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Optimization studies on mixing of curd and ingredients during *Lassi* (Stirred Curd) manufacturing

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Abstract: Consumption of the fermented milk is on the rise due to its therapeutic benefits to human health. Several processes and technologies evolved to further enrich the fermented foods. In the present study, an Indian fermented milk product *Lassi* (sweet stirred curd) was selected for the experimental trials and optimized for the different process conditions. *Lassi* was prepared by adding sugar and water in curd and mixing them in the prefabricated mixing vessel. Three different impellers were tested in the preparation of *Lassi*. Two independent factors, namely off-bottom clearance (OBC) and impeller speed (IS) were selected to optimize the process conditions to yield a uniform good quality product. The quality of the finished product was assessed by sensorial and rheological attributes. Performance of the impeller was estimated by evaluating the mixing time, mixing index and power consumption. Three levels of OBC (3, 6, 9 cm) and IS (300, 400, 500 rpm) respectively were selected. Experiments were designed as per response surface methodology and accordingly trials were conducted. Constraints were optimized by setting up the goal for each of them. Based upon the solutions provided by the software, the product was prepared keeping the impeller positioned at 4.8 cm OBC and rotational speed of 389 rpm. The prepared product was validated against the experimental values and found no significant difference between theoretical and experimental values. Results indicated that the multivane churn impeller was found to be suitable for the preparation of good

quality *lassi*. Mixing time and power consumption were optimized at 160 ± 17.32 and 34.21 ± 0.70 , respectively. Overall acceptability of the product was 8.16 ± 0.153 on 9-point hedonic scale. It can be concluded that the developed unit and impeller was able to deliver a uniform quality product with minimum mixing time and power consumption.

Keywords: *Lassi*, Mixing index, Mixing time, Multivane churn impeller, Stirred curd

Introduction

Fermented milks have been consumed around the world for thousands of years. In India, the consumption of butter milk and other dairy products have been described in Vedic literatures and are part of Indian diet from centuries. *Lassi* is a refreshing beverage derived from milk curd and is a popular drink in India. Earlier *lassi* production was confined to local vendors. Due to increase in consumer demand it gained commercial significance and is been produced in dairies at large scale. Significant advances have been made towards the industrial production of *lassi* through the application of ultra-high-temperature (UHT) treatment of milk (Aneja et al. 2002).

Lassi is a sweetened fermented dairy product obtained after the growth of selected culture, usually lactic *streptococci*. Generally, heat treated milk is used for curd preparation which is then followed by sweetening with sugar. It is characterised by creamy consistency, sweetish rich aroma and mild to acidic flavour, which makes the product refreshingly palatable. It is refreshing therapeutic beverage consumed as a cold, usually in summer.

Lassi has been studied extensively by various researchers to optimize the level of sweeteners, fat percent, starter cultures and other functional ingredients (Hussain et al. 2015; George et al. 2010; Padghan et al. 2017; Maurya and Aggarwal, 2019). However, very sparse study has been carried out from the engineering perspective in the preparation of *lassi*. The stirring action plays an important role on quality of *lassi* and very few studies have been carried out to check the influence of impellers and stirring speed on quality of stirred curd (Guénard-Lampron et al. 2019; Gilbert et al. 2020; Tribst et al. 2020).

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Sweetened *lassi* is prepared by breaking up the coagulum with the help of agitator driven by power with addition of sugar, water and flavor (George et al. 2010). In order to gain consumer confidence, the product delivered to the market should be of uniform quality which requires a meticulous technical process standardization. Commercial production necessitates optimum processing cost. The processing of milk and milk products encompass various unit operations such as stirring, atomization, homogenization and recombination (Walstra et al. 2006). Various unit operations in dairy processing are devoted to mixing i.e. solid-liquid mixing, liquid-liquid mixing, gas-liquid mixing and solid-solid mixing. Hence, specific and custom designed units for homogenization, blending, kneading, mixing and dispersion are widely employed in the dairy plant for processing the products. During manufacturing of *lassi*, mixing is one of the major unit operation, and therefore optimization of mixing time for reduction of the power consumption is vital. Hence, in order to optimize the mixing parameters at pilot scale, the present study was conducted.

In present study, a mechanical unit was developed to prepare the *lassi* of a uniform quality. Three designs of impellers viz., pitched blade, saw tooth and multivane churn impeller had been tested to evaluate their action on curd and the overall impact on mixing performance. The paper discusses the selection of impeller and effect of various processing conditions on physicochemical and sensorial properties of the *lassi*. The data obtained may be useful in process engineering of the stirred curd products.

