute shift. The retention time of each pesticide was evaluated for a possible shift, which was found satisfactorily meeting the criteria of ±0.1 minute as per SANTE/11321/2021. Calculation of retention time shift with the example of Phorate is as given below,

Example: Retention time of phorate in 5-point calibration curve is consistently 7.51 minute. For confirm positive determination of phorate in an unknown sample, RT of a peak shall fall within criteria of acceptable tolerance i.e. ±0.1, For constant retention time instrument conditions like flow of mobile phase, temperature,

Table 5 Results for Validation Study (Multiresidue GC-MSMS)

Sr. No.	Name of the Parameter	Optimised conditions Quantifier (Q) qualifier (q) transitions			Method validation results at different levels of spiking (µg/kg)			
		RT	MRM Transitions	CE (Ev)	% Mean Recovery (± RSD, n = 6) (RSDr)			Target LOQ µg/kg
					5	10	40	
1	Bifenthrin	13.94	181.2 > 165.2 (Q)	25	120.7 ±7.8*	109.8 ±4.9	99.5 ±3.3	10
			181.2 > 166.2 (q1)	10				
			166.2 > 165.2 (q2)	20				
2	Chlorothalonil	8.42	265.9 >133 (Q)	45	113.7 ±17.9	100.7 ±6.9	92.3 ±8.8	5
			265.9 > 230.9 (q1)	20				
			265.9 >168 (q2)	30				
3	Chlorpyriphos	9.85	196.9 > 169 (Q)	15	113.1 ±11.5	108.1 ±6.9	101.9 ±5.3	5
			198.9 >171 (q1)	15				
			313.8 > 257.8 (q2)	15				
4	Cypermethrin (sum of Four Isomers)	16.62	181.2 > 152.1 (Q)	25	200.0 ±76.2*	114.5 ±11.1	107.3 ±4.4	10
			181 > 152.1 (q1)	25				
			165.1 > 91.1 (q2)	15				
5	Deltamethrin	18.2	252.9 > 93 (Q)	15	112.8 ±7.8	115.3 ±4.6	109.0 ±3.6	5
			181 > 152.1 (q1)	25				
			250.7 > 172 (q2)	5				
6	Dichlorvos (DDVP)	4.65	184.9 > 93 (Q)	10	128.8 ±22.4*	74.2 ±11.5	77.3 ±16.2	10
			144.9 > 109 (q1)	10				
			109 > 79 (q2)	5				
7	Etofenprox	16.89	163 > 107.1 (Q)	20	120.6 ±7.9*	114.3 ±5.5	103.1 ±4.2	10
			163 > 135.1 (q1)	10				
			107 > 77 (q2)	15				
8	Fenproprathrin	14.12	181.1 > 152.1 (Q)	25	118.5 ±6.4	109.7 ±7.6	100.7 ±3.8	5
			125 > 55.1 (q1)	10				
			207.9 > 181 (q2)	5				
9	Fenvalerate (sum of Two Isomers)	17.46 (I) 17.66 (II)	167 > 125.1 (Q)	5	110.7 ±9.2	107.4 ±4.2	102.4 ±5.3	5
			167 > 88.9 (q1)	40				
			224.9 > 119 (q2)	15				
10	Fipronil	10.46	366.8 > 212.8 (Q)	25	118.2 ±13.0	112.6 ±6.6	101.7 ±3.0	5
			254.9 > 228 (q1)	15				
			350.8 > 254.8 (q2)	15				
11	Phorate	7.5	260 > 75 (Q)	5	123.2 ±13.1*	106.2 ±9.8	95.1 ±7.6	10
			121 > 47 (q1)	15				
			128.9 > 65 (q1)	10				
12	Pirimiphos Methyl	9.5	290 > 125 (Q)	20	113.6 ±8.8	107.0 ±6.7	97.1 ±5.1	5
			232.9 > 151 (q1)	5				
			232.9 > 125 (q2)	5				

*Due to % recovery outside the accepted range, target LOQ for these compounds was established at 10 µg/kg

Table 6 Results for Validation Study (Multi and Single Residue LC and GC-MSMS)

Multiresidue (MR-1) analysis of multiclass pesticides (LC-MS/MS)									
Sr. No.	Name of the Parameter	Optimised conditions Quantifier (Q) qualifier (q) transitions				Method validation results at different levels of spiking (µg/kg)			
		RT	MRM Transitions	Cone (V)	CE (Ev)	% Mean Recovery (± RSD, n = 6) (RSDr)			Target LOQ µg/kg
						5	10	40	
13	Acephate	1.54	183.9 > 142.8 (Q)	20	10	106.5	107.4	97.7	5
			183.9 > 124.9 (q)	20	12	±13.9	±10.8	±12.9	
14	Methamidophos	1.22	141.9 > 124.8 (Q)	30	14	97.3	114.9	103.2	5
			141.9 > 93.9 (q)	30	12	±9.2	±8.1	±7.3	
15	Acetamiprid	6.02	223.0 > 126.00 (Q)	30	20	99.0	95.5	76	5
			223.00 > 56.1 (q)	30	15	±1.6	±8.9	±5.9	
16	Azoxystrobin	11.6	404.1 > 372.0 (Q)	15	8	98.3	86.8	85.3	5
			404.1 > 328.9 (q)	15	30	±2.3	±14.1	±2.6	
17	Carbendazim	3.4	192.1 > 160.1 (Q)	10	15	94.7	85.7	96.1	5
			192.1 > 132.1 (q)	10	30	±2.0	±17.0	±9.2	
18	Bitertanol	13.9	338.1 > 98.9 (Q)	30	16	100.5	97.2	109.2	5
			338.1 > 70.1 (q)	30	8	±2.6	±13.7	±3.5	
19	Buprofezin	14.84	306.1 > 201.0 (Q)	31	12	95.9	101.1	114.2	5
			306.1 > 57.4 (q)	31	20	±3.3	±4.9	±3.9	
20	Carbaryl	9.13	202.1 > 145.1 (Q)	25	10	91.7	92	79.1	5
			202.1 > 127.1 (q)	25	25	±10.5	±12.2	±4	
21	Carbofuran	8.58	222.11 > 165.1 (Q)	5	10	114.50	106.3	113.8	5
			222.11 > 123.0(q)	5	20	±8.3	±5.7	±4.2	
22	Carbofuran hydroxy	5.75	238.0 > 163.00 (Q)	34	16	99.3	91.1	77	5
			238.00 > 107.0 (q)	34	16	±1.9	±10.9	±5.7	
23	Chlorantraniliprole	11.01	484.0 > 453.0 (Q)	18	17	92.9	90.6	73.4	5
			484.0 > 286.0 (q)	18	12	±2.7	±4.9	±4.4	
24	Chlothianidin	5.04	250.0 > 169.0 (Q)	25	10	99.0	90.9	76.4	5
			250.0 > 132.0 (q)	25	15	±4.0	±15.1	±5	
25	Difenoconazole	14.33	406.1 > 337.2 (Q)	35	12	94.9	91.6	81.6	5
			406.1 > 250.9 (q)	35	25	±3	±8.4	±4.8	
26	Dimethoate	5.4	230.0 > 198.8 (Q)	20	10	95.5	92.9	78.6	5
			230.0 > 124.8 (q)	20	22	±1.5	±7	±4.7	
27	Dinotefuran	2.53	203.2 > 129.1 (Q)	10	10	100.4	107.1	87.9	5
			203.2 > 114.1 (q)	10	15	±6.5	±9.2	±6.2	
28	Edifenphos	13.5	311.0 > 111.0 (Q)	23	26	98.3	101.4	93.7	5
			311.0 > 109.0 (q)	23	32	±1.5	±9.5	±2.3	
29	Emamectin Benzoate	15.68	886.6 > 158.0 (Q)	45	37	90.9	85.1	75.4	5
			886.6 > 82.0 (q)	45	35	±3.1	±8.2	±3.4	
30	Ethion	15.22	385.0 > 199.0 (Q)	30	10	94.5	94.4	92.3	5
			385.0 > 142.9 (q)	30	25	±3.2	±11.5	±4.1	
31	Flubendiamide	13.33	683.3 > 408.2 (Q)	5	5	98.5	103.9	103.1	5
			683.3 > 274.2 (q)	5	16	±10.7	±7.9	±10.8	
32	Flusilazole	13.02	316.0 > 247.0 (Q)	5	20	96.2	84.3	80.2	5
			316.0 > 165.0 (q)	5	25	±6.3	±9.1	±4.2	
33	Imidacloprid	5.14	256.1 > 209.0 (Q)	25	12	98.7	85.2	77.3	5
			256.1 > 174.9 (q)	25	20	±3.7	±27.8	±8.2	
34	Indoxacarb	14.4	528.1 > 217.9 (Q)	30	25	92.3	92.4	83.3	5
			528.1 > 202.9 (q)	30	40	±6.0	±6.2	±4.6	
35	Kresoxim Methyl	13.21	314.2 > 131.0 (Q)	30	25	105.5	110.9	112.6	5
			314.2 > 115.9 (q)	30	12	±5.4	±2.9	±1.3	
36	Methomyl	3.58	162.9 > 105.9 (Q)	15	10	97.0	91.3	103.3	5
			162.9 > 88.0 (q)	15	10	±3.2	±13.1	±5.9	
37	Metolachlor	12.83	284.1 > 252.1 (Q)	17	15	93.7	88.3	75.9	5
			284.1 > 176.10 (q)	17	25	±4.3	±11.1	±5.4	
38	Monocrotophos	4.31	224.10 > 127.1 (Q)	26	15	95.5	82.9	77.9	5
			224.1 > 98.0 (q)	26	12	±2.3	±3.2	±7.5	
39	Oxydemeton Methyl	5.41	263.0 > 169.0 (Q)	20	13	94.7	82.6	77.5	5
			263.0 > 120.99 (q)	20	14	±1.3	±2.7	±8.6	
40	Penconazole	13.32	284.0 > 159.0 (Q)	15	25	97.3	87.8	77.4	5
			284.0 > 70.1 (q)	15	15	±1.1	±16.3	±4.2	
42	Phenthoate	13.12	321.0 > 135.0 (Q)	9	20	112.1	118.9	110.4	5
			321.0 > 79.1 (q)	9	40	±6.1	±3	±1.5	
42	Phorate sulphones	9.9	293.2 > 171.2 (Q)	20	10	104.2	97.6	98.7	5
			293.2 > 97.1 (q)	20	10	±11.5	±9.5	±11.4	
43	Phorate sulphoxides	9.79	277.0 > 143.0 (Q)	24	20	105.3	110.3	99.3	5
			277.0 > 96.9 (q)	24	32	±10.9	±9.1	±8.5	

44	Propiconazole	13.67	342.1 > 158.9 (Q)	35	20	95.4	90.7	80.1	5
			342.1 > 69.1 (q)	35	30	±3.7	±13.8	±3.6	
45	Pyraclostrobin	13.85	388.1 > 193.9 (Q)	25	12	99.5	90.5	85.1	5
			388.1 > 163.0 (q)	25	25	±2.7	±13.4	±2.7	
46	Tebuconazole	13.35	308.2 > 125.1 (Q)	20	40	96.8	92.2	85.5	5
			308.2 > 70.1 (q)	20	24	±1.0	±11.4	±2.1	
47	Thiacloprid	6.88	253.0 > 125.8 (Q)	35	20	100.3	85	98.1	5
			253.0 > 90.0 (q)	35	40	±12.7	±4.7	±13.6	
48	Thiamethoxam	3.88	292.0 > 211.2 (Q)	25	10	97.1	90.2	73.4	5
			292.0 > 132.0 (q)	25	20	±2.3	±12.2	±1.2	
49	Thiophanate Methyl	8.56	343.0 > 151.0 (Q)	28	22	91.2	88..3	72.8	5
			343.0 > 93.0 (q)	28	40	±11.4	±7.1	±6.9	
50	Trichlorfon	5.23	257.0 > 109.0 (Q)	28	18	95.4	90.3	77.4	5
			257.0 > 79.0 (q)	28	30	±1.6	±7.8	±3.7	
51	Triadimefon	12.14	294.1 > 196.9 (Q)	30	16	95.8	89.2	73.4	5
			294.1 > 69.1 (q)	30	20	±2.2	±39.5	±1.2	
52	2, 4 D	3.46	218.9 > 125.0 (Q)	20	25	5*	10*	50*	5
						107.0	110.0	106.5	
						±2.4	±2.1	±1	
			218.9 > 160.9 (q)	20	15				
53	MCPA	3.46	199 > 140.9 (Q)	30	18	96.8	113.1	99.3	5
			199 > 154.9 (q)	30	18	±5.3	±1.6	±7.9	
54	Glufosinate Ammonium	2.92	180 > 85 (Q)	30	16	5*	10*	40*	10
						NA	112.4	99.8	
							±3.6	±2.8	
			180 >95 (q)	30	16				
55	Paraquat dichloride	3.46	171.1 > 155.0 (Q)	80	30	5*	10*	50*	5
						115.8	100.3	105.6	
						±7.7	±19.5	±5.9	
			185.1 > 170.1 (q)	10	18				
56	Dithiocarbamate as CS_2	1.89	76 (Q)	NA	NA	25*	50*	200*	25
						114.8	114.4	99.0	
						±14.7	±17.9	±15.6	
			78 (q)	NA	NA				

NA-Not Applicable as not validated at the concentration
*Concentration (µg/kg) for particular method validation study

condition of column, liner, injector port and any change in mobile phase plays an important role.

Conclusions

For complete confirmatory and accurate analysis of pesticide residues as per FSSAI in milk and milk products, a single laboratory validated method using liquid and gas chromatography with a mass spectrometer is presented. Out of all regulatory pesticides about 49 can be extracted using multi-residue extraction protocol followed by their detection on LC-MS/MS and GC-MS/MS. Because of the different chemical nature of the remaining pesticides, laboratory needs to perform another 5 different extraction and detection protocols. Further to ensure accuracy of testing, laboratory has to follow good laboratory practices (GLP). Standards should be prepared with accuracy and stored at recommended condition. Care must be taken to avoid cross-contamination of standards with test samples especially while reusing volumetric equipment, glassware, vials, and other chemicals. Each step of sample preparation that is extraction, clean-up, and evaporation should be fit for the purpose. During routine analysis quality control tools such as replicate determination, analysis of positive and negative blank, CRM analysis, Proficiency Testing (PT) participation, and monitoring of data through control chart should be used for continual performance verification of method.

All targeted methods of analysis meet the validation criteria of SANTE/11321/2021. The method validation study will help dairy industry and other laboratories to adopt methodology for regulatory compliances and ensure safety of consumers.

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RESEARCH ARTICLE

Comprehensive genetic analysis of linear type traits for characterization of the Sahiwal cattle in an organized herd

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Abstract: Sahiwal is one of the most important dairy cattle breeds of the Indian subcontinent. A holistic and balanced approach to selection and breeding strategies must be adopted to bring about desirable genetic improvement in livestock, including indigenous cattle. The inclusion of linear type traits for dairy cattle selection in any breed improvement program has not been carried out in India so far. Therefore, this study was taken up to characterize Sahiwal cattle, on the basis of linear type traits. Data was collected on adult Sahiwal cows up to their 10th parity, maintained at Institute herd of Indian Council of Agricultural Research- National Dairy Research Institute, Karnal, Haryana from 2006-2021. Data were analysed using Least Squares Maximum Likelihood (LSMLMW), considering a sire model for the present study. Parity, season and period of calving were taken as fixed effect and age at first calving as covariable. Sire or animal was considered as random variable. Least squares means of objective linear type traits *viz.,* stature was 125.97±0.68 cm, body length 136.58±1.01 cm, chest girth 173.04±1.14 cm, body depth 203.40± 1.39 cm, rump angle 14.50±0.39 cm, rump width 20.62±0.28 cm, udder depth 20.87±0.41 cm, rear udder height 18.38±0.33 cm, and teat length 6.16±0.24 cm. On the other hand, least square mean scores of subjective linear type traits (1-9 scale) viz. fore teat placement was 6.32±0.46, rear teat placement was 7.27±0.35, rear leg side view was 6.26±0.26, rear leg rear view was 6.28±0.27 score, foot angle 6.86±0.27, angularity 34.97±0.50-degree, central ligament 6.55± 0.39, fore udder attachment 5.91±0.32, fore teat placement 6.32±0.46, and rear teat placement was 7.27±0.35. Parity had a significant ($p<0.05$) effect on fore udder attachment and central ligament and teat length in Sahiwal cattle. Season of calving had the significant ($p< 0.05$) effect, observed in case of udder depth in Sahiwal cattle. Period of calving had a significant ($p<0.05$) effect on rump angle, udder depth, teat length, angularity and central ligament in Sahiwal cattle. Based on results of present study, it was observed that Sahiwal cattle establishes a balance between the scoring pattern of linear type traits, as the balance scoring of animals was desirable for better sustainability and production performance of a dairy cattle, on a longer run. Moreover, linear type traits have a better scope in selection process and genetic improvement programs that can be utilized as balanced and comprehensive approach for indigenous dairy cattle.

Keywords: Least-Squares Analysis, Linear type traits, LSMLMW, Sahiwal

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Introduction

India has a vast diversity of livestock species. According to 20th Livestock Census, India has 53.6 crores of total livestock population and 14.5 crores of cattle population (DAHD, 2019). Sahiwal, also known as Multani, is one of the most important indigenous breeds of milch cattle. Since many years, it has been observed that the selection of dairy sires in our country is mainly based on the production performance of their progenies, and this practice of sire evaluation is routinely followed till date in most of the dairy cattle breeding programmes. Many countries have moved towards more balanced selection objectives of dairy cattle by inclusion of previously underestimated and underrated traits like linear type traits, functional traits etc other than just the production traits (Miglior et al. 2005). This practice of sire selection is holistic, balanced and animal welfare oriented. Linear Type traits play an important role in selection, and breeding program of dairy cattle, which may enhance the reproductive performance of both sires and dams, which are the main contributors to involuntary culling. Linear type traits are the basis of all modern type classification systems, and are the foundation of all systems for describing the dairy cow. It has been found that selection only on the basis of production traits continuously has decreased the overall well-being of animal (Dadati et al. 1986). Scientists and animal breeders in the developed countries have

realized the important role of linear type traits towards the welfare of dairy cattle, but the application of such linear type traits is scanty in indigenous livestock breeding programs in our country. Hence, the present study was formulated with an objective to estimate various linear type traits in Sahiwal cattle and thereby, to characterize this prominent cattle breed of India.

Materials and Methods

Source of Data

The present study was conducted on the Sahiwal herd maintained at Livestock Research Centre (LRC), ICAR-National Dairy Research Institute, Karnal, Haryana. Linear type traits of 185 live adult cows belonging to the Sahiwal breed were recorded for the present study. Stature (STAT), Body length (BL), Chest girth (CG), Body Depth (BD), Angularity (ANG), Rump Angle (RA), Rump Width (RW), Rear Legs Rear View (RLR), Rear Legs Set-Side View (RLS), Foot Angle (FA), Fore Udder Attachment (FU), Front Teat Placement (TP), Teat Length (TL), Udder Depth (UD), Rear Udder Height (RUH), Central Ligament (CL), Rear Teat Placement (RTP) were recorded on each of the Sahiwal cows. To define Sahiwal breed on the basis of linear type traits, all these type traits were scored and measured as per the guidelines of International committee for Animal Recording, (2018) and National Dairy Development Board, (2017). Type traits can basically be classified into two broad categories with respect to their pattern of assessment *viz.* subjective assessment (for which no dimensions are made) and objective assessment (which can be measured by dimensions or unit). Fore udder attachment, front and rear teat placements, foot angle, rear legs side view and rear leg rear view are the traits that depend on subjective assessment on a scale of 1-9; while other traits like stature, body length, chest girth, body depth, rump angle, rump width, teat length, udder depth and rear udder height depend on objective assessment through measurement. For those traits which depend on subjective assessment, scoring was carried out through visual observations of live animals. For traits which depend on objective assessment, scoring was completed through actual measurement of traits (in centimeters). Stature, Body length, Chest girth, Body Depth, Rump Angle, Rump Width, Teat Length, Udder Depth, Rear Udder Height, were measured in centimeters. Angularity (Degree), Rear Legs Rear View, Rear Legs Side View, Foot Angle, Fore Udder Attachment, Front Teat Placement, Central Ligament, Rear Teat Placement were recorded subjectively on a 1-9 scale, while a score of 5 was considered being the intermediate value.

Instruments for Measurement Stature, Body length, Chest girth, Body Depth, Rump Angle, Rump Width, Teat Length, Udder Depth, Rear Udder Height, were measured using a rewindable, 5m long metal tape with a precision of 1.00 mm and a wooden flat strip was used for leveling. Angularity was measured using a wooden device with a protector to measure the angle of openness of the ribs. Rear Legs Rear View, Rear Legs Side View, Foot Angle, Fore Udder Attachment, Front Teat Placement, Central Ligament, and Rear Teat Placement were given scores on the basis of visual observation on a scale of 1 to 9, with 5 being the intermediate score.

Data Structure

To ensure the proper normalisation, the data set was standardized using mean ±2×standard deviation for each trait. The records of the animals with normal lactation were considered for this study. The records with less than 100 kg of lactation yield were discarded. The records of the adult Sahiwal cows with known pedigree were taken for the present study. Experimental and pregnant animals were also excluded from the study. Abortion, stillbirth and other pathological conditions which affected the lactation yield were considered as abnormalities and hence such records were not taken into consideration. The non-genetic factors *viz*; season of calving (4 levels) and period of calving (2 levels), parity of animal (4 levels) were classified into subclasses to assess the effects of non-genetic factors on different traits in order to get a precise estimate of genetic parameters of different traits under study. Age at first calving was taken as covariate.