Materials and Methods

Preparation of *lassi*

Fresh cow milk was procured from the Experimental Dairy of SRS of ICAR-NDRI, Bengaluru. The milk was standardized to 3.0% fat and 8.5% SNF using the Pearson square method (De, 1980). The standardized milk was heated to 85 °C for 10 min and cooled to 30-37 °C. Mixed starter culture at the rate of 2% was inoculated and mixed well. It was then incubated at 37° ± 1°C for 12 hour and the set curd was transferred to a refrigerator for cooling and stabilize the acid production.

A prefabricated unit having capacity of 5 liters was used for preparation of *lassi*. The unit is double jacketed vessel provided with heating and cooling mechanism. The unit is comprised of a removable process vessel having height of 20 cm and inner diameter of 18 cm to process the product. The setup was designed to accommodate a motor (120 W) (Speed range- 0-2000 rpm) mounted with impeller shaft for stirring. The rate of stirring can be controlled by the speed of the impeller which can be regulated with the help of inbuilt speed regulator connected to the motor. The height of impeller can be adjusted to set the required off-bottom clearance. A measured quantity of curd (2 kg) is taken into the processing vessel and water is added at the rate of 25%

(0.5 kg) to that of curd. Other ingredients like crystalline sugar at the rate of 12.5% (0.25 kg), and color and flavor were also added. The mixing time was set to 5 min and mixing was carried out at preset speed to dissolve the sugar in curd.

Selection of impeller

Three different impeller designs viz., pitched blade, saw tooth and multivane churn impellers as shown in Fig. 1 were selected for the test and were evaluated for *lassi* making. All impellers comply with sanitary standards and were fabricated with SS-316. The pattern of the mixing was studied by mapping the distribution pattern of food color introduced into *lassi* during mixing. A measured quantity food grade color was introduced at the peripheral surface of curd in the vessel and mixing was done with impeller at preset speed. The distribution of the color within the mix were recorded using a video camera and video grabs were analyzed at regular intervals to understand the mixing patterns generated by each impeller. Impeller with the better mixing pattern was identified and selected for further study.

From the temporal profiles plotted in Fig. 1, it can be deduced that the saw tooth disc impeller performed poorly during the mixing of the product; the streak of the colour dye remained unmixed and was clearly visible even after 5 min of processing at 500 rpm. Complete mixing was observed for both the multivane churn impeller and pitched blade impellers after 5 min of processing. However, a close perusal of the videographs clearly showed unmixed zones around the edges of the product at 3 min when processed by pitched blade, while the mixing appeared more uniform for the multivane churn impeller after the lapse of the same time. Thus, this further reaffirmed that multivane churn impeller performed better for mixing effectiveness for the processing of medium viscous products such as *lassi*. The details of the design of multivane churn impeller is shown in Fig. 2.

Determination of mixing index

The quality of mixing of real mixtures would fall between that of totally unmixed (segregated) state and that of a perfectly mixed (random) mixtures (Berk, 2018). The goodness of mixing is quantified in terms of mixing index or index of mixedness as follows (Lacey, 1954):

$$M = \frac{s_0^2 - s^2}{s_0^2 - s_R^2} \quad (1)$$

Where,

S^2 - variance in the real mixture,

S_0^2 - the variance in totally unmixed system,

S_R^2 - variance in completely randomized system.

S_R^2 is determined using Equation (2) as:

$$s_0^2 = q(1 - q) = qp$$

$$S_R^2 = \frac{q-p^2}{N} \quad (2)$$

p and q are mass fraction of two components, N is the total number of the readings from a sample. Berk (2018) stated that for large samples sizes, the variance S_R^2 approaches zero and equation (1) is simplifies as:

$$M = 1 - \frac{s^2}{s_{ii}^2} \quad (3)$$

Where S^2 is known as variance.

The mixing index M lies between 0 (totally segregated) and 1 (totally randomized). It would be logical to assume that complete mixedness, $M=1$, is approached asymptotically as mixing is continued and to seek a quantitative relationship between mixing time and mixing quality (Kuakpetoon et al. 2001).

In the present study, the mix was assumed as binary phase with suspended solids and liquid phase for evaluating the mixing index. Representative samples from different locations of the vessel were drawn while the mixing was under process. The product was analyzed for total solids content by standard gravimetric method at different instances of time during the period of mixing. The data was analyzed to evaluate the variance in the mix, during the mixing process and substituted in Equation (3) to compute the mixing index.

Determination of mixing time

The mixing time is defined as the time taken for the uniformity 'U' to reach the equilibrium concentration (Oshinowo et al. 2000). The mixing effectiveness was determined using the following equation with some modifications:

$$U_{(t)} = 1 - \frac{C_{\infty} - C_{(t)}}{C_{\infty}} \quad (4)$$

In this expression, C_{∞} is the equilibrium or the final concentration and $C_{(t)}$ is the concentration at a point at some instance of mixing time. The mixing time was read from the plot of Mixing effectiveness vs Time, as the time corresponding to the point when the mixing effectiveness reached a steady state.