Statistical Analysis

The Sahiwal breeding data were analyzed by Least Squares Maximum Likelihood (LSML) approach by using the Computer software package LSMLMW (Harvey, 1990). The effect of non-genetic factors (period of calving, season of calving, parity and AFC) was estimated using fixed effect model for linear type traits in Sahiwal cattle. The model was used with the assumption that different components being fitted into the model were linear, independent and additive. The following fixed effect model was considered to estimate the effect of non-genetic factors on the linear type traits in Sahiwal cattle.

$$\mathbf{Y_{ijkl} = \mu + S_i + P_j + L_k} + b\,(X_{ijkl} - \bar{X}) + \mathbf{e_{ijkl}}$$

where,

Y_{ijkl} = linear type traits of l^{th} animal calved in i^{th} season and j^{th} period and of k^{th} parity

μ = Population mean

S_i = Effect of i^{th} season of calving (i=1,2,3,4)

P_j = Effect of j^{th} period of calving (j=1,2)

Lk = Effect of k^{th} parity of animals (k=1,2,3,4)

X_{ijkl} = Age at first calving corresponding to Y_{ijkl}

b = Regression of variable on age at first calving

$\bar{X}$ = Average age at first calving

e_{ijk} = Random error associated with observation, assumed to NID $(0, \sigma^2_e)$

Duncan's multiple range test as modified by Kramer (1957) was used for testing differences among least squares means.

Results and Discussion

To define Sahiwal breed on the basis of linear type traits, all these type traits were scored and measured as per the guidelines of ICAR (2018) and NDDB (2017). The least squares mean of 17 linear type traits of Sahiwal cattle with their standard error were presented in the Table 1. Sahiwal cattle can be defined as a breed of dairy cattle having average stature of 125.97±0.68 cm, average body length of 136.58±1.01, chest girth of 173.04±1.14 cm, body depth of 203.40± 1.39 cm, rump angle of 14.50±0.39 cm and rump width of 20.62±0.28 cm. The average score of rear leg rear view, rear leg side view, foot angle, udder depth, rear udder height, fore udder attachment, teat length, front teat placement and rear teat placement 6.28±0.27, 6.26±0.26, 6.86±0.26,20.87±0.41 cm,18.46±0.33 cm, 5.91±0.32 score, 6.16±0.24 cm, 6.32±0.46 score,

Table 1 Least-Squares Means (LSM), Standard Error (SE) and Range of Linear Type Traits

Sr	Trait	LSM ± SE	Range	Interpretation
1	Stature (cm)	125.97 ± 0.68	116-134	Intermediate stature
2	Body Length (cm)	136.58 ± 1.01	115-155	Moderate body length
3	Chest Girth (cm)	173.04 ± 1.14	151-194	Intermediate chest girth
4	Body Depth (cm)	203.40 ± 1.39	168-242	Moderately strong body
5	Rump Angle (cm)	14.50 ± 0.39	8-22	High rump angle
6	Rump Width (cm)	20.62 ± 0.28	16-26	Intermediate rump width
7	Udder Depth (cm)	20.87 ± 0.41	14-37	Intermediate udder depth
8	Teat Length (cm)	6.16 ± 0.24	2-18	Moderate teat length
9	Rear Udder Height (cm)	18.46 ± 0.33	12-29	Intermediate rear udder
10	Angularity (angle in degree)	34.99 ± 0.51	20-45	Intermediate
11	Rear Leg Rear View (score;1-9 scale)	6.28 ± 0.27	1-9	Intermediate to high
12	Rear Leg Side View (score;1-9 scale)	6.26± 0.26	1-9	Intermediate to high
13	Foot Angle (score;1-9 scale)	6.86 ± 0.26	1-9	Intermediate to high
14	Fore Udder Attachment (score;1-9 scale)	5.91± 0.32	1-9	Moderate attachment
15	Front Teat Placement (score;1-9 scale)	6.32± 0.46	1-9	Centrally placed in majorityCases.
16	Central Ligament (score;1-9 scale)	6.55 ± 0.39	1-9	Intermediate to stronger central ligament
17	Rear Teat Placement (score;1-9 scale)	7.27 ± 0.35	1-9	Centrally and closely placed rear teats

Table 2 Effect of non-genetic factors on linear type traits in Sahiwal cattle

Sr	Traits	Parity	Season of calving	Period of calving
1	Stature	NS	NS	NS
2	Body Length	NS	NS	NS
3	Chest Girth	NS	NS	NS
4	Rump Width	NS	NS	NS
5	Rump Angle	NS	NS	S*
6	Udder Depth	NS	S*	S*
7	Rear Udder Height	NS	NS	NS
8	Teat Length	S*	NS	S*
9	Angularity	NS	NS	S*
10	Rear Leg Rear View	NS	NS	NS
11	Rear Leg Side View	NS	NS	NS
12	Foot Angle	NS	NS	NS
13	Fore Udder Attachment	S*	NS	NS
14	Front Teat Placement	NS	NS	NS
15	Central Ligament	S*	NS	S*
16	Rear Teat Placement	NS	NS	NS
17	Body Depth	NS	NS	NS

S* significant at $p<0.05$

and 7.27±0.35 score, respectively. The significant effect of various non-genetic factors on linear type traits were listed in the (Table 2).

Average linear type traits scores in Sahiwal cattle

During measurement of linear type traits, a wide variability in stature (116-134) of animals was observed with the average 125.97±0.68 cm. Chest girth was measured as the circumference around the chest at the proximal end of chest, while body depth was measured around the circumference of last ribs towards attachment of abdomen. The average score for chest girth and body depth were 173.04±1.14 cm and 203.40 ± 1.39 cm in Sahiwal cattle, respectively.

Angularity was measured as an angle made by last rib of the body to an imaginary line drawn perpendicular to the ground, and a wedge-shaped body indicates good milking ability of animal. The average score for angularity in Sahiwal cattle was 34.99±0.51 degree that depicted that Sahiwal breed is intermediate in angularity. Rump angle was measured as a distance between two imaginary lines drawn: one straight from hip bone in long axis of spine and the other one on long axis of pin bone. Mean estimate for rump angle was 14.50 ± 0.39 cm in Sahiwal cattle. There should be a little difference in levels of hip and pin bones, and there was very little difference in levels of hip and pin bones in Sahiwal as compared to Holstein Friesian (NDDB, 2017), it may be because of this reason, indigenous breeds have lesser reproductive or calving related difficulties like dystocia than most of exotic breeds. Godara et al. (2015) reported similar observations (5.55±0.47 score) in Tharparkar cattle (n=149) on a 1-9 scale of measurement. Rump width was measured as the distance between two pin bones. Mean estimate for rump width was 20.62±0.28 cm in Sahiwal cattle, indicating intermediate to wider pelvic area. This trait indicates the capacity of pelvis of cattle which is a fine indicator of calving ease. Udder depth was measured as the distance between the attachments of udder on the abdominal wall to the lowest portion of udder up to the level of hock joint, by a measuring scale. Pendulous and deep udders were scored least, as they were more prone to injury and damage. In present study, average udder depth was 20.87±0.41 cm, indicating intermediate depth, and shallow udder in Sahiwal cattle. Deep udders are more prone to mastitis and damage (Rogers, 1993). So, during selection procedures, a balance must be established, between deep udders and shallow udders. The mean estimate of teat length was 6.26±0.24 cm, which suggested that Sahiwal has medium to long teats. Rear udder height in Sahiwal cow was measured as the distance in centimetres from the lower tip of vulva to the point of attachment of udder of the animal. Mean estimate for rear udder height was 18.46 ± 0.33 cm in Sahiwal breed, indicating low to intermediate rear udder height.

On 1-9 points scale, average score for rear legs side view was 6.26±0.26 in Sahiwal cow, and rear leg rear view was scored as 6.28±0.27, indicating intermediate to higher score in Sahiwal, depicting the proper direction and alignment of hind feet in Sahiwal cattle, to bear and support the weight of the animal. Foot angle was the angle between the imaginary line drawn at level of hoof wall to the ground. It should be steep not flat, since flat foot is not desired as it causes walking difficulties and it can't bear the weight of animal, leading it susceptible to hoof injury. Average score for foot angle was 6.86±0.26 points, indicating intermediate to moderate angle and well-formed feet. Dubey et al. (2014); Godara et al. (2015); Khan and Khan (2015) reported similar observations in Sahiwal, Tharparkar and Sahiwal (n=310) cattle, respectively. Fore udder attachment indicates

Table 3 Least-Squares Means and influence of parity on linear type traits

Traits	Overall (185)	Parity1 (64)	Parity 2 (30)	Parity 3 (30)	Parity 4 (61)
Stature	125.97±0.68	126.42 ±1.17	126.96 ± 1.1	125.99 ± 1.1	124.37± 1.17
Body Length	136.58±1.01	136.72 ± 1.9	137.40 ± 1.9	135.46 ± 1.9	134.62 ± 1.9
Chest Girth	173.04±1.14	174.17 ± 2.3	176.9 ± 2.2	171.11 ± 2.3	168.9 ± 2.3
Body Depth	203.40± .39	203.2 ± 2.98	203.7 ± 2.97	202.10 ± 3.0	202.8 ± 3.0
Rump Angle	14.50 ± 0.39	13.91 ±0 .77	14.15 ± 0 .76	14.15 ±0 .66	15.4 ± 0.77
Rump Width	20.62 ± 0.28	20.98 ± 0 .49	21.02± 0.49	20.25± 0 .50	19.70 ± 0 .49
Udder Depth	20.87 ± 0.41	20.12 ± 1.0	20.11 ± 1.04	21.46 ± 1.06	21.46 ± 1.06
Teat Length	6.16± 0.24	4.49^{a} ± 0. 58	6.41^{b} ± 0 .58	7.41^{c} ± .58	7.61^{c} ± 0 .58
Rear Udder Hight	18.46± 0.33	18.78 ± .88	17.37±0 .88	18.74 ±0.88	18.73±0 .88
Angularity	34.99± 0.51	36.10 ±1.3	34.12 ± 1.3	33.10 ± 1.3	35.11 ± 1.3
Rear Leg Rear View	6.28± 0.27	7.47 ±0 .70	6.41± 0 .75	5.47 ± 0 .72	5.24 ±0 .72
Rear Leg Side View	6.26± 0.26	6.45 ± 0 .67	5.45 ± 0 .11	6.45 ±0 .32	6.45 ± 0 .55
Foot Angle	6.86 ± 0.26	7.32 ± 0 .22	6.32 ± 0 .55	7.32 ± 0 .44	6.32 ±0 .61
Fore Udder Attachment	5.91± 0.32	6.92^{b} ± 0 .85	6.96^{b} ±0.85	5.92^{a} ± .85	5.98^{a} ±0 .85
Front Teat Placement	6.32± 0.46	6.87 ± 0 .83	6.87 ±0 .83	5.87 ± 0.83	6.87 ± 0 .83
Central Ligament	6.55 ± 0.39	8.02^{d} ± 0 .82	7.32^{c} ± 0.74	6.13^{b} ±.78	4.73^{a} ± 0.77
Rear Teat Placement	7.27 ± 0.35	7.91 ± 0 .74	7.31 ± 0.67	7.31 ± 0 .67	6.31 ± 0.67

Values with different superscript differ significantly at $p<0.05$, Figures in parenthesis is number of observations

Table 4 Least-Squares means and influence of Season of calving on linear type traits

Traits	Overall (185)	Winter (48)	Summer (39)	Rainy (59)	Autumn (39)
Stature	125.97±0.68	126.56± 0.98	124.47 ± 1.0	124.68 ± 1.0	128.07± 1.0
Body Length	136.58±1.01	134.71 ± 1.6	135.52 ± 1.6	135.91 ± 1.6	138.17± 1.6
Chest Girth	173.04±1.14	172.81 ± 1.9	171.32 ± 0.9	170.01± 1.1	175.80 ± 1.9
Body Depth	203.40± 1.39	200.67 ± 2.4	203.17± 2.4	200.63 ± 2.9	206.13±0 .49
Rump Angle	14.50 ± 0.39	14.89± 0 .65	14.83 ± 0 .65	13.81 ± 0 .65	14.74 ± 0 .65
Rump Width	20.62 ± 0.28	20.86 ± 0 .42	20. 71 ± 0 .42	20. 91± 0 .42	20.18± 0 .42
Udder Depth	20.87 ± 0.41	20.32^{a} ±0.83	$20. 11^{a}$±0.83	22.88^{b}±0.83	20.37^{a}± 0 .83
Teat Length	6.16± 0.24	5.83 ± 0 .46	6.53± 0 .49	6.25 ± 0 .49	6.16± 0 .49
Rear Udder Hight	18.46± 0.33	18.09 ± 0 .70	17.52 ± 0 .70	18.52 ± 0 .70	18.02 ± 0.70
Angularity	34.99± 0.51	35.68 ±0.91	33.91± 0 .91	33.24 ± 0 .91	34.18± 0 .91
Rear Leg Rear View	6.28± 0.27	6.08 ± 0.48	5.77± 0.48	6.90± 0 .48	6.28± 0.48
Rear Leg Side View	6.26± 0.26	6.01 ± 0.46	5.41± 0.46	6.72 ± 0 .46	6.19± 0 .46
Foot Angle	6.86 ± 0.26	6.97 ± 0.46	6.85± 0 .46	6.69± 0 .46	6.99 ± 0 .46
Fore Udder Attachment	5.91± 0.32	6.18 ± 0 .57	5.16 ± 0.57	6.09 ± 0 .57	5.31 ± 0 .57
Front Teat Placement	6.32± 0.46	6.31 ± 0 .62	6. 01± 0 .62	6. 40 ± 0.62	6.32 ± 0 .62
Central Ligament	6.55 ± 0.39	7. 70 ± 0 .58	6.10 ± 0.58	5. 07 ± 0 .58	6. 70 ± 0 .58
Rear Teat Placement	7.27 ± 0.35	7.67 ± 0 .52	7.64 ±0 .52	7. 77 ± 0 .52	7. 07 ± 0.52

Values with different superscript differ significantly at $p<0.05$, Figures in the parenthesis were the number of observations

Table 5 Least-Squares means and influence of Period of calving on linear type traits

Traits	Overall (n=185)	2017-2019 (n=63)	2020-2021 (n=122)
Stature	125.97±0.68	125.46 ± 0.84	126. 08 ±0.84
Body Length	136.58±1.01	134.94 ± 1.38	137. 89 ± 1.38
Chest Girth	173.04±1.14	173.23±1.64	172. 43 ± 1.64
Body Depth	203.40± 1.39	203.27±2.1	202.87 ± 2.1
Rump Angle	14.50 ± 0.39	14.15^{a} ± 0 .57	$14. 86^{b}$ ± 0.57
Rump Width	20.62 ± 0.28	20.22 ±0 .37	20.47 ± 0.37
Udder Depth	20.87 ± 0.41	19.28^{a} ±0 .66	22.86^{b}± 0 .66
Teat Length	6.16± 0.24	5.67^{a} ± 0.36	$6. 55^{b}$± 0 .36
Rear Udder Hight	18.46± 0.33	18.05 ± 0 .55	18. 03 ± 0 .55
Angularity	34.99± 0.51	35.78^{b} ± 0 .75	34.71^{a}± 0.75
Rear Leg Rear View	6.28± 0.27	5.78 ± 0 .40	6. 26 ± 0 .40
Rear Leg Side View	6.26± 0.26	5.92 ± 0.38	6.42 ± 0.38
Foot Angle	6.86 ± 0.26	6.56 ± 0 .38	7. 78 ± 0 .38
Fore Udder Attachment	5.91± 0.32	6.06 ± 0 .47	5. 77 ± 0 .47
Front Teat Placement	6.32± 0.46	6.41 ± 0 .55	6. 46 ± 0 .55
Central Ligament	6.55 ± 0.39	6.55^{b}± 0 .50	$6. 17^{a}$± 0 .50
Rear Teat Placement	7.27 ± 0.35	7.28± 0 .451	7.80 ± 0.451

the strength of attachment of the udder to the abdominal floor of a cow. It should be tight and strong, loose attachment of udder is not desirable. The average score for fore udder attachment was 5.91 ± 0.32 points in Sahiwal which was intermediate to slightly higher in range. Other udder traits like front and rear teat placement and their positions were examined and scores were given to each cow; closer the front teats, higher the score was allocated. Rear teats should be closer to central axis of cattle and close together, which indicates better placement of teats for easy milking, good appearance and less susceptibility to mastitis. Mean scores for front and rear teat placement were 6.32 ±0.46 and 7.27±0.35, respectively indicating central positions of teats in Sahiwal breed. Central ligament indicates strength of ligamental attachment of udder of cattle. Tight ligament attachment makes udders less susceptible to mastitis, and other udder related disorders. Average score for central ligament was 6.65 ± 6.60, indicating a medium to higher score in Sahiwal.

Effect of non-genetic factors

Effect of parity

Least squares mean of 17 linear type traits scores based on parity of Sahiwal cows were presented in Table 3. Parity had a significant ($p<0.05$) effect only on fore udder attachment and central ligament

and teat length in Sahiwal cattle on the other hand other traits revealed no significant effect of parity in Sahiwal cows. Thompson et al. (1983) reported parity as a significant effect for all linear type traits in Holstein cattle. Lucas et al. (1984) reported significant effect of parity on fore udder attachment and udder depth in crossbreed cattle, similar to the present study. Least squares mean of teat length increased from first parity to fourth parity in a linear fashion in Sahiwal cattle. Marinov et al. (2015) reported significant effect of parity on teat length in Holstein cattle. Least squares mean of fore udder attachment was decreased from second to third parity, indicating, fore udder attachment loosened with increase in parity in Sahiwal cattle. Constant decrease in least square means of central ligament was observed from first to fourth parity, indicating, weakening or loosening of central ligament of Sahiwal cattle with increase in parity number.

Effect of season of calving

Season of calving was having the significant ($p< 0.05$) effect on udder depth in Sahiwal cattle while, other traits revealed no significant effect of season of calving in Sahiwal cows (Table 4). Udder depth in rainy season (22.82 ±0.83) was found significantly higher than other seasons *viz*., winter, summer and autumn. However, there was no significant difference between winter and summer season in Sahiwal.

Effect of period of calving

Period of calving had a significant ($p<0.05$) effect on different linear type traits viz., rump angle, udder depth, teat length, angularity and central ligament in Sahiwal cattle (Table 5). While, other traits revealed non-significant effect of period of calving in Sahiwal cows. Least squares mean of rump angle (14.86±0.57), udder depth (22.86±0.66) and teat length (6.55±0.36) were significantly higher ($p<0.05$) in 2020-21 year of calving in Sahiwal cows. While, LSM score of angularity (35.78±0.75) and central ligament (6.55±0.50) were significantly ($p<0.05$) higher in 2017-19 years of calving in Sahiwal cattle.

Conclusions

Based on the observations of the present study, it is concluded that Sahiwal cattle have established a balance between the scoring patterns of linear type traits, as the balance scoring of dairy animal for linear type traits is desirable for better sustainability and production performance. Dairy cattle exhibiting a proper balance in linear type traits should be recommended for future breeding. Sahiwal cattle is a breed of intermediate stature, medium to long body, wide and strong chest and intermediate body depth. It was observed that Sahiwal, being one of the most prominent indigenous cattle may have a better scope in the selection process and genetic improvement programs for linear type traits. Results in the present study, focused on the fact that incorporation, scoring and recording of linear type traits should be initiated at organized herds in different other indigenous breeds with larger sample size for efficient observations. This would provide more reliable estimates for inclusion of linear type traits in the selection criteria of the Indian dairy cattle.

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RESEARCH ARTICLE

Comparative study of multiple linear regression and artificial neural network for prediction of first lactation 305-days milk yield in Tharparkar cattle

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Abstract: The present investigation was undertaken on 3266 weekly test day milk yield records of first lactation Tharparkar cows spread over a period of 8 years (2012-2020) maintained at Livestock Research Station, Beechwal, Bikaner. The weekly test day milk yields (WTD) were used to develop best multiple linear regressions (MLR) and artificial neural network (ANN) model for prediction of first lactation 305-days milk yield (FL305DMY). Further, the comparison was made between MLR and ANN model based on coefficient of determination (R^2) and root mean square error (RMSE). Artificial Neural Network was trained using back propagation algorithms viz. Scaled conjugate gradient (SCG). It has been observed that the coefficient of determination of the models was increased with the addition of test day milk yields as input variables. It was inferred from the study that artificial neural network was better than the multiple linear regression to predict FL305DMY with more than 70% accuracy by almost all the input sets at early as 117th day of the lactation with lesser value of RMSE in comparison to MLR. Therefore, it is concluded that ANN is a potential tool for the prediction of the first lactation305-days milk yield in Tharparkar cattle than multiple linear regression.

Keywords: Artificial neural network, First lactation 305-days milk yield, Multiple linear regressions, Tharparkar cattle, Weekly test day milk yields

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Introduction

The ability to predict first lactation 305-days milk yield (FL305DMY) of a cow from its test-day milk yields would determine the success of dairy herd culling programs. In dairy cattle, a high rate of genetic improvement is only possible through the early culling of low-producing cows. The selection of dairy cattle based on test day milk yields is advantageous to the dairy farmer as it cuts down the cost of progeny testing, genetic evaluations studies specially, sire evaluation at an early age and also saves time in decision-making for selection. However, in developing countries such as India, there is a limited level of milk recording, and the use of test day models would result in lower recording costs because we could have longer intervals between milk recording and less frequent collection of milk samples. As a result, use of test day milk yields intervals is receiving more importance for prediction of milk yield in dairy cattle. Prediction of FL305DMY using test day milk yields in an early stage of lactation with maximum accuracy is one of the criteria of selection for lifetime profitability of dairy cows (Kannan and Gandhi, 2006). Currently, multiple linear regressions are using for predict lactation milk yield or first lactation 305 days milk yield at most of the dairy evaluation programmes. In multiple linear regressions, several explanatory variables are used to predict the outcome of the response variable. It estimates the coefficients of the linear relationship between input and output variable. The neural network is a set of non-linear data modeling tools and consisting of three layers i.e. input layer, output layer, and one or two hidden layers. The weights associated with the connections between neurons in each layer are iteratively adjusted by the training. The knowledge is acquired by the network through a learning process and synaptic weights are used to store the knowledge so it resembles the human brain.

Mostly, the prediction of 305-days milk yield is done on the basis of prediction equations constructed by multiple regression analysis, which does not consider co-linearity among explanatory variables and may lead to biased results (Pindyick and Rubinfeld, 1991). On the other hand, the connectionist models also known as artificial neural networks (ANNs) are algorithmic and mathematical models that mimic the learning process of the human brain and can be applied to non-linear and complex data, even if

it is imprecise and noisy, therefore nowadays it is receiving more importance for the prediction of milk yield.

It has found wide applications viz., prediction of second parity milk yield and fat percentage of dairy cows based on first parity information using neural network system (Edriss et al. 2008), prediction of first lactation 305-day milk yield in Sahiwal cattle (Dongre et al. 2012), comparisons of artificial neural network and multiple linear regression for prediction of first lactation 305-day milk yield in Murrah buffaloes (Rana et al. 2021). Hence, the present investigation was carried out to compare the relative efficiency of multiple linear regression and artificial neural network for prediction of first lactation 305-days milk yield in Tharparkar cows based on weekly test day milk yields.

Material and Methods

The data on 3266 weekly test day milk yields records of first lactation of Tharparkar cows maintained at Livestock Research Station, Beechwal, Rajasthan University of Veterinary and Animal Sciences, Bikaner, India over a period of 8 years (2012–2020). Data were analysed to predict first lactation 305-days milk yield (FL305DMY) using weekly test day milk yield records. The farm is located at 28° 1' N Latitude and 73° 1 9' E Longitude. It has an average elevation of 234.84 meters above mean sea level. The soil is sandy. The maximum temperature goes as high as 50 °C during the summer months and falls down to the level of 0 °C during the winter months. Low and erratic rainfall is also a common feature in this area. Thus, it is obvious that Tharparkar cattle maintained at this farm have been exposed to extreme climatic conditions.

The records on animals that dried up before 100 days of lactation were not included in the study. The statistical analysis was performed using Statistical Package for the Social Sciences version 20 software. The whole data was divided into four main training data -test data sets (%) as SET-A (66.67-33.33), SET-B (75-25), SET-C (80-20) and SET-D (90-10).

From each animal, a total of 43 test day milk yield records were collected at weekly interval starting from the 6th day of lactation onwards. Out of all test days, a total of three test day records were selected using backward elimination method of multiple linear regressions (MLR) to use as input variables for artificial neural network. MLR starts with all explanatory variables included in the model. The least significant explanatory variable, that is, the one with the highest p-value, is then removed at each step until all variables have been added. When the overall fit of the model is considered, variables were automatically removed until the optimum model was found. The optimum model has three test-day milk yield records i.e. 3th, 14th and 24th which were recorded on 20th, 97th, and 167th day of lactation.

Further, a total of three input sub-sets have been prepared with a total of three test days which were used as input variables. The first input set included two test day milk yields records viz. WTD 3 and WTD24, second input set included two test days namely WTD14 and WTD24, and finally, the last input set included three test days as, WTD3, WTD 14 and WTD24.

Multiple linear regression

$$v_i = a + b_i \text{ “ } X_i$$

Where,

v_i = Estimated first lactation 305-day or less milk yield of the ith animal

a = Intercept

X_i = First lactation weekly test day milk yield record of ith animal

b_i = Regression coefficient of first lactation 305-day or less milk yield on weekly test day milk yields

The accuracy of fitting the regression model was calculated by coefficient of determination (R^2) using the following formula:

Coefficient of determination R^2=

$$\frac{\text{Sum of squares due to regression}}{\text{Total sum of square}} X100$$

Artificial neural network (ANN)

Artificial neural network was used to predict the first lactation 305-days or less milk yield from weekly test day milk yield records, using SPSS (Statistical Package for the Social Sciences) software version 20.0. ANN model is basically an intelligent data processing system which learns the predictive ability automatically from the data set presented while training the network. The artificial neural network consists of input layer, hidden layer and output layer. Each layer has a specific role in execution of the neural network. In back propagation technique, input vector and the corresponding target vectors are used to train a network until it can approximate a prediction function. The network was trained using back propagation algorithm i.e. scaled conjugate gradient. Network parameters such as learning rate, momentum, and error goal was used as the default setting of the algorithms.

The performance efficiency of the artificial neural network model was calculated using the value of coefficient of determination (R^2-value) and Root Mean Square Error (RMSE) value.

Coefficient of Determination (R^2)

The proportion of the variance in the dependent variable that is predictable from the independent variable(s).

Calculated as

$R^2 = (\text{“}_i (Y_i - \acute{e})^2 \text{“}_i (Y_i - v_i)^2 / \text{“}_i (Y_i - \acute{e})^2)$

Where

Y_i = Observed value

é = Mean of observed values

v_i = Estimated value

Root Mean Square Error (RMSE)

The root-mean-square error (RMSE) is a frequently used measure of the differences between values (sample or population values) predicted by a model and the values observed.

$$RMSE = \sqrt{\sum_{i=1}^{n} \frac{\left(\hat{Y}_i - Y_i\right)^2}{n}}$$

Where,

v_i = Values predicted by the model.

Y_i = Actual values

n = Number of observations

Comparison of Multiple Linear Regression (MLR) and Artificial Neural Network (ANN)

The comparison of multiple linear regression and artificial neural network for prediction of first lactation 305-days or less milk yield was done based on R^2 value (coefficient of determination) and RMSE (Root Mean Square Error) value. The higher R^2 value and smaller RMSE value denoted a better fit model of prediction.

Results and Discussion

Multiple linear regression

The weekly test day milk yields were used to predict first lactation 305-day milk yield (FL305DMY) by using the multiple linear regression. The backward elimination method was used to find the optimum equation (Table 1). When all the weekly test day records (WTD1 to WTD43) were incorporated in an equation, the R^2-value was 98.80% and RMSE value was 145.25. The reported estimate of R^2-value was very close to the estimate reported by Garcha and Dev (1994) for prediction of lactation milk yield from all the ten monthly test day records in crossbred cattle with 99% R^2-value. On the contrary, Joshi et al. 1996 (93.07%) in Hariana cattle, reported lower estimate of R^2-value for prediction of lactation milk yield from all the ten monthly test day records.

Table 1 Prediction equations for first lactation 305-day or less milk yield on the basis of weekly test day milk yields by backward elimination method

S. NO.	Prediction equations	R^2-Value (%)	RMSE
1	Y= 43.148 + 140.809 WTD1 + 11.819 WTD2 - 25.901 WTD3 + 118.937 WTD4 + 9.356 WTD5 - 142.577 WTD6 + 17.683 WTD7 + 117.676 WTD8 - 145.657 WTD9 + .002 WTD10 - 117.109 WTD11 + 16.023 WTD12 + 52.899 WTD13 + 372.996 WTD14 - 317.096 WTD15 + 181.363 WTD16 + 19.019 WTD17 - 86.921 WTD18 + 34.447 WTD19 - 263.533 WTD20 + 150.524 WTD21 + 47.449 WTD22 + 154.210 WTD23 - 53.880 WTD24 -21.888 WTD25 - 27.284 WTD26 + 93.759 WTD27 + 134.732 WTD28 - 291.026 WTD29 + 325.472 WTD30 + 69.655 WTD31 -85.348 WTD32 + 308.736 WTD33 -90.686 WTD34 - 173.402 WTD35 - 529.390 WTD36 + 529.167 WTD37 - 64.459 WTD38 - 66.411 WTD39 - 49.851 WTD40 - 5.274 WTD41 + 28.088 WTD42 +40.344 WTD43	98.80	145.25
2	Y = 304.194 + 71.944 WTD3 - 56.944 WTD6 + 37.150 WTD7 + 86.935WTD14 - 69.248 WTD15 + 52.926 WTD20 -38.251 WTD23 + 206.351 WTD24	73.20	239.05
3	Y = 382.155+ 67.912 WTD3 - 55.416 WTD6 + 43.092 WTD7 + 87.076 WTD14 - 65.36015 + 202.462 WTD24	72.50	238.64
4	Y = 412.027+ 69.774 WTD3 - 42.371 WTD6 + 67.878 WTD14 + 178.606 WTD24	71.10	241.14
5	Y = 391.959 + 46.932WTD3 + 53.605 WTD14 + 171.223 WTD24	70.10	243.79

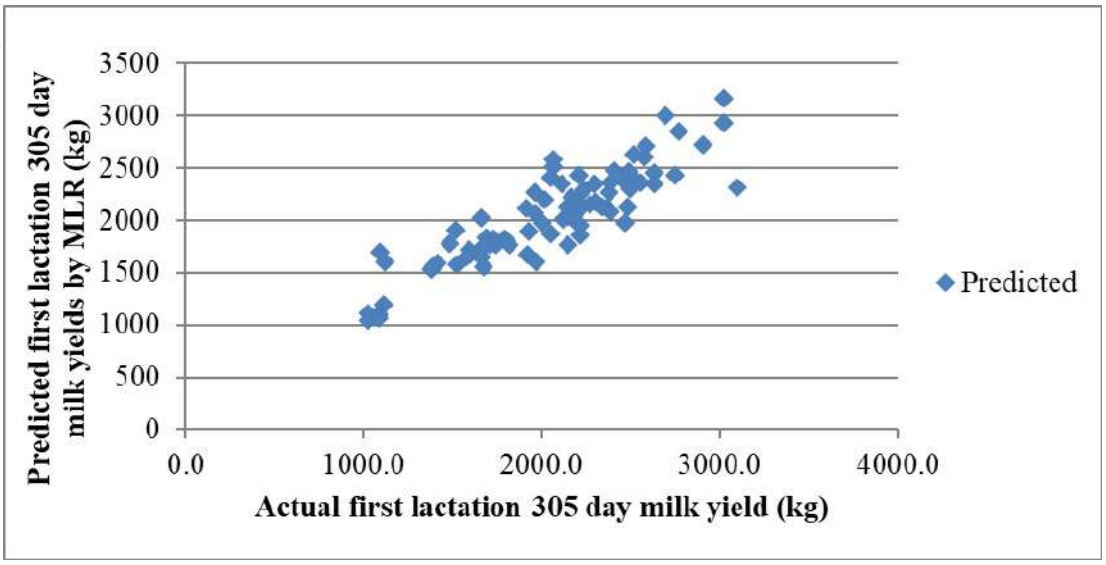

Fig 1. Predicted versus actual FL305DMY by Multiple linear regression in Tharparkar cattle

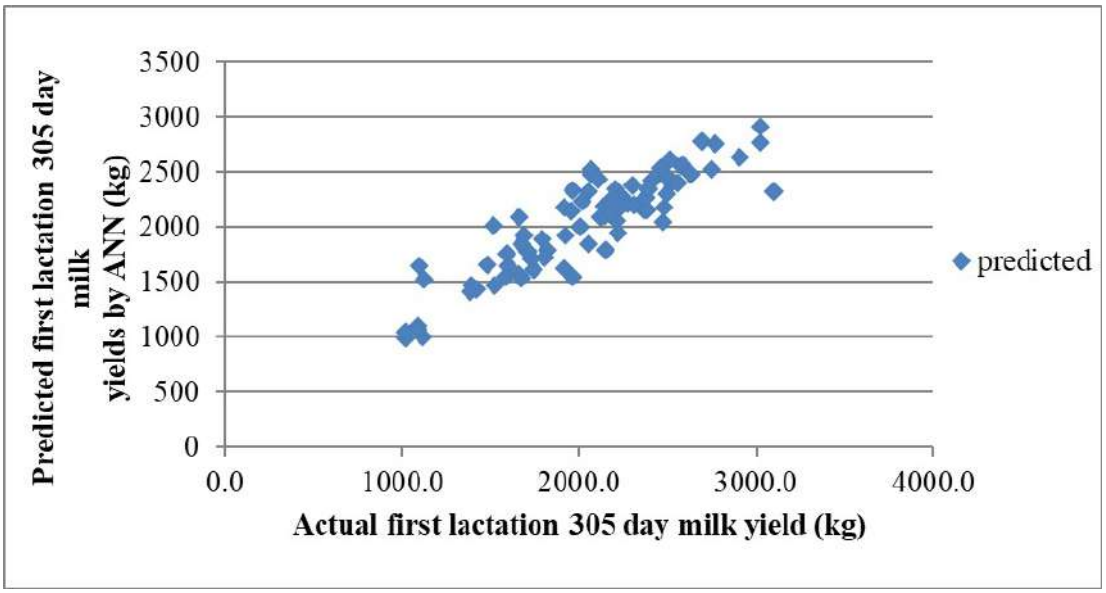

Fig 2. Predicted and actual FL305DMY by Artificial neural network in Tharparkar cattle

The optimum equation had total three variables (test days) viz. WTD3, WTD14 and WTD24. This equation gave an accuracy of prediction of 70.10% and 243.79 RMSE value. Kokate et al. (2014) reported, regression equation with 3 variables viz. BTDY-2, BTDY-3 and BTDY-5 was considered more appropriate for prediction of first lactation 305-day milk yield with 83 % accuracy and 7.54 percent error of prediction in 305-day milk yield in Karan Fries cattle.

Artificial neural network

The ANN was trained by a training data set with three test days, which was incorporated in the best equation from regression analysis. It was observed that the coefficient of determination (R^2) was increasing while the percentage of test data set was decreasing (Table 2). In SET-D (training data–test data: 90–10%), the artificial neural network explained highest (76.70 %) coefficient of determination and lower RMSE value 217.66. Bhosale MD & Singh TP (2015) repoted 85.07% accuracy as early as 126th day of lactation with Bayesian regularization neural network model and it has been also found that R^2 value of the models increases with increase in the number of test-day milk yield records in dairy cattle. Akilli & Hulya (2020) reported 79.18% accuracy with Scaled Conjugant Gradient (SCG) algorithm with decimal scaling normalization technique.The coefficient of determination and RMSE values for different input sets on test data for SET-A, SET-B, SET-C and SET-D have been presented in Table 2.

Comparison of Multiple linear regression and artificial neural network

In the present investigation, significant difference found between multiple linear regression and artificial neural network for predicting FL305DMY in Tharparkar cows. The artificial neural

Table 2 Comparison of R^2 values of different input sets with test data

Training-Test data (%)	Input Sets	R^2-value (%)	RMSE
SETA (66.67- 33.33)	1	73.70	233.12
	2	73.40	232.89
	3	70.20	261.90
SETB (75-25)	1	73.80	232.90
	2	74.10	230.07
	3	74.90	229.21
SETC (80-20)	1	72.60	236.43
	2	70.40	246.00
	3	75.70	222.79
SETD (90-10)	1	73.30	233.26
	2	72.80	239.84
	3	76.70	217.66

network model was found better than multiple linear regression for prediction of FL305DMY in Tharparkar cattle. Similar results were also reported by Sharma et al. (2007) in Karan Fries cattle, Njubi et al. (2010) in Kenyan Holstein- Friesian cattle, Dongre et al. (2012) in Sahiwal cattle, Gorgulu (2012) in Brown Swiss cattle, Bhosale MD & Singh TP (2015), Atil& Akilli (2016) in dairy cattle, Norouzian et al. (2021) in dairy cow and Singh et al. (2022) in Murrah buffalo. The FL305DMY predictions made by the best ANN model and the MLR model developed here are graphically depicted in Figs. 1 and 2, respectively.

Conclusions

The comparison was made between MLR and ANN on the basis of R^2 value and RMSE value. The arterial neural network using scaled conjugant gradient (SCG) algorithm achieved 76.70 % R^2 value and 217.66 RMSE value while in MLR it was found 70.10 % R^2 value and 243.79 RMSE value. Finally, it is concluded that artificial neural networks is better method for prediction of FL305DMY in Tharparkar cows.

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Indian J Dairy Sci 76(1): 69-72
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RESEARCH ARTICLE

Diversity analysis of DRB3 gene locus in indicus cattle- identification of novel PCR-RFLP allelic patterns

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Abstract: In the current study, genetic diversity analysis of MHC class II (DRB3) locus has been documented among indicus cattle breeds. Genotyping of 192 zebu cattle belonging to eight different breeds, was carried out to identify different allelic patterns by PCR-RFLP of BoLA DRB3 exon 2, revealing 43 different restriction patterns. Highest genetic diversity of BoLA-DRB3 gene was observed in Malnad Gidda and Gir and least in Mewati, Tharparkar and Sahiwal cattle. Allelic pattern, BM was the most frequently observed heterozygous pattern and with highest frequency among Mewati and Ladakhi cattle breeds. Few breed specific alleles also identified, which were found specifically in Tharparkar, Gir and Ongole breeds. This genetic information may be important for exploring the correlation between *BoLA*-DRB3 genetic diversity and disease susceptibility and overall allelic diversity status of the Indian cattle populations.

Keywords: Cattle, *Bos indicus*, BoLA DRB3, Allelic diversity, PCR-RFL

Introduction

The indigenous cows of India, scientifically known as *Bos indicus* or Zebu cattle, have different genetic attributes due to which they react differently to environmental stimuli. These responses are closely associated with physio-anatomical characteristics, which the animals have developed as the result of natural selection over the centuries. India is among very few nations having most of the variable natural agro-climatic conditions available within the same region. Zebu cattle are well adapted in diverse geo-climatic conditions of the country as well as under field conditions being quite refractory to several infectious diseases. In order to comprehend the distinctive disease resistance characteristics of these Zebu cattle it is important to understand the immune system of these animals. Immunity is the resistance of body against external etiological factors of disease, provided by the interaction of chemical, humoral and cellular reactions in the body. The major histocompatibility complex is one such important component of immune system responsible for modulation of innate as well as adaptive immune response.

The major histocompatibility complex (MHC) of cattle is known as bovine leukocyte antigen (BoLA), located on the short arm of bovine chromosome 23 and consisting of class I, IIa, IIb and III regions (Lewin et al. 1999). Major histocompatibility complex (MHC) class I and class II are cell surface molecules that play an important role in the intercellular recognition and self/non-self discrimination and trigger of humoral as well as cell-mediated immune responses. The *BoLA* Class IIa region comprises: *DRA*, *DRB*, *DQA* and *DQB* genes which encode the classical peptide presenting class II molecules (DR and DQ) in cattle (Lewin et al. 1999; Andersson and Davies, 1994). Three *DRB* genes, DRBP1, DRB2 and DRB3, have been identified in cattle. DRBP1 is evidently a pseudogene and functional expression of DRB2 has not been found, whereas DRB3 is functionally expressed and investigation of *BoLA-DRB3* gene of cattle is of special interest due to remarkable functional importance of the gene controlling the immune response to the viral and bacterial infections and a high level of polymorphism reported (Da Mota et al. 2002).

Genetic diversity of BoLA DRB3 gene has been studied by various researchers employing different methods. Among these, PCR-RFLP has many advantages over the other methods for the genetic analysis of populations, as it requires small amount of genomic DNA and being adaptable to crude DNA preparations (Van Eijk et al. 1992). It has been found to be a powerful tool for detecting the variation in DNA sequences of bovine lymphocyte antigens (Russell et al. 1997). Various molecular and immunological markers have been identified which are being associated with disease

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resistance and production traits, important for selection of animals with high immuno-competence or disease resistance and high performance status. It is a well-known fact that indigenous cattle breeds harbor some invaluable characteristics such as disease resistance, adaptation to heat stress, good libido and fertility, better feed conversion efficiency, compared to exotic and crossbred. Maintenance of genetic diversity at MHC loci is an important factor to ensure that population remains fit to fight against disease outbreaks and is capable of survival under continuous disease threats. This study therefore was envisaged with the objectives to explore the immune system of the Zebu cattle through investigating the allelic diversity of Major Histocompatibility Complex (MHC) DRB3 gene using simple PCR-RFLP technique among different cattle breeds adapted to varied agro-climate zones of India.

Materials and Methods

Blood samples were collected from 192 animals, 24 each of eight different cattle breeds (Konkani, Tharparker, Mewati, Gir, Ongole, Malnad-Gidda, Ladhakhi and Sahiwal), belonging to different geographical regions of India. A standard protocol, employing phenol/chloroform extraction and precipitation with ethanol, was used to isolate genomic DNA from the blood samples (Sambrook and Russell, 2001). The DNA quantification and quality was checked by using NanoDrop ND1000 (Thermo Scientific, Wilmington, DE). Agarose gel electrophoresis was also used to check the integrity of the DNA used. All samples were brought to the concentration of 50-100 ng/ul.

BoLA-DRB3 exon 2 region was amplified using primers (HL030; 5'- ATCCTCTCTCTGCAGCACATTTCC-3' and HL031; 5'-TTTAAATTCGCGCTCACCTCGCCGCT-3') as reported by earlier workers (Van Eijk et al. 1992). The primers designed were meant for complete DRB3 exon 2 amplification. PCR amplification was carried out in 20 µl of reaction volume, containing 50-100 ng of genomic DNA, 0.5 µl of 10 pmol of each primer, 0.5 µl of 10mM dNTPs mix, 10X PCR buffer containing 15 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (New England Bio Labs, USA). The thermal cycling conditions were set for an initial denaturation at 95°C for 2.5 min followed by 32 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min with final extension at 72°C for 5 min. Amplified PCR products, were confirmed on agarose gel. All PCRs were performed in a 96-well C1000 thermal cycler (Bio-Rad Laboratories, Inc. USA).

The amplified products were subjected to PCR-RFLP using 5 µl of PCR products, digested for approximately 6 h at 37°C with 2 units of RsaI (New England Bio Labs, USA) in a total volume of 20 µl. RsaI restriction enzyme was selected for PCR-RFLP on the basis of earlier reports using it for the cattle and bison sequences to identify allelic patterns. For the analysis of different allelic patterns at *BoLA*-DRB3 region through differential restriction patterns the digested products were resolved and differentiated by 3% Metaphor high resolution agarose gel electrophoresis (Sigma, USA) with 50 bp ladder (GenRuler, Fermentas) in 1X TAE buffer at 80V for 90 min. After staining with ethidium bromide, the fragments were visualized on a UV trans-illuminator and analyzed for genotyping on the basis of different restriction patterns recorded manually. Nomenclature of these allelic patterns were done simply by alphabetic order to avoid any confusion because of the complexity generated through high allelic diversity and high heterozygosity at DRB3 locus.