Determination of power consumption

The power consumption was determined using a digital wattmeter (Model: PM03, Make HTC). The input of motor was connected to the socket of digital wattmeter which was connected to main power supply. The power consumed during the processing of

the product in the developed unit was monitored inline by recording the data from the wattmeter.

Chemical analysis

Total solid content of *lassi* was measured as per the method (IS: SP: 18, part XI, 1981), fat content of milk was determined using Gerber method. The pH of curd and *lassi* was measured using digital pH meter.

Sensory evaluation

The prepared *lassi* was subjected to sensory evaluation by trained sensory panelist who were well acquainted with the product. The 9-point Hedonic scale was used to adjudge the sensory quality of *lassi* for its sensory attributes in terms of color and appearance, mouthfeel and overall acceptability.

Apparent viscosity of *lassi*

The apparent viscosity of *lassi* sample was determined using a rotational viscometer i.e. Brookfield Viscometer (Model: RV DV2T, TC500) at spindle (RV-02) speed of 200 rpm. The viscosity measurements were carried out at 15 °C. The spindle was lowered into the sample to a depth indicated by the notch on the spindle. The spindle depth was kept constant throughout the measurements. The samples for viscosity measurement were taken in 500 ml beakers and the viscosity value was recorded every thirty seconds for a period of 5 min.

Experimental design and statistical analyses

Optimization study was carried out with multivane churn impeller (Fig. 2) during the preparation of *lassi*. Based on preliminary investigation, two process factors (independent variables) were identified for the study i.e. i) impeller speed (IS) and ii) off bottom clearance (OBC). OBC is the distance of impeller tip from the bottom of the vessel. The total height of cylindrical vessel was 20 cm. The product was filled up to 12 cm level in the vessel. Based on the preliminary study, three different levels of impeller speeds (300, 400 and 500 rpm) and OBC's (3, 6, 9 cm) were selected to find out the optimized combination for the preparation of the product. Four dependent variables viz., mixing time, mixing index, power consumption and overall acceptability were selected to optimize the processing parameters. Experimental design was setup according to face centered central composite design (FCCCD) with five replicates at the center point to develop predictive models to study the effect of independent variables on various dependent variables.

The combination of process variables, for the 13 runs of experiments, were conducted as per the selected design in terms of actual values of the independent variables as tabulated in Table 1. Replications of experiments in the center of design are applied to estimate the pure error that could occur due to

systematic errors during experimentation (Qiu et al. 2010). The experimental design as per Table 1 was conducted and the responses for each run were recorded in terms of mixing index, mixing time, power consumption and overall acceptability. The main and interaction effects of independent variables on the different responses were assessed using response surface methodology (RSM) by Design-Expert v.10.0 software (Stat Ease Inc., Minneapolis). The optimal combinations of process parameters (impeller speed and OBC) were determined using the numerical optimization by setting suitable constraints to the model.

Validation of the optimized process parameters

The optimization of the process parameters was done on the basis of setting the goals for each response variable. During optimization, the mixing index and overall acceptability was set to maximize while mixing time and power consumption was set to minimize. Based upon the solutions generated by the software, the solution with highest desirability (>0.8) was selected. The process conditions optimized in the study was validated against experimental values obtained by preparing the product under the recommended process conditions and recording the values of all the responses, i.e. mixing time, mixing index, power consumption and sensory scores in terms of overall acceptability. The obtained values were compared with the predicted value (generated by the software) and statistical difference between the experimental and predicted values were tested using the Students' t test ($\alpha = 0.05$).

Results and Discussion

Effect of OBC and IS on mixing index of *lassi*

The mixing index provides the idea of mixedness of a product processed with a given set of parameters. It is broadly described as the ratio of actual mixedness obtained to the mixedness that was expected (Berk, 2018). The value zero (0) indicates the totally segregated condition while value of one (1) is representative of total randomness of mixture or an ideally mixed state. Representative samples were drawn at regular intervals and total solid content was determined during the processing of *lassi* in the universal disperser assembled with the multivane churn impeller. The mixing index was calculated according to Equation (1) and the results obtained are summarised in Table 1.

From the data presented in Table 1, it can be concluded that there was no significant difference in the mixing index, irrespective of the speed of rotation of the impeller when it was mixed for the span of 5 minutes. The process exhibited a mixing index of nearly 1. Further, mixing index was used to calculate the optimum mixing time of *lassi*.

Effect of OBC and IS on mixing time of *lassi*

Mixing time was determined as the time at which certain degree of uniformity was achieved in the vessel. Mixing time is an important operational parameter and lower time is associated with better mixing performance (Zhang et al. 2012). The mixing time recorded during the processing of *lassi* in the developed unit at different values of OBC and IS of impeller is shown in Table 1. The response surface graphs of mixing time influence by OBC and IS are represented in Fig. 3.