Results and Discussion

Most of the polymorphism of the BoLA-DRB3 gene is located in exon 2, which encodes the peptide binding cleft, and its sequence variation plays an important role in the variability of immune response and disease resistance (Baxter et al. 2008). In the present study 310 bp fragment of exon 2 region of BoLA-DRB3 was successfully amplified in 192 animals (Figure 1). The BoLA-DRB3 exon 2 alleles in cattle have been found to be associated with resistance or susceptibility to various diseases as well as different milk protein traits (Starkenburg et al. 1997). Genotyping of BoLA-DRB3 was carried out to identify different alleles by PCR-RFLP, it was first done by Van Eijk and co-workers (1992).

The amplified BoLA-DRB3 exon 2 PCR products were digested with RsaI restriction enzyme, revealing a large number of restriction patterns (Figure 2) as combinations of different DRB3 alleles, present in either homozygous or heterozygous at single locus. Total 43 different restriction patterns or alleles were identified across 192 zebu cattle, out of this 22 (51%) were homozygous and 21 (49%) were heterozygous. Highest genetic diversity of BoLA-DRB3 gene was observed in Malnad Gidda and Gir and least in Mewati, Tharparkar and Sahiwal (Table 1). Results obtained also suggest that within breed genetic variation across breeds is higher than between breeds. This genetic information will be important for investigating the relationship between BoLA DRB3 and disease incidences in various cattle breeds. It has an implication on designing breeding programs that will aim at monitoring overall genetic diversity and herd health status and planning breeding programs to keep allelic diversity at this locus sufficient to combat the diseases.

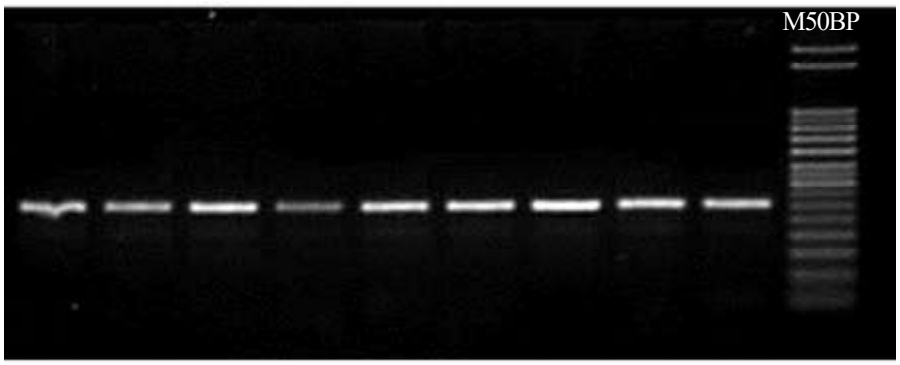
High variation for standard diversity indices were observed among the cattle breeds studied. These results corroborated with the previous findings (Takeshima et al. 2015) reporting, a total of 46 alleles of BoLA-DRB3.2 in the cattle breeds in this study. Previous workers (Wang et al. 2012) have reported that DRB3 gene is the most widely studied class II gene as it is extremely polymorphic. Among various alleles identified in this study, BM allelic pattern was the most frequently observed heterozygous pattern in indicus population and it was highest among Mewati and Ladakhi. Some breed specific alleles, AU, AG and AL were also identified, which were only found in Tharparkar, whereas KG allelic pattern was observed only in Gir and Ongole breeds.

Table 1 Genetic diversity of BoLA-DRB3 locus in different *Bos Indicus* breeds

S. No.	Breed	Total number of alleles	Number of homozygous alleles	Number of heterozygous alleles	Percentage of alleles	Heterozygous alleles with highest frequency*
1.	Mewati	9	7	2	19%	BM (21%)
2.	Ladakhi	12	8	4	26%	BM (21%)
3.	Malnad Gidda	14	10	4	30%	BL (8%)
4.	Gir	14	5	9	30%	KG (21%)
5.	Ongole	13	7	6	28%	KG (12%)
6.	Konkani	11	6	5	24%	BJ (12%)
7.	Tharparkar	9	2	7	19%	AU (25%)
8.	Sahiwal	9	4	5	19%	RG (17%)

*Alphabetical nomenclature given to each unique allelic patterns is as shown in representative agarose gel pictures (Figure 2).

Fig.1 PCR amplified products (310bp) of BoLA-DRB3 gene exon 2, resolved on agarose gel. M50BP- 50bp ladder marker



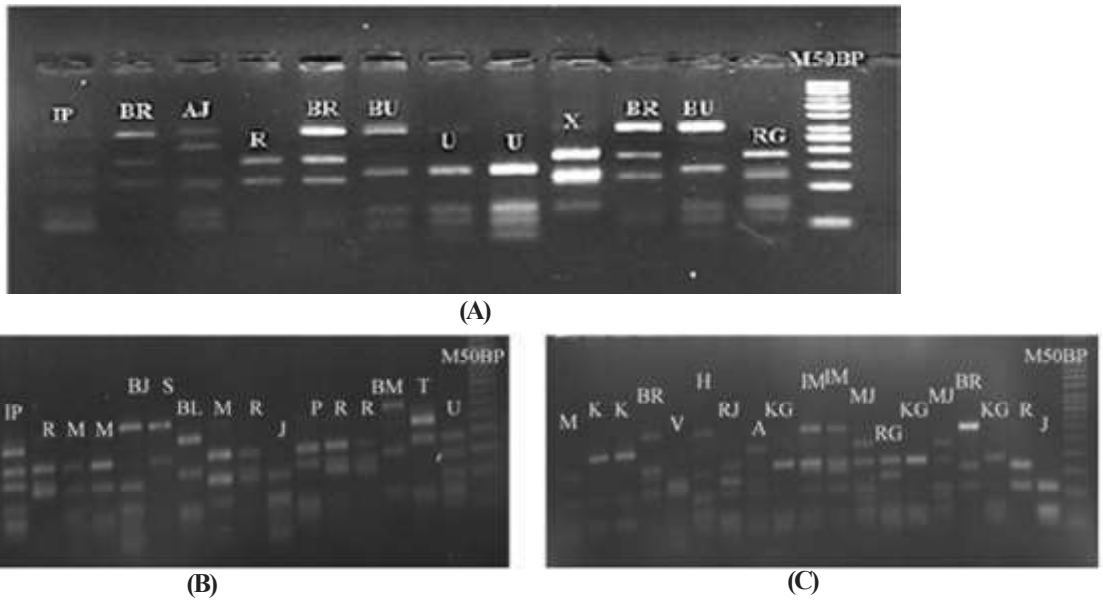


Fig. 2 PCR-RFLP allelic patterns of DRB3 gene 310 bp exon 2 using RsaI restriction enzyme on different cattle breeds. (A) Holstein Friesian, (B) Malnad Gidda and (C) Ongole, are the representative allelic patterns generated on specific breeds. Nomenclature of the patterns given is arbitrary alphabetically. M50BP- 50bp ladder marker

Earlier workers (Das et al. 2012), also identified breed specific alleles in three different breeds of cattle indicated their breeding structure or equilibrium and possible reservoir of rare alleles. In another study, Giovambattista et al. (2020), reported 71 distinct alleles, including three new variants of BoLA-DRB3 in local Myanmar cattle populations, while exotic Holstein-Friesian population, as a result of the different degrees of native admixture demonstrated a high degree of dispersion.

Conclusion

The higher heterozygosity values and greater genetic diversity of BoLA-DRB3 alleles observed in the present study among indicus cattle populations may be visualized as a positive genetic adaptations, which could be largely influenced by greater exposures to natural environmental conditions, pathogenic organisms and hot humid tropical climatic conditions, faced by them during free grazing in their native breeding tracts.

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RESEARCH ARTICLE

Effect of rumen-protected choline supplementation on production performance and haemato-biochemical profile of Kankrej cows

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Abstract: A study was conducted to examine the effect of supplementation of rumen-protected choline chloride (RPC) on production performance and haemato-biochemical parameters of Kankrej cows. Twenty Kankrej cows were randomly allocated to two treatment groups: control (CON: fed basal diet without supplement) and treatment (RPC: basal diet supplemented with 50 g/d/cow of RPC) from 4-weeks pre-partum through 8-weeks post-partum. Results showed that average body weights and dry matter intake were not affected (P<0.05) by the supplementation of RPC. There was significant (P<0.05) improvement in yields of milk (11.10 *vs.* 12.45 kg/d), FCM (10.93 *vs.* 12.49 kg/d) and ECM (12.07 *vs.* 13.76 kg/d) with RPC supplementation when compared with cows fed on the control diet. The milk fat, SNF, total solids, protein and lactose contents did not differ (P>0.05) between the treatment groups.Supplementation of RPC improved feed efficiency in terms of kg of 4% FCM/kg of DMI and kg of ECM/kg of DMI in lactating cows.The higher (P<0.05) serum glucose concentration (65.72 *vs.*62.05 mg/dL) was observed in RPC than the CON. Supplementation of RPC reduced (P<0.05) concentration of serum triglycerides (27.27 *vs.*31.38 mg/dL) as compared to the CON group. Other estimated blood metabolites were not influenced (P>0.05) by the supplementation of RPC. In conclusion, supplementation of 50 g/d rumen-protected choline during peripartum period improved milk production, feed efficiency and blood glucose in Kankrej cows.

Keywords: Blood metabolites, Choline, Kankrej cow, Milk yield, Transition phase

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Introduction

Nutritional care during the transition period influences productivity, health and fertility of dairy cows. Due to the reduced dry matter intake during periparturient period, the energy intake is usually insufficient in dairy cows to meet the high energy needs for milk production, resulting in a negative energy balance (Ospina et al. 2013). This leads to production of non-esteriûed fatty acids (NEFA)as a source of energy through the mobilization of fat from adipose tissue. The excessive amounts of NEFA overwhelm hepatic oxidation, leading to ketosis because of increased production and secretion of ketone bodies and “fatty liver” syndrome due to accumulation of triacylglycerol in the liver (Morrison et al. 2018). The elevated levels of ketone bodies and NEFA further contribute to oxidative stress, inûammatory responses, and a compromised immune system, therefore enhancing the susceptibility to infectious diseases, impairing fertility and reduced productive performance (Shahsavari et al. 2016).

Choline is a nutrient required for the synthesis of phosphatidylcholine, aphospholipid found in the membranes of VLDL (NRC, 2001). Choline improves lipid metabolism, increasing the VLDL synthesis, availability of fatty acids for the mammary gland, and its incorporation into phospholipid membranes around fat globules (Lopreiato et al. 2020). The dietary choline is rapidly degraded by rumen microbes, which lead to less than 20% bioavailability ofcholine in the ruminants (Bollatti et al. 2020a), the only effective method of increasing choline availability to dairy cows is to feed it in a form that is protected from ruminal degradation (Arshad et al. 2020). Recent studies have shown that supplementation of rumen-protected choline (RPC) during the periparturient period has led to increase in milk yield (Bollatti et al. 2020b; Potts et al. 2020; Holdorf and White, 2021). However, the effects of RPC supplementation in Indigenous dairy cows

have not been studied widely yet. Kankrej cows maintained at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Gujarat had average standard (305 days) lactation yield of 2479 litres during the year 2020 (Anonymous, 2021). To test the hypothesis that RPC supplementation during periparturient period in Kankrej cows improves yields of milk and milk components without affecting feed intake, a study was conducted to evaluate the effect of feeding RPC from 4-weeks pre-partum through 8-weeks post-partumon milk production, composition and haemato-biochemical profile of Kankrej cows.

Materials and Methods

The use of the animals and the experimental protocol of this study were approved by the Institutional Animal Ethics Committee of College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India (VETCOLL/IAEC/2019/13/PROTOCOL-08).

Twenty Kankrej cows (BW,457.1±6.22 kg;parity,3.2±0.28;average previous lactation milk yield,8.30±0.18 kg/d)from Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India were randomly divided into two groups: 1) control (CON), fed with basal diet (consisting of concentrate mixture, green oat fodder and jowar hay) and 2) treatment (RPC), fed with basal diet supplemented with 50 g/cow daily of rumen-protected cholinechloride (CholiPEARL™, Kemin Industries South Asia Pvt. Ltd., Chennai, India). RPC was mixed thoroughly into concentrate and provided once daily from 4-weeks pre-partum through 8-weeks post-partum. The basal diet was formulated to meet ICAR (2013) nutrient requirements. The chemical composition of feeds and fodders fed to the experimental animals (Table 1) was within the normal range for Indian feed stuffs (ICAR, 2013).

The offered feeds and subsequent left-over were recorded at fortnightly interval for each animal in order to calculate feed intake. The samples of feeds and fodders were collected, composited and dried at 60°C in a forced air oven for 48 h, ground to pass through a 1-mm screen using a Wiley mill (Star Scientific Instruments, Delhi, India). The feeds samples were analyzed for dry matter (DM, method 934.01), ash (method 942.05), crude protein (method 976.05) and ether extract (method 973.18) according to AOAC (2007). Neutral detergent fibre and acid detergent fibre were determined as per Van Soest et al. (1991). Non-fiber carbohydrate was calculated according to NRC (2001). Cows were milked twice a day and individual milk yield for each cow was recorded daily by using electronic weighing balance. The 4% fat corrected milk (FCM) was calculated using formula: milk yield (kg) x 0.4 + fat yield (kg) x 15. Energy corrected milk (ECM) was determined using formula: 0.327 × milk yield (kg/d) + 12.86 × fat yield (kg/d) + 7.65 × protein yield (kg/d). Milk samples were collected at fortnightly interval for analysis of milk composition (fat, solids-not fat (SNF), protein and lactose) using EKOMILK Ultra Pro Milk Analyzer (Everest Instruments Pvt. Ltd.).

On 56th day of experimental feeding, blood samples from jugular vein were collected from each animal in the vials (BD Vacutainer® Spray-coated K2EDTA Tubes, BD Franklin Lakes, NJ, USA) with and without EDTA (BD Vacutainer® Plus Plastic Serum Tubes, BD Franklin Lakes, NJ, USA). The fresh blood samples were analyzed for haemoglobin, haematocrit, erythrocytes and leucocytes using Exigo EOS Vet Haematology Analyser(Boule Medical AB, Sweden).The serum samples were analyzedfor concentrations of glucose, total proteins, albumin, urea, triglycerides and cholesterol using Randox Monaco Analyser (Randox Laboratories Ltd., UK).

All the experimental data obtained were statistically analyzed by using statistical software SPSS v.16.0 (SPSS Inc., Chicago IL). One-way ANOVA was used to test the level of significance. Significant differences between means of treatments were assessed by the Duncan's test, and the differences among treatments were declared significant at $P<0.05$.

Results and Discussion

The effect of rumen-protected choline chloride supplementation on production performance of Kankrej cows is given in Table 2. Supplementation of RPC increased yields of milk (1.35 kg/d; P=0.046), 4% FCM (1.69 kg/d; P=0.022) and ECM (1.81 kg/d; P=0.024) as compared to the CON group. The percentages of

Table 1 Chemical composition (% DM basis) of feeds and fodders fed to Kankrej cows

Composition	Concentrate mixture	Oat forage	Jowar hay
Dry matter	94.91	17.44	11.83
Crude protein	19.57	8.92	6.39
Crude fibre	6.58	27.67	32.25
Ether extract	4.88	3.12	1.34
Ash	8.02	7.34	9.07
NFC	42.38	23.81	21.79
NDF	25.15	56.81	61.41
ADF	15.27	32.06	40.98

NFC: Non-fiber carbohydrate, calculated by equation: NFC (% of DM) =100 - (CP + NDF + EE + Ash); NDF: Neutral detergent fiber; ADF: Acid detergent fiber

milk fat, SNF, total solids, protein and lactose were not influenced (P>0.05) by the supplementation of RPC. There were increased yields of milk fat (18.2%), SNF (11.4%), total solids (13.6%), protein (12.8%) and lactose (13.7%) due to feeding of RPC when compared to the CON group. The improved production performance in dairy cows due to supplementation of RPC may be because of role choline in intermediary metabolism, in particular as a component of phospholipids and lipoproteins, which are critical for lipid absorption and transport, and enhancing uptake of fatty acids in early lactation, thereby making transition cows responsive to supplementation (Bollatti et al. 2020b). Choline also influences nutrient partition in the mammary gland toward milk synthesis mediated by increases in growth hormone (Kawamura et al. 2012). Choline kinase, an enzyme involved in the conversion of choline to phosphocholine, regulates mammary cell proliferation (Ramirez de Molina et al. 2004). Thus, an increased supply of choline in early lactation might have stimulated the enzyme choline kinase to enhance mitosis in mammary cells in RPC supplemented cows. These results are in agreement with those obtained by Potts et al. (2020) who found that feeding of 60 g/d of RPC in primiparous Holstein cows increased milk yield by 3.1 kg/d when compared to the control. Bollatti et al. (2020b) reported that yields of milk (36.5 *vs.* 34.8 kg/d), 3.5% FCM (43.1 *vs.* 39.6 kg/d) and ECM (42.0 *vs.* 38.9 kg/d) were improved due to supplementation of 12.9 g/d of choline ion (60 g/d of RPC) in Holstein cows. Arshad et al. (2020) in their meta-analysis reported a 1.6 kg/d response in milk production in multiparous cows fed 12.9 g/d of choline ion (60 g/d of RPC). Recently, Holdorf and White (2021) reported that milk yield tended (P<0.10) to increase when 60 g/d of RPC was supplemented in multiparous Holstein cows.

Feeding of RPC did not affect (P>0.05) average body weight and DM intake (Table 3). Feed efficiency in terms of 4% FCM/DMI (P=0.044) and ECM/DMI (P=0.049) was significantly improved in RPC supplemented group than the CON. Similar to the present finding, recent studies also reported that there were no difference

Table 2 Effect of rumen-protected choline chloride supplementation on production performance ofKankrej cows (n=20)

Parameters	CON	RPC	P value
Yield (kg/d)			
Milk	11.42^{a}±0.51	12.77^{b}±0.41	0.046
4% FCM	11.21^{a}±0.53	12.90^{b}±0.42	0.022
ECM	12.39^{a}±0.58	14.20^{b}±0.46	0.024
Fat	0.44^{a}±0.02	0.52^{b}±0.02	0.013
Solids not fat	0.88±0.04	0.98±0.03	0.056
Total solids	1.32^{a}±0.06	1.50^{b}±0.05	0.028
Protein	0.39^{a}±0.02	0.44^{b}±0.01	0.042
Lactose	0.51^{a}±0.02	0.58^{b}±0.02	0.046
Milk composition (%)			
Fat	3.93±0.10	3.97±0.05	0.702
Solids not fat	7.62±0.12	7.65±0.09	0.846
Total solids	11.55±0.16	11.63±0.11	0.698
Protein	3.37±0.02	3.40±0.02	0.354
Lactose	4.46±0.09	4.50±0.01	0.635

FCM: fat corrected milk; ECM: energy corrected milk.
CON: Basal diet without additive; RPC: Basal diet + 50 g/animal/day of rumen-protected choline
^{ab}Means in a row with different superscripts differ significantly (P≤0.05)

Table 3 Effect of rumen-protected choline supplementation on body weight, dry matter intake and feed efficiency in Kankrej cows (n=20)

Parameters	CON	RPC	P value
BW (kg)	456.10±8.80	458.00±10.15	0.889
DMI (kg/d)	11.18±0.22	11.22±0.25	0.888
Feed efficiency			
Milk (kg)/DMI (kg)	1.03±0.06	1.14±0.03	0.114
4% FCM (kg)/DMI (kg)	1.01^{a}±0.06	1.15^{b}±0.03	0.044
ECM (kg)/DMI (kg)	1.12^{a}±0.06	1.27^{b}±0.03	0.049

DMI: dry matter intake; FCM: fat corrected milk; ECM: energy corrected milk
CON: Basal diet without additive; RPC: Basal diet + 50 g/animal/day of rumen-protected choline
^{ab}Means in a row with different superscripts differ significantly (P≤0.05)

Table 4 Effect of rumen-protected cholinesupplementation on haemato-biochemical profile of Kankrej cows (n=20)

Parameters	CON	RPC	P value
Haematological parameters			
Haemoglobin (g/dL)	10.41±0.28	10.48±0.18	0.835
Hematocrit (%)	33.53±1.26	33.93±1.54	0.843
Erythrocytes (10^6/µL)	6.82±0.16	6.89±0.20	0.780
Leukocytes(10^3/µL)	8.00±0.46	8.12±0.31	0.830
Blood biochemical parameters			
Glucose (mg/dL)	62.05^a±1.40	65.72^b±0.58	0.027
Total protein (g/dL)	6.48±0.08	6.40±0.10	0.547
Albumin (g/dL)	2.96±0.07	2.89±0.06	0.461
Globulin (g/dL)	3.52±0.14	3.51±0.13	0.940
Urea (mg/dL)	42.08±1.71	38.68±1.82	0.190
Triglycerides (mg/dL)*	31.38^b±0.85	27.27^a±0.95	0.005
Cholesterol (mg /dL)	149.03±3.29	154.14±4.17	0.349

CON: Basal diet without additive; RPC: Basal diet + 50 g/animal/day of rumen-protected choline
^{ab}Means in a row with different superscripts differ significantly (*P<0.05)

(P>0.05) in DM intake and body weight in Holstein cows fed 60 g/d of RPC (Bollatti et al. 2020b; Potts et al. 2020; Salman and Alan, 2020).Improved conversion efficiency of DMI into FCM and ECM in the present experiment might be related to improved nutrient absorption due to better gastrointestinal function with RPC supplementation. Moreover, less disrupted gastrointestinal barrier due to choline supplementation might have reduced the nutritional costs necessary to support an activated immune system (Kvidera et al. 2017), which would have spared more nutrients for milk synthesis.

The effect of supplementation of rumen-protected choline on haemato-biochemical parameters of Kankrej cows is given in Table 4. There was no significant (P>0.05) difference in the concentrations of haemoglobin, haematocrit, erythrocytes and leucocytes between the CON and RPC groups. The supplementation of rumen-protected choline in Kankrej cows significantly (P<0.05) increased glucose concentration (65.72 *vs*. 62.05 mg/dL) as compared to the CON group. Higher blood glucose levels in RPC group may be resulted because of feeding RPC increases mRNA for GLUT2, a hepatic protein that facilitates glucose release from the liver into blood (Zhao and Keating, 2007). Similar to the present findings, Arshad et al. (2020) in their meta-analysis reported that feeding of 12.9 g/d of choline ion (60 g/d of RPC) increased (P<0.05) blood glucose concentrations in dairy cows. In contrast to the present findings, previous studies observed that plasma glucose concentration was unaffected by RPC supplementation in cows (Zhou et al. 2016; Zenobi et al. 2018; Bollatti et al. 2020c; Potts et al. 2020). The concentration of triglycerides was significantly (P<0.05) reduced in RPC (27.27 mg/dL) group when compared to the CON (31.38 mg/dL) group. This may be attributed to role of choline which acts in intermediary metabolism, in particular as a component of phospholipids and lipoproteins, which are critical for lipid absorption, transportation and has been shown to attenuate triacylglycerol infiltration into the liver (Zenobi et al. 2018). In agreement with our results, Mohsen et al. (2011) reported that RPC supplementation led to a significant decrease (P<0.05) in the concentrations of plasma triglycerides in dairy cows. Also, Holdorf and White (2021) observed that 60 g/d of RPC supplementation tended (P<0.10) to reduce blood triglycerides in Holstein cows. The concentrations of serum total proteins, albumin, globulin, urea and cholesterol did not differ (P>0.05) between the CON and RPC groups. Previous studies also observed no change in other blood biochemical parameters in dairy cows supplemented with RPC (Zenobi et al. 2018; Bollatti et al. 2020c; Potts et al. 2020).

Conclusions

Based on the results of the present study, it may be concluded that dietary inclusion of 50 g/day of rumen-protected choline in Kankrej cows from four weeks pre-partum up to eight weeks post-partum for improved milk yield with higher concentration of serum glucose and lower concentration of triglycerides.

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RESEARCH ARTICLE

Effect of sprinkler with fan on growth, physiology and behaviour of Murrah buffalo calves

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Abstract: The aim of the present study was to investigate the effect of sprinkler with ceiling fans on the growth performance, physiological responses and behaviour of Murrah buffalo calves. For this study, a total of twelve Murrah buffalo female calves were selected based on the age and body weight and allocated to two treatments (n=6 each). In the first treatment (T1), the calves were provided with sprinkler with fan. While, in second treatment called control (T0), the calves were not provided with any cooling system. The average daily gain was greater in T1 than T0 calves on week (w) 3 (P=0.01), w 4 (P=0.03), w 5 (P=0.04), w 6 (P=0.02), w 7 (P=0.01) and w 8 (P=0.05). While it was similar between the calves of two groups on the w 1 (P= 0.32), w 2 (P=0.31), w 9 (P=0.16), w 10 (P=0.19), w 11 (P=0.12), w 12 (P=0.1).The overall eye temperature was significantly (P<0.05) higher in control group (37.42±0.04 ºC) than treatment group (36.42±0.13 ºC) calves. The overall muzzle (P=0.01) and skin temperature (P=0.001) was also significantly higher in control calves as compared to treatment calves. The average respiration rate was lower (P<0.05) in T1 (22.83±0.18 breaths/min) than in T0 (30.83±0.12 breaths/min) calves. Similarly, overall pulse rate was also lower (P<0.05) in T1 (38.36±0.11 No./min) as compared to T0 (60.19±0.13 No./min) calves. The average time spent on eating, ruminating and resting was significantly greater in T1 than in T0 calves. In conclusion, the provision of sprinkler with fan reduces physiological response along with body temperatures. Consequently, it improved the growth performance and helped in normal behavioural expression of the buffalo calves.

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Introduction

The domestic riverine buffaloes (*Bubalus bubalis*) are the main dairy animals in India. Where, the performance of these animals is a great challenge due to high ambient temperature especially in summer months (Bah et al. 2021). Further,buffaloes are relatively more sensitive to solar radiation due to dark coat colour, comparatively lesser density of sweat gland and dense epidermis (Marai and Habeeb 2010).The exposure of buffaloes to the hot conditions strongly affects their bioenergetics, with adverse effects on the dry matter intake (9–13%), growth rate, metabolism of water, protein, energy and mineral balances (Kumar et al. 2018). These series of changes cause impairment of reproduction and productive performances (21% reduction in milk production) in buffaloes (Das et al. 2014). Further, young calves of buffaloes are reported to be more vulnerable leading to lower feed intake, decreased feed utilization efficiency which results in poor growth rate and lower life time productivity (Adin et al. 2009). Therefore, in order to combat thermal stress, for survivability and to abate heat load, these animals wallow in water ponds (Napolitano et al. 2013). Since, traditions at small buffalo farms in India and Pakistan, farmers splash the water on the buffalo body during late morning, afternoon and in early evening hours in order to reduce the effect of thermal stress (Bah et al. 2022). Moreover, buffaloes wallow in the community ponds at village level after grazing but over time, reduction in wallowing ponds in villages have led to limited or no access to water for wallowing (Bah et al. 2021). Further, traditional buffalo production system is shifting to intensive buffalo production system where water splashing to individual animal is not possible. Moreover, there has been an emphasis on reducing water use on cooling dairy animals with nominal effect on productivity and welfare (Tresoldi et al. 2018; Bah et al. 2021). So, in order to ameliorate thermal stress in buffaloes and to reduce water consumption for cooling various managemental methods *viz.,* sprinkling (Khongdee et al. 2011), showering, fanning, forced ventilation, foggers and misters have been tried with varied success. Similarly, Anderson et al. (2013) and Tresoldi et al. (2018) had reported that the most common method of cooling in dairy cows during summer is sprinklers/soakers or misters. Earlier various studies on the buffaloes also shown that the provision

of sprinklers dropped respiration rate and body temperature, and improved dry matter intake and milk yield as compared to non-cooled buffaloes during summer (Aggarwal et al. 2008; Yadav et al. 2016; Ahmad et al. 2019). Furthermore, evaporative cooling through foggers, sprinklers and fans has turn out to be a common practice to improve milk production (Avendano-Reyes et al. 2006), feed utilization and to decrease rectal temperature, pulse rate and respiration rate (Rahangdale et al. 2012) in dairy animals as a tool to combat heat stress during hot environment. However, to our best knowledge there is no available information on the effect of cooling system on growth performance and physiological variable of the buffalo calves.

Further, Rajasthan state in India have different environmental condition with highest temperature may reaches to 49 ºC during summer months, however, humidity ranges 45% to 47%. The state is having very low rainfall (32.7 to 64.9 cm). In Jaipur, temperature may reaches to 35 ºC during September and October months. While, the thermoneutral zone and comfort zone for the buffaloes is 15º to 25ºC, leading to higher thermal stress, poor milk production and reproductive efficiency. Considering all this information, we hypothesized that use of sprinkler with fan may reduce the physiological responses and improve the growth rate of buffalo calves. Therefore, the aim of the present study was to investigate the effect of sprinkler with fan on the growth performance, physiological responses and behaviour of Murrah buffalo calves in hot summer in Rajasthan region of India.

Materials and Methods

The Institutional Animal Ethics Committee (IAEC, Registration No. PGIVER/IAEC/2022-16) approved this study, which was conducted following IAEC established standards as per Article Number 13 of the committee for the Control and Supervision of Experiments on Animals (CPCSEA) policies of the Government of India. The present study was conducted at the farm of Post Graduate Institute of Veterinary Education & Research (PGIVER), Jaipur, Rajasthan, India (longitude=75°522 15.062 2 E, latitude= 26°532 34.662 2 N and altitude= 431 m above the mean sea level). The summer in Jaipur, Rajasthan, India is very hot while winters are extremely cold. The maximum temperatures hover at 40 °C to 47 °C in May and June Months. Heat wave prevails for a few days in the season, when day temperature rises to 4-6 ° C above normal. The winter minimum temperatures remain about 4-9 °C and fall below zero degrees. The experiment began in 01 August 2021 and ended in 31 October 2021. During this period, temperature may reaches to 35 °C in Jaipur.

Experimental design

For this study, a total of twelve Murrah buffalo female calves were selected based on the age (4 to 12 month) and body weight (106.33±14.72) and allocated to two treatments (n=6 each). In the first treatment (T1), the calves were provided with sprinkler with fan. The six sprinklers and two ceiling fans (8 feet height at 7 feet distance) were fitted at the height of 2.10 m from the ground level. The sprinkler with fan cooling system was operated at 11 am to 4 pm with gap of 5 minutes. While, in second treatment called control (T0), the calves were not provided with any cooling system.

Housing and feeding of calves

The experimental calves of both treatments groups were allocated to a loose housing system with a covered shed and an adjoining open paddock with a total floor space of 3 m^2 per calf and a shared feeder through a fence line feed barrier, and a shared drinker. The allocation of floor space and feeding space was consistent with the standards of the Bureau of Indian Standards for buffaloes in loose housing systems (BIS: 1223-1987). The floors of the calves' enclosures were made of concrete, with grooves under the covered and open area. The roof was flat type with average height of 2.65m from the ground.

Calves were offered concentrate at 1 % of their body weight, and *ad libitum* chopped green and dry fodders. Clean and fresh water with salt lick was offered *ad libitum*. Calf concentrate was composed of maize 35%, wheat bran 20%, gram 10%, Groundnut cake 32%, mineral mixture 2%, and common salt 1%.

Body weight and average daily gain

The body weight of the experimental calves was recorded on weekly (w) basis until the end of the experiment. Weights of all calves were recorded in the morning between 6:00 a.m. and 6:30 a.m., before feeding and watering, using an electronic weighing machine with a precision of 500g. The average daily gain (ADG) was calculated in grams as (Final body weight-Initial body weight)/ weekly intervals and expressed in gm/day.

Physiological parameters

Physiological parameters viz., rectal temperature (RT), muzzle temperature (MT), eye temperature (ET), skin temperature (ST), pulse rate (PR) and respiration rate (RR) were recorded on daily basis at 3:00 PM and the average of these recorded values are presented in weeks. The eye, muzzle and skin temperature were taken by using digital thermometer at the distance of one meter from the calves. The RT (°C) was measured from rectum with the help of digital thermometers. The PR (beats per min) was taken from the coccygeal artery. The RR (times per min) was recorded from the distance without disturbing the animals observing the flank movement. The RR was recorded first followed by PR and RT for getting actual observation.

Recording of behaviour of calves

Behaviors parameters of calves were observed by using CCTV (CP Plus) video cameras installed to record the activity of animals

for 24 hours per day. The CCTV cameras were installed in such a way as to have a complete view of animals inside the covered area as well as in open area. The camera had 8x digital zoom for closer viewing. The cameras were enabled with array infrared technology for best night vision. Cameras were installed at different places and different angles in the experimental shed so that whole shed can be covered in viewing angle. The images and video were stored in 16 channel digital video recorder (DVR) having hard disk of 4 TB space. All the parameters were recorded in hours: minutes format initially which were later changed to hours according to needs of parameters.

Statistical analysis

The Body weight, ADG and various physiological parameters, time spent on eating, rumination, resting and standing were compared using a mixed model (IBM SPSS Statistic 22.0 computer software). Treatment, time, and their interaction were included as fixed effects, and the individual calves were included as random effects. Differences were considered statistically significant when *P dH* 0.05. Results are presented as LS means ± SEM.

Results and Discussion

Growth performance

Body weight and average daily gain

There were non-significant effects of group and their interaction (P=0.822), while there was significant effect of time on the calves' body weight (P=0.001). The average body weight of the calves did not differ between the two groups on all the sampling weeks (P=0.979, 0.955, 0.933, 0.903, 0.884, 0.854, 0.882, 0.798, 0.782, 0.766, 0.749, 0.735, 0.719, respectively) (Fig 1 A). There was an effect of time (p <0.01), group (p <0.01), and an interaction between group and time (p <0.01) on the calves ADG. The average daily gain was greater in T1 than T0 calves on w 3 (P=0.01), w 4 (P=0.03), w 5 (P=0.04), w 6 (P=0.02), w 7 (P=0.01) and w 8 (P=0.05). While it was similar between the calves of two groups on the w 1 (P= 0.32), w 2 (P=0.31), w 9 (P=0.16), w 10 (P=0.19), w 11 (P=0.12), w 12 (P=0.1) (Fig 1 B). The results of this study support the initial hypothesis that provision of sprinkler along with ceiling fans was effective in reducing the thermal stress and in improving the growth performance of the buffalo calves. The core body temperature of animals rises primarily because of reduced heat loss due to the dropping of thermal gradient between the skin surface and environment. So, an ideal thermal gradient between the animal body and the surrounding environment is essential for the effective dissipation of heat energy. Once ambient temperature rises well beyond the upper critical limit, thermoregulation is incapable to avoid an elevation of core body temperature. However, in this study, animals remained under sprinkler with ceiling fans, therefore, probably had a lower body temperature. The results of the present study on ADG are reinforced by previous studies which revealed the positive impact of sprinkler with fan (Vijayakumar et al. 2009), four times washing (Das et al. 2011), fan-cum-mist cooling (Singh et al. 2014) and modified roofing (Khongdee, 2016), on weight gain in buffaloes. A higher ADG in T1 group of calves may be due to improved feed intake as a result of better cooling through provision of sprinkler along with ceiling fans. In Addition, efficient utilization of feed under calm micro-environments might have triggered in more weight gain in cooled calves. The lower average daily weight gain in control calves indicated higher tissue catabolism and reduced anabolic activity due to extra energy requirements to dissipate excess body heat (West, 2003) in order to sustain homeothermy in heat-stressed animals.

Body temperature

The overall rectal temperature was slightly higher in control group of (101.94±0.13 ºF) calves as compared to treatment calves (101.19±0.11 ºF), although the difference was not statistically significant (P=0.19).The overall eye temperature was significantly (P<0.05) higher in control group (37.42±0.04 ºC) than treatment group (36.42±0.13 ºC) calves. Similarly, the overall muzzle (P=0.01) and skin temperature (P=0.001) (Table 1) was also significantly higher in control as compared to treatment calves.

Rectal temperature was lower in the calves of the treatment group than in the control group. Owning to dark skin color, sparse hair coat and very few sweat glands, buffaloes absorb a profound quantity of solar radiation and are considered more susceptible to heat stress (Kishore et al. 2016; Kapila et al. 2016; Lakhani et

Table 1 Mean± SEM of overall average body temperature and physiological parameters of two different groups of calvesData are presented as LS means ± SEM. a, b indicates differences between the mean values of different groups.

Parameter	Control (To)	Treatment (T1)
Body temperature		
Overall RT (ºF)	101.94±0.13	101.19±0.11
Overall ST (ºC)	36.75±0.06^{b}	33.64±0.10^{a}
Overall MT (ºC)	36.59±0.02^{b}	36.22±0.01^{a}
Overall ET (ºC)	37.42±0.04^{b}	36.42±0.13^{a}
Physiological parameters		
Respiration rate (breaths/min)	30.83±0.12^{b}	22.83±0.18^{a}
Pulse rate (beats/min)	60.19±0.13^{b}	38.36±0.11^{a}

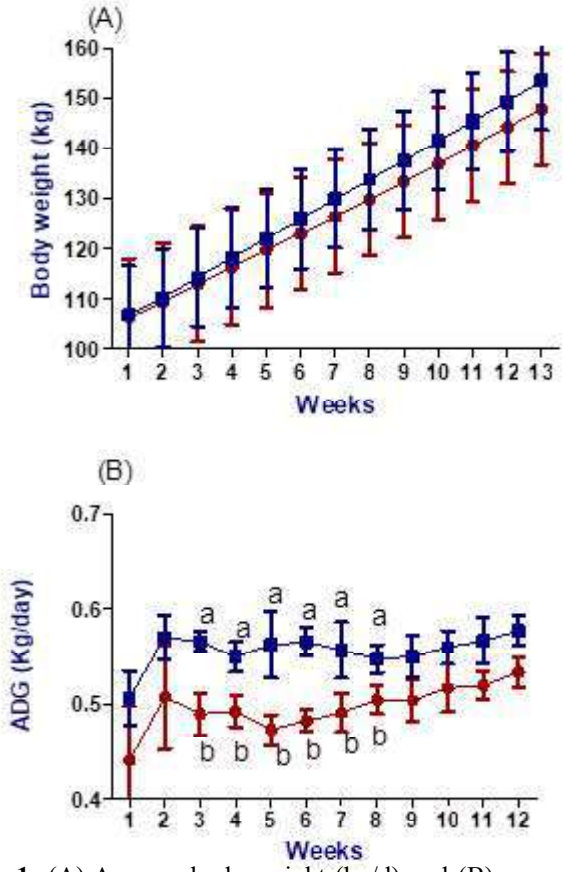

Fig 1: (A) Average body weight (kg/d) and (B) average daily gain (kg/day)of Murrah buffalo calves had provision of sprinkler with fan (▲ ; T1), no sprinkler with fan group (Ï% ; T0). Data are presented as LS means ± SEM. Different letters indicate differences between the mean values of the groups at that time point ($p<0.05$).

al. 2018; Prasad et al. 2020). The provision of sprinkler with fan results in evaporative cooling in the treatment calves of the present study, which results in lower environmental temperature and consequently body temperature of the calves (Avendaño-Reyes et al. 2006; Ambulkar et al. 2011). Further, higher eye temperature in control groups may be because of dilation of ocular blood vessels and increased visual alertness in response to stress (Stewart et al. 2007). It is probably because the eyes have rich capillary beds that are innervated with sympathetic and responsible for changes in the eye blood flow and further rhythmic changes in eye temperature (Uddin et al. 2019; Prasad et al. 2020).

Basically, skin temperature rises with increase in solar radiation (Das et. al. 1999). In our study, lower skin temperature in T1 groups is due the fact of lower environmental temperature due to provision of time bound sprinklers with ceiling fans, which have cooled the animals through conduction process (Ahmad et al.2017). The present study results are in general agreement with the previous finding of Singh et al. (2005) on Nili-Ravi buffalo; Das et al. (2011) on Nili-Ravi calves and Singh et al. (2014) on Murrah buffalo, who had reported significant decrease in skin temperature when buffaloes were subjected to water splashing during hot summer.

Physiological parameter

The average respiration rate was lower (P<0.05) in T1 (22.83±0.18 breaths/min) than in T0 (30.83±0.12 breaths/min) calves (Table 1). Similarly, overall pulse rate was also lower (P<0.05) in T1 (38.36±0.11 beats/min) as compared to T0 (60.19±0.13 beats/min) calves (Table 2). All through hot and humid climatic conditions, the environmental temperature is generally higher than the body temperature of calves, and they cannot release additional body heat to the environment. Therefore, in order to release extra body heat, animals evaporate heat from the body through increased respiration rate. Therefore, the provision of sprinkler with fan in the treatment group of present study have prevented in rising of environmental temperature in surrounding of the calves. On the similar line, Seerapu et al. (2015) had reported lower respiration rate among the animals provided with foggers and foggers plus fans during the summer season than the animals of the control group. Moreover, Singh et al. (2014) in Murrah buffalo calves reported significant decreased in respiration rate when these animals were subjected to water splashing during hot summer season.

Further, provisions of sprinkler with ceiling fan have resulted in higher pulse rate in the control group. This is possibly because of higher environmental temperature, heat stress, stimulate hypothalamic-pituitary-adrenal axis to secrete adrenaline and cortisol. This higher adrenaline hormone constricts blood vessels resulting in higher blood flow within the blood vessels. Similarly, present study results are in support with the findings of Ganaie et al. (2013) and Das et al. (2014) who had reported lower pulse rate in the treatment group having provision of fogger with fans.

Behaviour of calves

Time spent on eating, rumination, resting

The data pertaining to average time spent on eating, rumination and resting by calves are graphically depicted in fig 2 A, B, C. The average time spent on eating (373.09±5.64min/d *vs* 338.48±4.22min/d), rumination (520.73±6.98 min/d *vs* 504.36±6.18min/d) and resting (778.62±5.58 min/d *vs* 683.83±6.19min/d) was significantly greater (P<0.05) in T1 than T0 calves, respectively. The time spent on eating and rumination was higher in calves which were having provision of sprinkler with fans (treatment group). It has been reported earlier that the season can be a critical factor in altering the feeding behaviour of calves (Grant and Albright 2001). Further, any temperature higher than their thermo-neutral zone reduces the voluntary feed intake of animals (Hooda et al. 2010). Likewise, Magrin et al. (2017) stated that young bulls exposed toeH74 temperature-

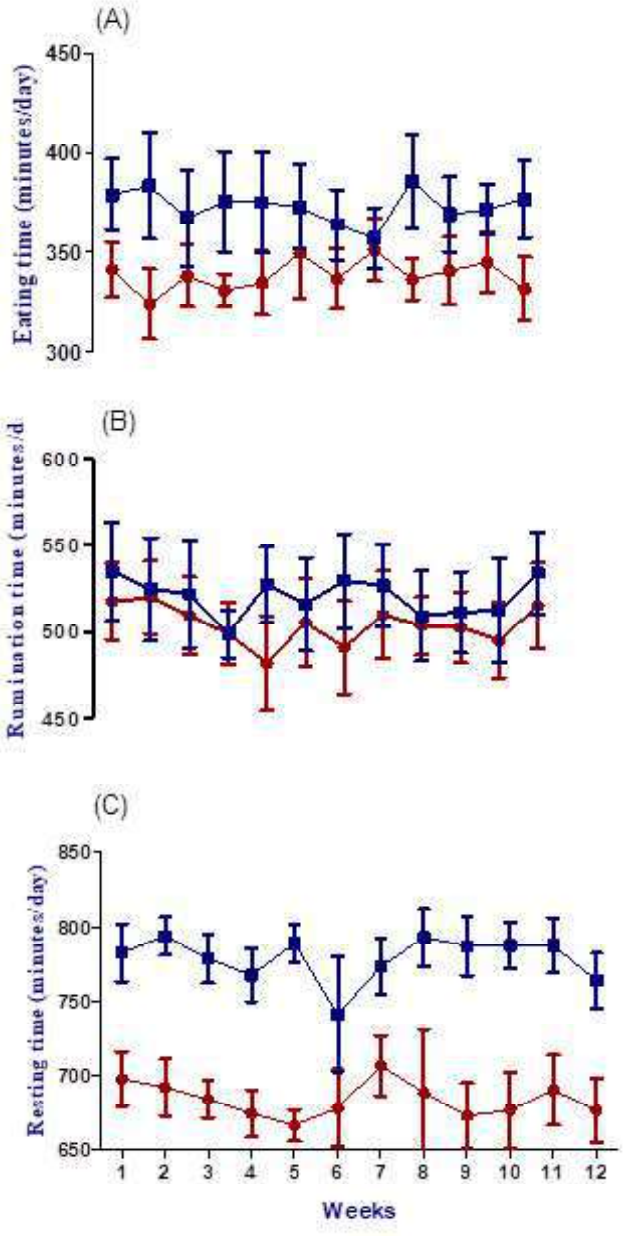

Fig 2: (A) Average time spent on eating (min/d) (B) average time spent on rumination (min/d) and (C) Average time spent on resting behaviour of Murrah buffalo calves had provision of sprinkler with fan (▲ ; T1), no sprinkler with fan group (Ï% ; T0).

humidity index (THI) decreases their time spent on eating and ruminating compared to their counter parts under optimum THI conditions, however the provision of ceiling fans augmented time spent on rumination and exploring activities in young calves. The calves in our study under control group spent less time for eating and ruminating, possibly in an effort to decline metabolic heat and to maintain their core body temperature (Nardone et al. 2010). The time spent on resting was significantly ($P<0.05$) lower in the calves of the control group compared to the treatment group. The lower time spent on resting among calves housed in the control shed might be due to concrete flooring and also the animals might have spent most of their time standing for heat dissipation as the body surface area exposed to air is more when they keep standing. The evaporation from the body surface increases by standing in calves or there may be a chance of sun-heated floor, which might have decreased the resting time in the present study (Kamal et al. 2018). Further, the increased standing time is the most common behavioural change observed in animals under heat stress (Widowski 2001). Tucker et al. (2008) observed 10% increase in standing time when heat load increased by 15% in dairy cattle, might be due to increased heat loss by exposing the more amount of skin to airflow.

Conclusions

The provision of sprinkler with fan reduces physiological response along with body temperatures. Consequently, it improved the growth performance and helped in normal behavioural expression of the buffalo calves.

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RESEARCH ARTICLE

Impact of COVID-19 pandemic on household consumption pattern of dairy products in India

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Abstract: Income loss, fear of infection and movement restrictions during the COVID-19 pandemic not only altered the amount and pattern of spending but also changed the shopping behaviour of the consumers. In the case of food commodities, dairy products seem to be more susceptible to such changes owing to their perishability and comparatively higher income elasticity. However, there were also some speculations that household dairy consumption in India might have increased during lockdown due to greater number of meals at home and immunity boosting qualities of milk. Any change in dairy consumption in India has a direct bearing on household nutritional security. The present study attempts to capture the impact of COVID-19 pandemic on household consumption pattern of dairy products across geographical regions and income class on the basis of a pan-India survey covering around 1000 households. Data was analysed using tabular analysis, frequency analysis and paired t-test. The results revealed that there was a significant decline in the household consumption of milk, paneer, butter and ice-cream during lockdown whereas no significant change was observed in the consumption of ghee, curd and buttermilk. The fall in consumption was comparatively higher in the milk deficit eastern zone and among the lowest income quintile which indicates that external support is required by the marginalized section during such crisis for ensuring the nutritional security. Moreover, a shift was also observed towards packaged products and online delivery services which can be further boosted for strengthening organized dairy sector.

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Keywords: COVID-19, Consumption, Dairy, Shopping behaviour

Introduction

Viral pandemics and the restrictive measures imposed to control them often lead to demand uncertainties and supply disruptions, ultimately influencing the product prices and consumption pattern of the households. Moreover, the containment measures like isolation, social distancing, shut down of businesses, closure of borders etc. result in steep decline in economic activities which distresses the household income and that is again reflected in the household expenditure. COVID-19 crisis has also affected the household income and thereby the consumption pattern in an unprecedented way. Though, food is a basic necessity but even then, it does not remain untouched by the waves of crisis. Food consumption during disease outbreak may decline as a result of the inability to work or reduced income (Piwoz et al. 2000; Kallon et al. 2017), fragmentation of savings into health care and consumption (Caroline, 2009) and fear of infection (Wolle et al. 2020). The change can be noticed not only in quantity but also in the quality and type of food consumed- consumption of cheaper substitutes (Mutangadura, 2000), less frequent consumption of high value foods like fruits and dairy products (Hirvonen et al. 2020), substitution of animal source foods like dairy, meat, poultry (Tesfaye et al. 2020; Hirvonen et al. 2020), use of more shelf-stable packaged foods (Masters, 2020) etc. Likewise, alterations can be noticed in the mode and source of purchase. The consumers may prefer those channels which provide option for online order placement and door-step delivery, choose the ones that they consider as safer and can maintain higher home inventories to limit the number of visits to market. These changes can be more prominent in household consumption of dairy products which not only come under high value food category and have high income and price elasticity, but are of animal origin and also perishable. During HIV epidemic in Zimbabwe around 71 and 61 percent households in urban and rural households, respectively, reported a decrease in the consumption of milk after a death in the household (Mutangadura, 2000). A recent survey in Addis Ababa (Ethiopia) reported that share of households consuming dairy products dropped by 11 percentage points since the COVID-19 crisis due to perceived

risk of infection (Tesfaye et al. 2020). Around 14 per cent dairy consumers reported a decline in household consumption of milk during COVID-19 in the city of Nagpur due to loss of income (Uikey & Thakkar, 2021).

Dairy constitutes an important part of daily diet in India, the world's largest dairy producer and consumer. Importance of milk and milk products in the Indian diet can be observed from the fact that private consumption expenditure on milk and milk products (21%) occupies second highest share in the total household expenditure on food next only to bread, cereals and pulses group (23%) and its share in the total household expenditure is around 8 per cent (MoSPI & WFP, 2019). This is why even during the lockdown dairy being an essential commodity was given certain relaxations in the country but it still faced setbacks due to supply issues and demand irregularities. Though, there was a definite fall in the overall demand of dairy products amid COVID-19 crisis in India as well but this was mainly attributed to closure of HoReCa (Hotels, Restaurants and Cafes). Still, little is known about the changes in household demand of dairy products and the consumer behaviour during the crisis. Quite contrary to the experience of Ethiopia (Tesfaye et al. 2020), it was reported that the household dairy consumption in India has in fact increased during lockdown as more people are staying at home in urban areas and are having less number of meals outside while in rural areas more surplus milk is available (Bhosale, 2020; Jha, 2020; Shashidhar, 2020). Similarly, there were speculations that household dairy consumption might have increased as milk is considered as an immunity booster. But, till now there is no systematic scientific literature for the same. With this backdrop, the current paper attempts to study the changes in household consumption pattern of dairy products and the shopping behaviour of consumers in India in the wake of a pandemic.

Methodology

Study Area

The study was conducted in India- the leading milk producer and consumer in the world. The first case of COVID-19 in the country was reported on January 30, 2020 and the nation-wide lockdown was imposed from March 25, 2020 to May 31, 2020. Despite, complete closure of offices and businesses and restrictions on travelling and gathering there was a constant increase in the cases of COVID-19 and it spread throughout the country. Some of the states like Maharashtra, Gujarat and Tamil Nadu which also happen to be the major milk producing states were more severely affected.

The production and consumption pattern of dairy products is quite diverse within India just like its geographical diversity. Most of the northern and western states are high milk producers and rank high in dairy progressiveness while the southern states are moderately dairy progressive and the central and eastern ones rank the least (Kale et al. 2016). The states differ considerably with respect to milk consumption also. Per capita annual milk consumption ranges from as low as 14.5 kg in Chhattisgarh (Central Zone) and 16.4 kg in Odisha (Eastern Zone) to as high as 160.5 kg and 140.7 kg in Northern states of Haryana and Punjab, respectively (Kumar et al. 2014). Thus, in order to get a good representation, pan-India study was conducted and data was collected from consumer households of all the zones.

Data Collection

Data required for the study were collected through an online survey. Well-structured questionnaire was designed in the form of google form and its link was circulated widely throughout the country by using social media. Similar method of data collection have been used in the past also for collecting primary data at the time of crisis (Jribi et al. 2020; Jung et al. 2016; Hirvonen et al. 2020; Harris et al. 2020). This method is particularly useful during infectious disease outbreak when limited face-to-face human interaction is recommended for controlling the spread of disease. Though, field survey might reduce the biasness in data but in the prevailing scenario such surveys are not possible. The survey was administered to consumers across the country exactly after two months of lockdown i.e., on May 26, 2020 and responses were collected till June 2, 2020. The questionnaire tried to capture basic information pertaining to the household consumption of dairy products, their market prices, availability of different dairy products during lockdown, home inventory, shopping behavior of the consumers and their perception about job and income security. Around 1014 responses were received from across the country out of which complete survey responses were available for 993 consumer households which were finally selected for the study. The number of households selected from North, South, East, Central & Western region of India was respectively 388, 189, 250, 54 and 112. Besides zonal classification, the collected data was also post-stratified on the basis of income quintiles.

Data Analysis

Data collected for the study was analyzed in two stages- In the first stage, descriptive statistics was calculated for all the sample consumer households and simple tabular analysis was done by using frequency counts, mean, percentages and difference to obtain a basic idea about the impact of lockdown on household dairy consumption, expenditure, availability of dairy products and shopping behavior of the consumers. The analysis was conducted zone-wise (North, South, East, West and Central) and income class-wise. For income classes, total respondents were divided into five income groups (from lowest income to highest income) so that approximately 20 per cent of the total respondents was included in each group. After this, paired t-test was used in order to check the statistical significance of the differences obtained at 1 per cent and 5 per cent level. The null hypothesis of

no significant difference in household dairy consumption before and during lockdown was tested against the alternative hypothesis of significant difference between the two time periods.

Results and Discussion

Socio-economic profile of the sample households

Socio-economic conditions of the consumer have direct bearing on the household consumption pattern. Table 1 presents the socio-economic profile of sample respondents in brief. The age

Table 1 Socio-economic characteristics of sample households

Particulars	Categories	North n=388	South n=189	East n=250	West n=112	Central n=54	Overall n=993
Age of the respondent	< 30 years	198	116	134	67	38	553
		(51.03)	(61.38)	(53.60)	(59.82)	(70.40)	(55.69)
	30-50 years	145	53	83	37	13	331
		(37.37)	(28.04)	(33.20)	(33.04)	(24.10)	(33.33)
	50-70 years	43	20	27	6	4	100
		(11.34)	(10.58)	(10.80)	(5.36)	(5.60)	(10.07)
	>70 years	1	0	6	2	0	9
		(0.26)	(0.00)	(2.40)	(1.79)	(0.00)	(0.91)
Educational qualification of the respondent	Primary education	0	0	33	3	1	37
		(0.00)	(0.00)	(13.2)	(2.68)	(1.90)	(3.73)
	Secondary	3	0	10	0	3	15
		(0.77)	(0.00)	(4.00)	(0.00)	(5.60)	(1.51)
	Higher secondary	14	6	23	0	13	56
		(3.61)	(3.17)	(9.20)	(0.00)	(24.10)	(5.64)
	Graduate & above	371	183	184	109	37	885
		(95.88)	(96.12)	(73.60)	(97.32)	(68.50)	(89.12)
Family Size	-	3.91	3.74	4.29	4.09	4.17	4.00
Location of house	Rural	52	53	84	25	14	228
		(13.40)	(28.04)	(33.6)	(22.32)	(25.90)	(22.96)
	Urban	336	136	166	87	40	765
		(86.60)	(71.96)	(66.40)	(77.67)	(74.10)	(77.04)
Major source of household income	Agriculture	23	28	51	12	14	128
		(5.93)	(14.81)	(20.40)	(10.71)	(25.90)	(12.89)
	Labour	0	1	6	2	0	9
		(0.00)	(0.53)	(2.4)	(1.79)	(0.00)	(0.91)
	Government Employee	202	85	111	57	28	483
		(52.32)	(44.97)	(44.40)	(50.89)	(51.90)	(48.64)
	Private employee	121	52	37	31	3	248
		(31.19)	(27.51)	(14.80)	(27.68)	(5.60)	(24.97)
	Pension	9	3	7	0	2	20
		(2.32)	(1.59)	(2.8)	(0.00)	(3.7)	(2.01)
	Self -employed	27	12	10	8	1	58
		(6.96)	(6.35)	(4.00)	(7.14)	(1.90)	(5.84)
	Fellowship	5	8	28	2	6	47
		(1.29)	(4.23)	(11.20)	(1.79)	(11.10)	(4.73)
Average monthly household income quintile	Quintile 1	53	38	68	20	19	198
		(13.66)	(20.11)	(27.20)	(17.86)	(35.19)	(19.94)
	Quintile 2	67	43	62	16	12	200
		(17.27)	(22.75)	(24.80)	(14.29)	(22.22)	(20.14)
	Quintile 3	86	40	48	17	7	198
		(22.16)	(21.16)	(19.20)	(15.18)	(12.96)	(19.94)
	Quintile 4	93	28	39	28	10	198
		(23.97)	(14.81)	(15.60)	(25.00)	(18.52)	(19.94)
	Quintile 5	89	40	33	31	6	199
		(22.94)	(21.16)	(13.20)	(27.68)	(11.11)	(20.04)

Note: Figures in parentheses represent the percentage of their respective column total

of the majority of the respondents (55.69 %) was less than 30 years and a very high percentage (89.12%) of the total respondents were graduates or possessed a higher educational degree. This was expected as around 65 percent of population in India is below 35 years and further young and more educated people have higher access to internet and social media. Despite this, it is anticipated that it will not lead to much bias in the data as information was sought for entire household and not on individual basis. The average family size was almost same across the zones. About 77 per cent of the sample households were located in urban area whereas the remaining 23 per cent were in rural area.

Around 48 per cent of the sample households were earning major chunk of their income from government job and are thus, expected to face relatively less fluctuations in their income as compared to the 44.61 per cent employed in private sector, agriculture, as daily wage earners and the ones having their own business set-ups.

Impact of COVID-19 lockdown on household consumption of dairy products

Household consumption of milk and milk products for before lockdown and during lockdown period was estimated zone-wise and income-wise which is shown in Table 2 and Table 3, respectively. Overall, there was a significant decrease in the consumption of milk, paneer and butter during lock-down. There was no significant change observed in the case of ghee, curd and butter-milk. Maximum amount of change was seen in the consumption of butter while it was least for ghee. This may be because consumers mostly buy packaged butter which was not freely available during lockdown whereas ghee even during normal times is often stocked as home inventory and can also be prepared at home due to which there was no major change in its consumption. Despite increase in temperature, there was a considerable decline in the consumption of ice-cream as the households having zero consumption increased by around 36 percentage points. This might be either because of unavailability of ice-cream in the market or apprehensions of catching cold which was also one of the symptoms of COVID-19.

Table 2 shows the monthly consumption of milk and milk products zone wise. Among all the zones, the average monthly consumption of liquid milk was highest in north zone and the least in south zone both before and during lock down. Except for central zone where the decrease was significant, all other zones witnessed a non-significant decrease in the consumption of liquid milk after lock down. This indicates that liquid milk supply was intact to a good extent even during lock-down and majority of people were able to purchase it. It is astonishing that consumption of paneer declined significantly in the milk surplus states of northern and western India. In northern India, there was small but significant increase in consumption of buttermilk which may be mainly because of approaching summers during lockdown. There was a decrease in consumption of all the dairy products in milk deficit eastern zone and the decline was specifically significant in the case of ghee, curd and butter. Eastern zone depends on supply from other zones for meeting its demand of dairy products. Thus, apparently breakdown of supply chain might have affected this zone more. A thing which was common among all the zones is significant decline in the consumption of ice-cream and butter. Hence, it shows that lockdown has severely hit the ice-cream and butter industry in India.

Income is the most important variable that affects consumption. Table 3 depicts the change in monthly consumption pattern of milk and milk products in different income quintiles. Decrease in consumption of milk was highest and significant in the lowest income quintile (Quintile I) while it wasn't much conspicuous in higher income quintiles. Along with liquid milk, consumption of paneer, ghee and butter also declined significantly in the case of households of Quintile I. This indicates that already distressed

Table 2 Impact of COVID-19 on monthly consumption of milk and milk products in different zones (unit change per consumer household)

Products		North n=388	South n=189	East n=250	West n=112	Central n= 54	Overall n= 993
Milk (L)		-0.42^{NS}	-0.99^{NS}	-0.27^{NS}	-0.84^{NS}	-3.48*	-0.71*
Paneer (kg)		-0.32**	-0.03^{NS}	-0.12^{NS}	-0.29**	-0.07^{NS}	-0.20**
Ghee (kg)		-0.02^{NS}	0.01^{NS}	-0.04*	0.06^{NS}	0.05^{NS}	-0.01^{NS}
Buttermilk (L)		0.59*	0.21^{NS}	-0.26^{NS}	-1.09^{NS}	0.44^{NS}	0.11^{NS}
Curd (kg)		0.35^{NS}	0.77**	-0.37*	-0.17^{NS}	0.26^{NS}	0.19^{NS}
Butter (g)		-14.69*	-7.74^{NS}	-22.00*	-11.11^{NS}	-38.89*	-3.40**
Ice Cream (Percentage of respondents)	Once in 3 days	-4.38	-3.18	-7.60	-7.21	-12.90	-5.74
	Once in a week	-15.72	-12.70	-15.20	-27.93	-9.30	-16.01
	Once in a month	-10.05	-23.81	-23.20	-9.91	3.70	-15.21
	Nil	30.15	39.68	53.00	45.04	18.60	36.96

Note: **significant at 1%, *significant at 5% and ^{NS} not significant.

group was affected more due to the crisis which increases the concern about nutritional security. The consumption of paneer decreased in all the income quintiles and the change was significant for all except Quintile II. The consumption of ghee was more or less constant for the income quintiles- II, III, IV and V. Except for the Quintile II, all the other groups reported an increase in the consumption of buttermilk/lassi. The consumption of curd also increased in all the income quintiles but non-significantly. Butter was another dairy product whose consumption was affected considerably during the lockdown. The decline in consumption of butter was found to be highest in the case of Quintile I followed by Quintile IV and Quintile II. The demand for ice cream decreased almost evenly for all the income groups.

Thus, consumption of ghee was least affected while ice-cream and butter faced major setback during the lock-down period. Contrary to the normal years, consumption of curd, butter-milk and ice-cream didn't increase considerably in response to the rise in temperature. With respect to geographical zones, consumption of majority of the dairy products declined significantly in the milk-deficit eastern zone while lowest income quintile faced the major brunt among the income classes.

Effect of COVID 19 pandemic on monthly expenditure of consumer households on dairy products (Table 4) was estimated by using collected data on monthly consumption and market prices of milk and milk products for before lockdown as well as during lockdown period.

Household monthly expenditure on milk and milk products declined during the lockdown. Thus, change in expenditure was found to be negative for all the categories except for south zone and richest quintile of income class for whom the change in monthly expenditure on milk products was found to be positive but non-significant. In the case of MERS outbreak also a decline by 7 per cent was observed in overall consumption expenditure of households (Jung et al. 2016) which shows that pandemics do affect the household consumption expenditure. Srivastava et al. 2020 also predicted that the decline in consumption expenditure

Table 3 Impact of COVID-19 on monthly consumption of milk and milk products by income-class (unit change per consumer household)

Products		Quintile I	Quintile II	Quintile III	Quintile IV	Quintile V	Overall
Milk (L)		-2.02**	-0.34^{NS}	-0.63^{NS}	-0.04^{NS}	-0.52^{NS}	-0.71*
Paneer (kg)		-0.24**	-0.02^{NS}	-0.25**	-0.26**	-0.22**	-0.20**
Ghee (kg)		-0.07**	-0.02^{NS}	0.02^{NS}	0.00^{NS}	0.02^{NS}	-0.01^{NS}
Buttermilk (L)		0.01^{NS}	-0.07^{NS}	0.36^{NS}	0.12^{NS}	0.12^{NS}	0.10^{NS}
Curd (kg)		0.01	0.30^{NS}	0.18^{NS}	0.00^{NS}	0.43^{NS}	0.19^{NS}
Butter (g)		-25.76**	-10.00*	-12.62^{NS}	-18.18*	-0.51^{NS}	-13.40**
Ice-Cream (Percentage of respondents)	Once in 3 days	-5.56	-7.00	-8.59	-5.05	-5.80	-5.74
	Once in a week	-3.03	-15.00	-13.13	-21.72	-16.00	-16.01
	Once in a month	-29.29	-7.00	-17.17	-10.10	-15.20	-15.21
	Nil	37.88	29.00	38.89	36.87	37.30	36.96

Note: **significant at 1%, *significant at 5% and ^{NS} not significant.

Table 4 Effect of COVID-19 pandemic on monthly expenditure of consumer households on milk and milk products

Particulars	Categories	Change (Rs. per month)
Zones	North	-36.69^{NS}
	South	27.62^{NS}
	East	-42.96**
	West	-141.47^{NS}
	Central	-17.52^{NS}
Income Quintiles	Quintile I	-123.09**
	Quintile II	-21.18^{NS}
	Quintile III	-14.12^{NS}
	Quintile IV	-79.11^{NS}
	Quintile V	52.86^{NS}
Overall	-	-36.80^{NS}

Note: **significant at 1%, *significant at 5% and ^{NS} not significant.

of Indian households on high value food commodities like milk will be comparatively higher than the decline reported in case of staple foods (cereals, pulses and edible oil). Absolute decline in monthly expenditure on milk and milk products was highest for west zone (Rs. 141) but it was only the eastern zone where change was found to be significant. This indicates that most of the households in zones other than the east adjusted the quantity of dairy products that were being consumed but kept their expenditure almost same. On the contrary, a clear decline was observed in the household expenditure on dairy products in the case of eastern zone and lowest income quintile. Change in monthly expenditure on dairy products was found to be positive only for the richest income quintile which might be because of additional amount spent by the households for ensuring food safety and lowering disease risk.

Impact of COVID-19 pandemic on consumer behaviour

Disease outbreak and the fear of infection not only affect the consumption level but it can also change what, how and from where people shop. Shopping behaviour of the sample households for dairy products was studied and the results are discussed in the following paragraphs. Prior to the lockdown, majority of the consumers (66%) were purchasing milk and milk products from milk vendors and retail outlets selling packed products. While during lockdown there was a decline in the percentage of consumers buying products from these two points, a strong surge (+4 percentage points) was observed in the percentage of consumers opting for online order and delivery. Reduction in percentage of consumer households purchasing from retail outlets and milk vendors may be due to their reduced operating hours. Preference for getting packed products and that too at door-steps can be the other reason for this. Our results were found to be in line with other studies conducted at times of crisis that have shown an increase in e-commerce (Robertson, 2020; Jung et al. 2016; Jribi et al. 2020) to prevent exposure to the disease. This is a new and emerging trend in dairy marketing for a developing country like India where network of e-commerce for selling dairy products is just limited to few cities. This platform can be made available to a greater number of people particularly at this time when India is emphasizing on more and more digitalisation.

It is also noteworthy that during lockdown period most of the consumers switched from fresh-dairy products to packed one. This shows that perception of food safety was stronger for packaged products. Around 20 per cent of the respondents switched to door-step delivery during lockdown period for avoiding issues in movement and for limiting direct contact with outsiders. Travel and transport restrictions were an important component of regulations during lockdown period which severely reduced the movement pace of goods. Thus, availability of products in the market was a major problem. Supply of liquid milk suffered least and it was always available to most (91%) of the respondents. The supply of value-added products specifically paneer, butter and ice-cream suffered a major set-back. Low availability of paneer can be one of the reasons behind significant decline in its consumption which was common across the zones. Only 23 per cent of the respondents said that ice-cream was always available in the market. Low availability of ice-cream may be due to its slashed demand (by 85 per cent during March- April period) and lower sales during lockdown (Vora, 2020). Other than restricted movement, the apprehension of catching cold after eating ice-creams might have fuelled the reduction in its demand. As demand and supply cycle works, since there was less demand, the availability or supply of ice-cream was also less. Availability of dairy products was almost similar in all the zones. Effect of availability of products is clearly visible on consumption also. We noticed that decline in consumption of liquid milk was significant only for central zone and in the same zone lowest percentage of people stated that milk was always available. Similarly, significant decline in butter consumption is also reflected in its lower availability. Availability of curd and butter was lower in the eastern zone and there was a significant decline in their consumption also.

Conclusions

Pandemics do affect household food consumption pattern. Though, there were some speculations in India that crisis like COVID-19 can in fact increase the household consumption of dairy products owing to their immunity boosting qualities and people spending more hours at home but the results of online consumer survey portray a different picture. A significant decline in the household consumption of most of the dairy products like milk, paneer, ice-cream and butter was observed during lock-down period. Decline was higher in the case of value-added products and that too more in the case of ones which are majorly bought from market and have lower shelf life. Milk deficit zones and the lower income class faced the major brunt of the crisis. Significant decline in quantity consumed and consumption expenditure of dairy products in lower income groups re-validate the fact that any type of crisis hits the bottom-most section of income pyramid badly. What is more disturbing is that around 15 per cent of the respondents in lowest income group stated that their household consumption of milk and milk products is going to further decline in the post-lockdown period. This calls for external support for ensuring nutritional security of these households. In order to make sufficient quantity of milk available to poorer section of society, milk can be included in ration scheme for some time. On the positive side, pandemic crisis has increased the awareness regarding food safety among the dairy consumers. Thus, better prospects lie ahead for packaged commodities. Similarly, there has been a shift towards online ordering and door-step delivery services. Better network of door-step delivery services will further strengthen the dairy supply chain in India. Up to some extent, it will also help in arresting the drop in

household demand of dairy products due to fear of infection in case of any such future pandemic.

Conflict of Interest

The authors declare no conflict of interest in the preparation of this paper.

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RESEARCH ARTICLE

Entrepreneurial behaviour of dairy farmers under Dairy Business School model

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Abstract: Entrepreneurship has become the need of the hour with good potential to improve livelihood and generate employment. Most of the small and marginal farmers are dependent on dairy farming and there is a need to enhance the income of the farmers as per government priority to double their income. Hence entrepreneurship development in production, processing, and marketing is essential. So with the aim to inculcate entrepreneurial behaviour among dairy farmers, the Dairy Business School (DBS) was set up on an experimental basis at NDRI, Karnal a premier institute working on Dairy. The present study was conducted in 12 villages in six adjoining districts of NDRI, Karnal. An action research design was used with a sample size of 180 farmers during 2021-22. The data were collected through a pre-tested interview schedule by holding the personal interview. The results of the study revealed that the majority (61.11 %) of the farmers had a medium level of entrepreneurial behaviour. All the thirteen components of entrepreneurial behaviour like risk taking, hope of success, persistence, feedback usage, self confidence, knowledgeability, persuasability, manageability, innovativeness, achievement motivation, decision making ability, cosmopoliteness and profit orientation were at a medium level among dairy farmers. Knowledgeability was ranked first which contributed most to the entrepreneurial behavior of farmers while decision making ability at last.

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Introduction

In 2022, India is accelerating its road to progress and emerge as the world's strongest economy. India has become third largest ecosystem with home for startups and entrepreneurship development with improved Global Innovation Index from 81st place to 46th place (www.economictimes.com). Entrepreneurship plays a pivotal role in the economic development and effective tool to fight against unemployment and poverty. Entrepreneurship development in Agriculture and Dairy is an important way out to bring a transformation in our rural areas.

The agriculture sector has showed growth rate of 3.6 per cent in 2020-21 and 3.9 per cent in 2021-22. The major drivers of overall growth are contributed by high growth in allied sectors including livestock, dairying and fisheries. The livestock sector has grown at a CAGR (compound annual growth rates) of 8.15 per cent over the last five years ending 2019-20. As per latest SAS, the sector has been stable source of 15 per cent of their average monthly income groups of agricultural households. The Committee on Doubling Farmers' Income (DFI, 2018) recognized the increasing importance of allied sectors including dairying, livestock, poultry, fisheries and horticulture considered as engines of high growth. Development of livestock sector has led to improvement in per capita availability of milk (gram per day) from 319 in 2014-15 to 427 in 2020-21, eggs (number per annum) from 62 in 2014-15 to 91 in 2020-21 and meat (kg per year) from 5.32 in 2014-15 to 6.52 in 2020-21 (Economic Survey, 2020-21).

The single largest agricultural commodity contributing 5 per cent of the national economy and employing more than 8 crore farmers directly is Dairy. India is ranked number one in milk production in world contributing 23 per cent of global milk production. Milk production in the country has grown at a compound annual growth rate of about 6.2 per cent to reach 209.96 million tonnes in 2020-21(Economic Survey, 2020-21). In rural areas, Dairy farming has the highest potential of generating income and employment through improved productivity of milch animals, animal trading, processing and marketing of milk and milk products. It is one of

the promising sectors for entrepreneurship development in India for small and marginal farmers.

Entrepreneurship development of dairy farmers ensures optimal utilization of available resources and facilitates value addition of milk to products and services. A sustainable and financially viable dairy farming which generate income and self employment through entrepreneurship is the need of the day (Gamit *et al*, 2015).For developing an entrepreneurship and to help the dairy farmer to start a dairy business enterprise, National Dairy Research Institute (NDRI), Karnal being Expert Institute has conducted Dairy Business School (DBS) with financial support from CCS NIAM, Jaipur.

DBS aims to inculcate the entrepreneurial behavior among the dairy farmers. DBS involves the capacity building of the farmers with respect to production, processing and marketing aspects and linking to the value chains. The farmers who were interested in starting dairy enterprise and dairy business were selected as participants. To select the participant farmers for DBS who were interested in starting dairy enterprise and dairy business, the study was conducted to know the entrepreneurial behavior of dairy farmers in the adjoining districts of locale of NDRI, Expert Institute in Haryana.

Methodology

The present study was conducted in Haryana in 2021-22. Haryana has been purposively selected based on the presence of Expert Institute (NDRI) in the field of dairy production and processing. The six peripheral districts of Expert Institute, namely Panipat, Kurukshetra, Jind, Kaithal, Karnal and Yamunanagar were selected for study. From each district, two villages were randomly selected. From each village, 15 respondents were selected based on the criteria's like having more than two milch animals, interested in dairy business and age less than 50 years which leads to sample size of 180. The data was collected with the help of a pre-tested structured interview schedule through personally interviewing the respondents by incorporating all the items pertaining to the specific objectives of the study. The collected data was scored, tabulated and analyzed by using frequency, percentage, mean and standard deviation.

The entrepreneurial behaviour of dairy farmers was measured by developing Entrepreneurial behaviour index (EBI). The entrepreneurial behaviour of dairy farmers was operationally defined as cumulative outcome of thirteen components namely, Risk Taking, Hope of success, Persistence, Feedback usage, Self confidence, Knowledgeability, Persuasability, Manageability, Innovativeness, Achievement Motivation, Decision making ability, Cosmopoliteness and Profit Orientation. Entrepreneurial behaviour index (EBI) was also calculated by using formula:

Entrepreneurial Behaviour Index (EBI) = Obtained mean score/ Maximum obtainable score x100

Result and Discussion

Entrepreneurial behaviour of dairy farmers:

The data in Table 1 depicted that entrepreneurial behaviour of dairy farmers comprised thirteen components such as Risk Taking, Hope of success, Persistence, Feedback usage, Self confidence, Knowledgeability, Persuasability, Manageability, Innovativeness, Achievement Motivation, Decision making ability, Cosmopoliteness and Profit Orientation.

Risk taking

Risk taking was the ability of the farmers to handle risk and uncertainty in facing problems in dairy enterprise. The majority (62.78 %) of dairy farmers belonged to medium level of risk taking followed by high (22.22 %) and low (15.00 %) levels. This might be due to the reason that dairy enterprise being the major source of income for livelihood and value addition of milk will improve the profit levels, the dairy farmers might take risk and face uncertainties. These findings are in accordance with Porchezhiyan et al. (2016), Raina et al. (2016) and Chaurasiya (2015).

Hope of success

The degree to which an individual is prepared to face the consequences of his/her dairy enterprise/venture includes hope of success and fear of failure. In the present study, majority of the farmers (60.56%) had medium level of this particular attribute. Nearly one-fifth were found having high level and remaining 19.44 per cent been having low level of hope of success. The result confirms that most of the respondents have prepared themselves to face the consequences raised in their dairy enterprise. The good number of farmers possessed this attribute indicating high level success in their dairy enterprise. These findings are in line with Palmurugan et al. (2008) and Sah (2005).

Persistence

Persistence is to take repeated or different actions to overcome obstacles in enterprise. It is evident from Table 1 that 60 per cent of respondent farmers belong to medium level of persistence followed by high (20.56 %) and low (19.44%) levels. This shows that most of the farmers were able to take alternative options or different actions to solve the difficulties faced by them in dairy enterprise. The past studies guide that obstacles does not easily demotivate farmers; rather they carry on with more hope of success and self confidence. These findings are being supported by Palmurugan et al. (2008).

Feedback usage

Feedback usage is the ability of farmer to seek and use information/feedback on his dairy enterprise to manage his resources efficiently to earn higher profits. The findings of study revealed that nearly half (53.89 %) of the respondent farmers belong to medium level of feedback usage followed by high (25.56 %) and low (20.56 %) levels. This clearly indicate that most of the farmers who are good at using feedback for improving decision making , performance and management of dairy enterprise, can be more successful. The studies done by Palmurugan et al. (2008) and Sah (2005) have also found the same results.

Self confidence

It is one of the important attribute should be possessed by every individual but especially here farmers and entrepreneurs who wish to run enterprise. The data on this attribute shows that almost cent farmers belonged to medium (82.22 %) and high (16.11 %) level of self confidence. Very few respondent farmers (1.67 %) belonged to low level of self confidence. The probable reason for such findings may be respondent farmers experience in dairy farming, knowledge levels, their ability to complete the different tasks and challenges in dairy enterprise. The findings are supported by Porchezhiyan et al. (2016), Gamit et al. (2015) and Raut and Sankhala (2014) who found majority of the respondents belonged to medium level of self confidence.

Table 1 Entrepreneurial behaviour of dairy farmers (n=180)

Sl. No	Components	Category	Mean	SD	Frequency	Percentage
1	Risk Taking	Low	8.68	3.00	27	15.00
		Medium			113	62.78
		High			40	22.22
2	Hope of success	Low	8.23	3.14	35	19.44
		Medium			109	60.56
		High			36	20.00
3	Persistence	Low	8.98	2.62	35	19.44
		Medium			108	60.00
		High			37	20.56
4	Feedback usage	Low	8.72	3.23	37	20.56
		Medium			97	53.89
		High			46	25.56
5	Self confidence	Low	7.28	2.30	3	1.67
		Medium			148	82.22
		High			29	16.11
6	Knowledgeability	Low	9.48	3.38	25	13.89
		Medium			128	71.11
		High			27	15.00
7	Persuasability	Low	6.58	3.21	39	21.67
		Medium			109	60.56
		High			32	17.78
8	Manageability	Poor	8.51	4.27	56	31.11
		Moderate			88	48.89
		Good			36	20.00
9	Innovativeness	Low	3.86	1.34	26	14.44
		Medium			129	71.67
		High			25	13.89
10	Achievement Motivation	Low	7.51	3.34	56	31.11
		Medium			84	46.67
		High			40	22.22
11	Decision making ability	Poor	3.17	1.82	49	27.22
		Moderate			91	50.56
		Good			40	22.22
12	Cosmopoliteness	Low	6.93	3.52	48	26.67
		Medium			102	56.67
		High			30	16.67
13	Profit Orientation	Low	8.18	4.18	42	23.33
		Medium			106	58.89
		High			32	17.78

Knowledgeability

Knowledgeability is the degree to which a farmer/entrepreneur perceives himself to be competent in the production, processing and marketing aspects of dairy to run and manage a dairy enterprise. More than two-third i.e., 71.11 per cent of dairy farmers had medium level of knowledgeability, followed by high (15.00 %) and low (13.89 %) levels. This shows that farmers were having strong technical base and experience to run a dairy enterprise. The respondents have also expressed the strong desire to gain knowledge on value addition, milk processing and marketing aspects.

Persuasability

The ability of entrepreneur/ dairy farmer to perceive himself to capable of convincing others to accept their own ideas, thoughts and information overtly or covertly**.** The data concluded that majority (60. 56 %) had medium level of persuasability, followed by 21. 67 per cent with low level and 17.78 per cent with high level of persuasability. The data on this parameter conclude that most of them were good at convincing others due to their good knowledgeability and self-confidence. The results are in similarity with Sah (2005) who observed that majority of respondents had medium level of persuasability.

Manageability

The results on this attribute showed in Table 1 indicate that nearly less than half of the respondent farmers (48.89 %) had moderate level of manageability. Whereas more than one fourth (31.11 %) of farmers had poor level of manageability and only 20.00 per cent farmers had good manageability. Most of the farmers had moderate manageability which can be improved through Dairy Business School with different interventions. The results are in line with Jhamtani et al. (2003).

Innovativeness

The important attribute which distinguish entrepreneurs or progressive farmer is innovativeness. Innovativeness is to explore for new technologies developed from time to time in new areas, products and services. The pooled analysis of data on this attribute reveals that 71.67, 14.44 and 13.89 per cent of farmers belonged to medium, low and high level of innovativeness respectively. The results concluded that most of the respondent farmers were innovative and satisfied with new technologies developed in dairy sector at study area. This may be due to presence of Expert Institute in study area i.e., NDRI, Karnal which is the premier institute to develop and communicate innovative technologies in to target group in dairy sector. The role of mass media and social media has also made farmer respondent to access the new technologies at their door steps. The findings are supported by Porchezhiyan et al. (2016), Chaurasiya et al. (2015), Gamit et al. (2015) who reported medium level of innovativeness in majority of the respondents.

Achievement Motivation

Achievement motivation is the urge of farmer/entrepreneur to improve oneself and excel to achieve their goals. Less than half (46.67 %) farmers are having medium level of achievement motivation followed by low (31.11 %) and high (22.22 %) levels. The reason for this may be due to selected respondents were small dairy farmers having 2-5 milch animals and were interested in dairy business who wished to become entrepreneur but due to limited resources, they were in confused mind which made them to have medium motivation. The results are in agreement with Baindha et al. (2014).

Decision making ability

It is the ability of the dairy farmer to choose among different alternatives to run and manage his dairy enterprise effectively**.** Maximum number of dairy farmers (50.56 %) had moderate decision making ability. Whereas more than one fourth of farmers had poor and 22. 22 per cent of them had good decision making ability. The probable reason for most farmers to be in medium to low levels is due to less ownership in joint family to take the decisions in the study area. The above findings are in line with Porchezhiyan et al. (2016) and Raina et al. (2016).

Cosmopoliteness

The data on this attribute depicted in Table 1 revealed that half (56.67 %) of the farmers had medium level of cosmopoliteness, followed by more than one fourth of farmers in low level and 16.67 per cent in high levels respectively. This cosmopolitan outlook may be due to moderate resource base, information seeking behaviour and economic conditions of respondents, leading to moderate participation in various organizational, extension and social events like field visits by NDRI scientist in study area, training programmes, demonstrations, clinical camps and dairy mela. Similar results are reported by Patel et al. (2014).

Profit Orientation

The profit orientation is the ability of farmer/entrepreneur to orient towards making good profit out of dairy enterprise in the present study. Majority farmers (58.89 %) had medium level of profit orientation. About 23.33 and 17.78 per cent farmers had low and high level of profit orientation respectively. The results conclude that most of the farmers have still potential to exploit the opportunities in dairy enterprise to earn profit. The Dairy Business School provides an opportunity for farmers to learn about the unexplored areas of dairy and help the farmers to follow the scientific management practices of dairy to obtain maximum benefit.

Table 2 Distribution of respondents according to their overall entrepreneurial behaviour(n=180)

Sl. No	Categories			Frequency	Percentage
1	Low			34	18.89
2	Medium	Mean= 96.12	SD=32.49	110	61.11
3	High			36	20.00

Table 3 Extent of entrepreneurial behavioral attributes possessed by dairy farmer (n=180)

Sl. No	Attributes	EBI	Rank
1	Risk Taking	54.24	IV
2	Hope of success	51.46	VI
3	Persistence	56.15	II
4	Feedback usage	54.51	III
5	Self confidence	45.52	IX
6	Knowledgeability	59.27	I
7	Persuasability	41.15	XII
8	Manageability	53.16	V
9	Innovativeness	44.13	X
10	Achievement Motivation	46.94	VIII
11	Decision making ability	19.79	XIII
12	Cosmopoliteness	43.30	XI
13	Profit Orientation	51.15	VII

Overall entrepreneurial behaviour of dairy farmers:

The data on entrepreneurial behaviour level revealed in Table 2 that majority (61.11%) of dairy farmers had medium level of entrepreneurial behaviour, followed by 20.00 per cent farmers having high level of entrepreneurial behaviour. Whereas, 18.89 per cent of farmers had low level of entrepreneurial behaviour. The reason behind most of the farmers to be in medium to high category of entrepreneurial behaviour is due to medium level of financial condition, medium land holding and medium educational levels. However, the medium level of all the components of entrepreneurial behaviour of dairy farmers led to medium entrepreneurial behaviour. Similar results are obtained by Mariammal and Seethalakshmi (2017), Raina et al. (2016), Porchezhiyan et al. (2016) , Chaurasiya et al. (2015) and Patel et al. (2014).

Contribution of entrepreneurial attributes towards Entrepreneurial Behaviour Index:

Appraisal of the data given in Table 3 concluded that 'Knowledgeability' ranked first with EBI-59.27 indicating highest contribution towards entrepreneurial behaviour of dairy farmers. Whereas, 'Decision making ability' has been ranked last with EBI-19.79 indicating lowest contribution towards entrepreneurial behaviour. On the other hand, all other eleven components like Persistence, Feedback usage, Risk Taking, Manageability, Hope of success, Profit Orientation, Achievement Motivation, Self confidence, Innovativeness, Cosmopoliteness, Persuasability ranked II, III, IV, V, VI, VI, VIII, IX, X, XI, XII respectively. The possible reason for knowledgeability and persistence to rank I and II may be due to strong technical knowledge and skills of farmers in dairy and they were able to opt for different options or actions to overcome the obstacles in dairy enterprise. The farmers of study area had constant contact with extension people, KVKs, NDRI which made them to have better knowledge on scientific dairy management practices of dairy. The probable reason for decision making to be at last may be due to joint family and joint ownerships to take decision in enterprise restricted them to take decisions.

Conclusion

The findings of the study concluded that majority of the dairy farmers were having medium level of entrepreneurial behaviour. This gap gave a way to empower the dairy farmers towards entrepreneurship development. Most of the farmers in study area were interested to start a dairy enterprise but lacked the required skills and guidance. So, effective entrepreneurship development programme and model trainings must focus on dairy farmers to help them to take risks and to tackle the uncertainties to improve their livelihood. The medium level of contribution of all the components of entrepreneurial behaviour indicated that these attributes needs to be improved to achieve in dairy entrepreneurship. The findings helped to select the most interested and entrepreneurial farmer for Dairy Business School where they were regularly monitored by Scientist and Experts of NDRI, Karnal by using different teaching methods like classes, field visits, exposure visits, demonstrations, practical classes etc., to impart the knowledge, skill and motivation to start a dairy enterprise i.e., dairy startup.

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SHORT COMMUNICATION

Breeding and healthcare management practices of dairy animals followed by farmers in Varanasi district of Uttar Pradesh

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Abstract: A field survey was conducted in the Varanasi district to find out the status of existing breeding and healthcare management practices followed by dairy farmers. A total of 80 farmers were selected from the 4 randomly selected blocks of the district and survey was done with the help of prepared interview schedule throughout November 2021 to April 2022. Majority of the farmers (73.75%) always practised Artificial Insemination (A.I.) as a method of breeding for their dairy animals while mucus discharge was mostly used for detecting heat. About 24.00 per cent of farmers always followed deworming and 26.25 per cent always vaccinated their dairy animals against FMD and HS. Repeat breeding was a major reproductive problem in dairy animals faced always by about 75.00 per cent of the farmers and pregnancy diagnosis of dairy animals was practised by only about 42.50 per cent of the farmers.

Keywords: Breeding, Healthcare, Artificial Insemination, Varanasi, Vaccination, Deworming

Livestock production is an important component of the agricultural economy, a contribution that goes beyond direct food production. India's livestock sector is one of the largest in the world with a livestock population of 535.78 million and hence plays an important role in developing India's economy. The breeding and healthcare management practices plays a very important role in influencing the productivity of dairy animals.

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The awareness of the different cow management practices used by farmers is crucial because it could bridge the gap between the current methods used and the scientifically advised methods. In order to determine the strengths and weaknesses of the rearing systems and to develop effective intervention strategies, it is essential to understand the livestock management practises used by farmers. Therefore, the present study was conducted to document the existing breeding and healthcare management practices followed by dairy farmers in the study area.

The present study was conducted in Varanasi district which is located in the eastern part of Uttar Pradesh. The state of U.P. was purposively selected as it has the highest milk production as well as highest livestock population and the district was selected on the basis that it was the least developed district in terms of dairy farming in the eastern part of the state. A total of 80 farmers who were having at least 1 Milch animal were randomly selected and data were collected with the help of structured and semi structured interview schedule. 4 blocks from the district were randomly selected and from each block 2 villages were randomly selected while 10 farmers were selected from each village. The collected data were analyzed statistically using frequency and percentage.

The herd composition of the dairy animals mainly comprised of indigenous cattle (Sahiwal, Gir), Jersey and Murrah breed in case of buffalo. The information regarding various breeding management practices are presented in Table 1 and Table 2. From the study it was concluded that majority of the respondents (73.75%) were practicing artificial insemination (AI) for breeding their cattle which is similar to the results obtained by Sabapara et al. (2010) in South Gujrat and Prajapati et al. (2015) but in contrast with the results obtained by Yadav et al. (2016) according to which 26.8 per cent use AI, which may be low due to the insufficient availability of AI coverage and poor policy implementations. The respondents having buffaloes mostly prefer natural service which is 26.25 per cent and all of Artificial Insemination was done by Para-vets in the study area. The findings were in line with the results obtained by Malik et al. (2005) that natural service was preferred for buffaloes and all of the A.I. in dairy animals was done or performed by para-vets. About 57.50 per cent of the farmers do not follow pregnancy

diagnosis to determine whether their dairy animals were pregnant. The results are similar to the results obtained by Yadav et al. (2016), which stated that (59.2%) of respondents did not follow pregnancy diagnosis for their livestock. The remaining 42.50 per cent of respondents adopted pregnancy diagnosis and the pregnancy diagnosis was done by Para-vets for 47.00 per cent of the respondents who adopt pregnancy diagnosis.

In the Table 2, according to the distribution of respondents 46.25 per cent of the respondents always use mucus discharge as the method to detect heat and bellowing was used always as the method of heat detection by 28.75 per cent of farmers while 41.25 per cent of respondents used it a method occasionally and mounting was never used by majority (71.25%) of the farmers. The problem of repeat breeding in dairy animals was faced by majority (75.00%) of the respondents and another problem faced occasionally by 26.25 per cent of respondents was infertility. About 55.00 per cent of respondents faced delayed onset of cycle occasionally. The breeding problems were solved by para-vets always for 30.00 per cent of farmers, while 63.75 per cent of the respondents consulted para-vets occasionally for breeding problems. Regarding veterinarian or veterinary hospitals only 27.50 per cent of respondents consulted them on occasional basis while for majority of the respondents (72.50%) the breeding problems was never solved by neither veterinarian nor veterinary hospitals.

The information regarding various breeding management practices are presented in Table 3. Deworming was practiced by 62.50 per cent of farmers which was contradicting to the results obtained by Sabapara et al. (2010) which was that 25 per cent of respondents always followed deworming in South Gujrat. The results obtained indicated moderate level of knowledge and awareness about deworming and its benefits among the respondents. The people who practised deworming about 67.50 per cent preferred para – vets for deworming of their dairy animals while 32.50 dewormed their animal by themselves. Vaccination for dairy was followed by 48.75 per cent of the respondents and for vaccination all of the respondents were always dependent on

Table 1 Breeding management practices of dairy animals followed by dairy farmers in Varanasi

Particulars	Frequency	Percentage
Method of Breeding followed		
Artificial Insemination	59	73.75
Natural Method	21	26.25
Artificial Insemination done by		
Veterinary Surgeon	00	00.00
Para-vets	80	100
Pregnancy Diagnosis done		
No	46	57.50
Yes	34	42.50
Pregnancy diagnosis done by		
Self – assessment	18	53.00
Para-vets	16	47.00

para-vets. Mainly the vaccination was done for diseases such as Foot and Mouth Disease (FMD) and Hemorrhagic septicemia (HS). The above results for vaccination were similar to the results obtained by Prajapati et al. (2015) which stated that about 90.3 per cent followed vaccination against FMD and HS. Vaccination against Brucellosis and Black quarter was not available or accessible, while vaccination against rabies is selective in nature and respondents sampled had never encountered scenario where they had to let their animals vaccinate for rabies.

Consultation for the treatment of sick animals was done primarily with para-vets always by majority of respondents (77.50%), followed by 22.50 per cent of respondents who occasionally consult para-vets. The obtained results were similar to the results obtained by Prajapati et al. (2015) in South Gujrat where only 19.5 per cent of respondents consulted veterinarians for sick animal treatment and 80.5 per cent respondents consulted with livestock inspectors which can be substituted in terms of para-vets. The vaccination schedule was followed properly by only about 15 per cent of the respondents while the remaining majority of the respondents (85.00%) did not follow any such schedule which can be due to unavailability of sufficient human resources (para-veterinary professionals), lack of awareness and knowledge about

Table 2 Other breeding management practices followed by dairy farmers in Varanasi

Particulars	Always	Occasionally	Never
Method to detect heat			
Mucus discharge	37(46.25)	30(37.50)	13(16.25)
Bellowing	23(28.75)	33(41.25)	24(30.00)
Mounting	3(3.75)	20(25.00)	57(71.25)
Reproductive problems faced by dairy animals			
Repeat breeding	60(75.00)	20(25.00)	0(00.00)
Infertility	0(00.00)	21(26.25)	59(73.75)
Delayed onset of cycle	9(11.25)	44(55.00)	27(33.75)
Breeding problems of dairy animals solved by			
Veterinarian/ Veterinary hospital	00(00.00)	22(27.50)	58(72.50)
Para vets	24(30.00)	51(63.75)	5(6.25)

Table 3 Health management practices of dairy animals followed by dairy farmers in Varanasi

Particulars	Frequency	Percentage
Deworming practised		
Yes	50	62.50
No	30	37.50
Deworming done by		
Self	26	32.50
Para-vets	54	67.50
Vaccination practised		
Yes	39	48.75
No	41	51.25
Vaccination done by		
Veterinary doctor	00	00.00
Para-vets	39	48.75
Consultation for with sick animal done with		
Veterinary doctor	18	22.50
Para - vets	62	77.50
Deworming interval followed		
3 months (Recommended)	29	36.25
6 months	28	35.00
Yearly	14	17.50
Vaccination schedule followed		
Yes	12	15.00
No	68	85.00
Sick animal isolated for		
7 days	55	68.75
15 days	18	22.50
1 month	5	6.25
Not isolated	2	2.50

vaccination. The recommended deworming interval for the cattle is once in three months which was followed by 36.25 per cent of respondents, and an interval of 6 months was followed by 35 per cent of the respondents while about 17.50 per cent practiced deworming on yearly basis. Majority of the respondents (68.75%) isolated their sick animals for a week (7 days) from the rest of the herd, while 22.50 per cent of respondents isolated their animal when sick up to 15 days and only about 6.25 per cent had isolated their animal for 1 month in case of serious sickness or disease. About 2.50 do not isolate their sick animals at all which can be due to the reasons such as small herd size (up to 2 animals) or insufficient space for separate area in case of sick animals.

Conclusion

A field study was conducted where primary information was collected from 80 farmers regarding the existing breeding and healthcare management practices followed by dairy farmers in Varanasi. The data was collected with the help of structured and semi-structured interview schedule. Artificial Insemination was practiced by majority (73.75%) of the respondents and heat was detected by mucus discharge mostly in the morning. Pregnancy diagnosis was only opted by about 42.50 per cent of the respondents and the major problem faced by the farmers in the study area was of repeated breeding for which farmers consulted para-vets. Deworming and vaccination was practiced by only 62.50 per cent and 48.75 per cent of respondents respectively, mostly by para-vets and vaccination was done against FMD and HS in the dairy animals. For sick animals, majority of the respondents (72.50%) always consulted para-vets for their treatment. Proper schedule of deworming as well as vaccination was not followed by the respondents, while the sick animals were isolated for 7 days by about 68.75 per cent of the farmers. Adoption of scientific breeding and healthcare practices was found to be satisfactory but awareness was still lacking regarding vaccination and following proper vaccination schedule in the study area.

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SHORT COMMUNICATION

Effect of replacement of concentrate feed with Moringa leaves on dietary nutrient utilization in non-descript Chhattisgarh goats

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Abstract: An experiment was conducted to analyse the effect of replacing commercially available concentrate mixture with dried Moringa oleifera leaves on the nutrient utilization pattern of diet in non-descript goat breeds of Chhattisgarh. Eight non-descript yearling goats allotted randomly into 2 groups (group1 and group 2) based on their BW (13.48±1.07 kg). Animals in group 1 were offered concentrate @ 1.5% of body weight and hybrid Napier grass ad lib. In the group 2, 50% of concentrate was replaced by Moringa leaves on dry matter basis. Dried leaves were analyzed for their chemical composition. The CP content was 27.52% and Fe and Zn were 125.4 ppm and 29.8 ppm, respectively. There was no significant effect on palatability of feed that was evident from similar DM intake in both the groups. Intake of individual nutrients also did not differ significantly among the groups. There was no effect of moringa leaves inclusion on the nutrient utilization. However significantly higher N retention was observed in Moringa supplemented group compared to commercial concentrate supplemented group. Thus, Moringa supplementation was helpful in reducing the quantity of commercial concentrate mixture inclusion in the diet of non-descript goats.

Keywords: Digestible energy, Moringa leaves, Non-descript goat, Nutrient utilization

Chhattisgarh is a state with typical tropical Indian climate because of its proximity to the tropic of cancer. Major population of the state lives in rural areas and are dependent upon agriculture or related occupations. With the small land holdings of small and marginal farmers, the income from agriculture is not sufficient for their living (Bhakar et al. 2007). In this context livestock gains socio economic and cultural importance in the state. The state is rich in livestock populations with maximum cattle population followed by goat populations. In rural Chhattisgarh rural, cattle are more used for draught purpose, obtaining manure and cow dung for fuel than for milk. Conversely, small ruminant like goat due to its good economic prospects becomes the choicest animal for rearing by rural landless/ marginal farmers. Their small size makes it affordable for their shed construction and management as well. Even the ladies and children of the house are capable to manage them when male of the hose are busy with agriculture related activities. Due to high demand of chevon, farmers hardly face any problem in their marketing. But the main problem faced by them in goat rearing is the feeding cost those alone accounts for about 60% of total expenditure in a goat rearing (Kumar 2007). In the above context a very nutritious leafy tree can act as a game changer and minimize the expenditure on feed cost to great extent. *Moringa oleifera* belong to the family Moringaceae, distributed in all regions of Chhattisgarh. It provides year-round source of nutritious food for the family that grow it (Swati et al. 2018).

Now a days government of the state also encouraging landless or marginal farmers to grow drumsticks/ moringa in the house premises to curb undernourishment in the state. Being a perennial tree, once planted Moringa yields leaves and drumsticks for several years. The leaves, pods, and flowers of Moringa can be used as human food, nevertheless the surplus leaves may also be used as a feed alternative for their goats. So, a trial was conducted in which nutritive value of diet was evaluated by replacing concentrate feed with Moringa leaves.

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Moringa (*Moringa Oleifera*) saplings were prepared from Moringa seed by incubating them in polybag by putting soil and cow dung manure. The saplings were transplanted after a about a month of sowing in rows. The ground for planting the sapling was prepared by mixing goat pellets manure of the goat Unit of the College of Veterinary Science and A.H., Anjora, Durg, Chhattisgarh. They reached a height of about 5 ft within 6 months. To facilitate more branching, they were cut regularly at a height of 4 ft. The leaves from the branches were collected and dried in sun and stored for the experiment. Dried Moringa leaves were analyzed for their chemical composition (AOAC, 2005) and trace mineral (Fe, Zn) profile (atomic absorption spectrophotometer).

The trial was conducted in the Goat Unit of the Anjora Veterinary College, Durg. Eight non-descript yearling goats were selected and allotted randomly into 2 groups (group1 and group2) based on their BW (13.48±1.07 kg). Before conducting the trial, the animals' faecal pellets were collected and tested for the parasitic load and the animals were dewormed. Feeding was done as per ICAR (2013). The animals of group1 were offered concentrate and grasses. The DM offered from concentrate in group 1 was 1.5% of BW and in group 2, 50% of concentrate was replaced by Moringa leaves on dry matter basis. Hybrid Napier grass was provided ad libtum. The animals were fed for a duration of 21 days on the above diet. Then they were kept in metabolic cages for 3 days adaptation followed by conduction of metabolic trail for 5 days. Feed was offered in the morning (8.00 AM) and faeces and urine were collected in the next day morning (8.00AM). Urine was collected into a container with 50 ml of 25% sulphuric acid solution to prevent volatilization of N compounds. The feed offered and faecal sample were analysed for their proximate principles (AOAC, 2005) and the urine samples were analysed for N composition (AOAC, 2005). The Group 1 was fed on commercial concentrate mixture whereas in group 2, 50% of the commercial concentrate mixture was replaced with Moringa. The chemical composition and nutrient composition of Moringa leaves and hybrid Napier grass is provided in Table 1. The crude protein content of dried Moringa leaves was 27.52% that is even higher than oil cakes like sesame cake, sunflower cake (Prusty et al. 2013). High CP content (29.4%) was reported by (Gopalakrishnan et al. 2016). However, Rajput et al. (2019) reported comparatively low CP (20.42%) of dried Moringa powder. The CF, EE and minerals were reported 21.5, 6.6 and 11%, respectively. Rajput et al. (2019) reported crude fibre 22.03%, ether extract 12.47% and total minerals 9.53% in dried Moringa leaves. Ajantha et al. (2018) reported following crude protein (26.01 %), crude fibre (7.08 %), ether extract (6.58 %) and total ash (9.41%) in dried Moringa leaves. Meel et al. (2018) reported CP-23.31%, EE-4.7%, CF-9.26% and minerals-9.76% in Moringa leaves. Damor et al. (2017) reported 26.3% CP, 8.8% CF, 5.7% EE and 14.1% total ash in moringa leaves. Table 1

There was no significant difference ($p>0.05$) in the DM intake among the 2 groups (Table 2). The DMI (g) was 451.42±10.95 and 391.82±8.02 in group 1 and group 2, respectively. No significant different ($P>0.05$) in intake of the nutrients were observed among the 2 groups. Better foliage intake capacity of goats has been reported by Salem et al. (2006). On replacing basal diet with tree leaves no effect on nutrient intake was observed in Surti kids (Patel et al. 2018). No significant effect on DM intake in goat was observed when dietary protein was replaced by Moringa leaves mixture (Patra et al. 2002). The DMI per metabolic weight was fell within reported range (Ndemanisho et al. 1998; Kearl et al. 1982) and somewhat lower than ICAR, 2013 values (70 g/kg $W^{0.75}$). *Moringa oleifera* leaf meal when included in the diet of Anglo Nubian goats replacing sesame meal at 50, 75 and 100% levels increased intake of feed and all nutrients. Damor et al. (2017) and Aregheore (2002) observed no significant effect of Moringa leaves supplementation on DMI in growing goats. A significantly higher DMI was observed when Moringa leaves replaced sunflower seed cake at 75 and 100% levels in goat feed (Sarwatt et al. 2002). The inclusion of MLM at 15% of diet replacing sesame meal increased feed intake, enhanced nutrient digestibility and ruminal fermentation, increased milk yield and modified milk fatty acid profile positively in lactating goats (Kholif et al. 2015). Moringa oleifera leaf meal as a protein source in lactating goat's diets: Feed intake, digestibility, ruminal fermentation, milk yield and composition, and its fatty acids profile. Crude fibre content of Moringa leaves was comparable to those of soybean meal, deoiled mustard cake and groundnut cake (NDDB, 2012; Sharma, 2011) may be the factor for its comparable intake to that of concentrate-based feeds. Table 2

The nutrient digestibility was similar among both the groups (Table 3). No adverse effect of Moringa leaves supplementation replacing 50% of concentrate mixture was observed on nutrient digestibility of the diet. When concentrate was replaced by

Table 1 Chemical composition of concentrate feeds and grass (% DM basis)

	Group1 (concentrate)	Group2 (Concentrate+MLM)	Hybrid napier grass	Moringa leaf meal
DM%	90.12	91.5	20	20.05
OM	92.5	90.75	90.5	89
CP	17.5	22.51	7.1	27.52
CF	8.5	8.31	25	8.12
EE	2.4	4.5	4.3	6.6
NFE	64.1	55.43	54.1	46.76

Table 2 Nutrient utilization in goats offered Moringa leaves

Parameter	Group 1	Group 2
Nutrient intake		
Dry matter (g)	451.42±10.95	391.82±8.02
Dry matter (g/kg $W^{0.75}$)	63.78±2.57	56.06±2.42
Organic matter (g)	413.00±10.48	357.35±7.61
Crude protein (g)	49.79±3.80	48.08±3.29
Ether extract (g)	15.85±2.02	19.061.71
Crude fibre (g)	83.75±4.60	75.44±3.41
Nitrogen free extract (g)	263.61±8.38	214.77±5.89
Nutrient digestibility (%)		
DM	67.53	69.91
OM	72.04	73.93
CP	60.63	64.60
CF	52.12	59.56
EE	78.90	82.68
NFE	80.09	80.21
Digestible nutrient (%) and N retention		
Digestible CP (%)	6.67	7.94
DCP (g/kg MBW)	4.30	4.54
Digestible EE (%)	2.78	4.04
Digestible CF(%)	9.70	11.48
Digestible NFE (%)	46.76	43.98
Total digestible nutrient (%)	69.38	72.49
Total N retention (g)	0.75	0.94
N retention (% intake)	9.99	12.64
N retention (% absorption)	16.71	20.45

*DE is calculated from equation 1kg TDN-4.4 Mcal DE

graded levels of Moringa leaves (0, 25%, 50%, 75% and 100%) in diet, except for ADF, no significant difference in digestibility of other nutrients were observed in bengal goats (Sultana, 2015). Feeding of *Moringa oleifera* leaves replacing commercially available concentrate feed at 75% level improved digestibility of dry matter and other nutrients in Sirohi goat kids. Sultana (2015) found higher DM, OM and CP digestibility in goats fed solely on Moringa foliage diet than fed a mixed diet of Moringa and napier grass. Meel et al. (2018) reported improved body weights gain, feed intake and overall health when *Moringa oleifera* leaves replaced concentrate feed.

The TDN of feed was derived from the total digestible individual nutrients (Table 2). DE is calculated from equation 1kg TDN is equivalent to 4.4 Mcal DE. No significant difference was observed in digestible CP, CF, NFE among the groups. A significantly higher DEE% was observed in moringa supplemented group than control ($P<0.01$).There was no significant difference in the total digestible nutrient and digestible energy value among the two groups. The TDN of Moringa based diet was 72.49%, that is optimum for maintenance and growth of goats. Moringa supplementation did not affect the DE value of the diet. The digestible energy (12.76 MJ/kg and 13.33MJ/kg) observed in present study are higher than reported values for growing goats (Sauvant et al. 1991 and Aregheore 2002). The DCP intake was higher than that of maintenance requirement of goats as recommended by ICAR, 2013 (3.32 g/ kg $W^{0.75}$) and Mandal et al. 2005 (3.22g/ kg $W^{0.75}$) for Indian goats. Similar DCP intake (4.32 g/ kg $W^{0.75}$) for goats have been reported by Aregheore (2002) when Moringa leaves replaced 50% of concentrate mixture in growing goat's diet. Fadiyimu (2010) obtained high CP digestibility (84.96%) in goats when fed solely on fresh Moring leaves.

Positive N retention was observed in either group whereas a significantly higher ($p<0.05$) N retention was observed in Moringa leaves supplemented group (Table 4). It indicates the protein in both the diets was adequate to meet the requirement for maintenance and growth of experimental goats. Sultana et al. (2015) observed higher N retention when Moringa leaves when it replaced concentrate mixture at 50% and 75%. There was no significant ($P>0.05$) differences in N retention when expressed on percentage of absorbed N or intake N basis. Higher N retention in sole Moringa leaves diet was observed compared to other fodders like Leucaena and Gliricidia combinations with Moringa leaves (Asaolu et al. 2011). Sultana (2015) and Damor (2017) reported significant increase in average daily body weight gain in goats on supplementing *Moringa oleifera* leaves in their diet. Though CP value of Moringa leaves is comparable to other tree fodders Gliricidia or Leucaena, it has a high content of bypass protein, 47% than others (Becker, 1995) and Moringa leaves are

generally rich in two important amino acids methionine and cysteine (Makkar and Becker, 1996).

Conclusion

Moringa leaves could replace 50% of concentrate mixture on DM without affecting nutrient intake, digestibility of nutrients and body weight gain in non-descript goat breeds of tropical climate.

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