From the investigation, it was found that both the factors, i.e. OBC and IS significantly influence the mixing time; the effect of IS was more pronounced than the effect of OBC. At 3 cm and 6 cm of OBC, the effect was observed only at the lower rpm (i.e., at 400 and 500 rpm). The impeller speed negated the influence of OBC and the process recorded near identical mixing at times of 180 and 120 s, respectively. Higher mixing times were recorded when the impeller was placed at an OBC of 9 cm for all impeller speed. The effect of speed of impeller was inversely proportional to the mixing time; as the impeller speed increased, the mixing time was found to decrease. Kasat & Pandit (2004) reported similar observation.

Effect of OBC and IS on power consumption of *lassi*

Power consumption, along with the mixing time, is an important criterion to evaluate the performance of a mixing equipment. In food mixing applications, it is always endeavoured to minimize power consumption for the given application. The power consumed during the processing of *lassi* in the developed unit is presented in Fig. 3. It was observed that both OBC and impeller speed significantly influenced the power consumption. The impeller speed was the dominant factor and power consumption recorded in the range of 33 - 36 W. The power consumption is directly proportional to the speed (rpm) of the impeller.

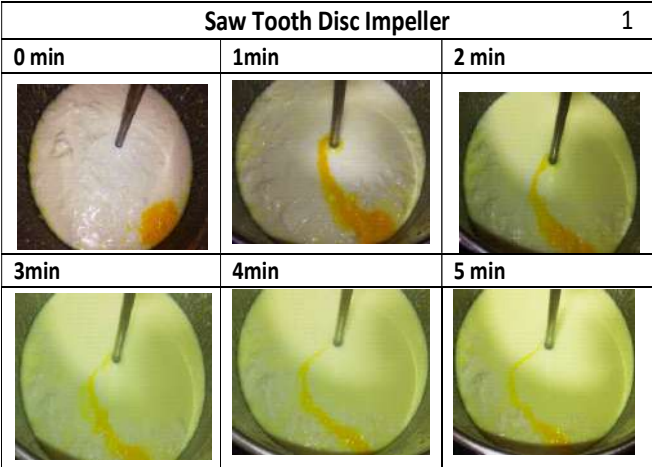
Effect of OBC and IS on sensory quality of *lassi*

The sensory quality of *lassi* was adjudged by 9-point hedonic scale with quality attributes like color and appearance, mouthfeel, flavor and overall acceptability. The serving temperature of *lassi* was around 7 °C.

Effect of OBC and IS on color and appearance

Lassi prepared at various off-bottom clearance and impeller speed showed significant effect on color and appearance as perceived by the sensory analysts. Stirring speed significantly affect the mixing quality. At lower speed, the curd was not broken evenly while at higher speed curd got evenly dispersed in the mixture. *Lassi* prepared at 300 rpm and 9 cm OBC scored highest CA value (7.92) while *lassi* prepared at 500 rpm and 9 cm OBC showed lowest CA value (7.58).

Effect of OBC and IS on mouthfeel



Good quality *lassi* should not have curd particles or any flakes. A good mouthfeel and acceptable consistency represent good body and texture. From the sensory data obtained in this study, it was observed that the *lassi* prepared in the developed unit fitted with the impeller placed at an OBC of 6 cm scored better (8.19) than the other samples (OBC 3cm and 9 cm). Moreover, it was observed that the body and texture of *lassi* prepared at the OBC of 9 cm was distorted (i.e. with unusual whey separation). The samples processed at OBC of 3 cm and 6 cm were intact even when stored at refrigerated condition for 3 days.

The stirring speed had significant effect on body and texture of the *lassi* prepared at different level of off-bottom clearance. Everard et al. (2008) discussed the effect of stirring speed on the quality of cheese curd. Among 3 levels of stirring speed, *lassi* prepared at 400 rpm scored high followed by 300 rpm and 500 rpm, irrespective of the off-bottom clearance. The maximum score of 8.19 was obtained when processed with an impeller positioned at an OBC of 6 cm and impeller speed of 400 rpm. When the *lassi* was stirred at 300 rpm, a thicker consistency was perceived while when stirred at 500 rpm, the product reported a thin consistency which are not liked by many judges. *Lassi* prepared at 400 rpm was neither too thick nor thin and in general, was preferred by the judges.

Effect of OBC and IS on overall acceptability of *lassi*

The overall acceptability scores recorded for the *lassi* sample processed at different off bottom clearance and impeller speed is presented in Table 1 and response surface plot is depicted in Fig. 3. Both parameters were observed to have significant effect on overall acceptability of *lassi*. The overall acceptability corresponded with the trend reported for the mouthfeel scores, samples prepared at 400 rpm and OBC of 6 cm was more acceptable than the other samples of *lassi*.

Effect of OBC and IS on viscosity of *lassi*

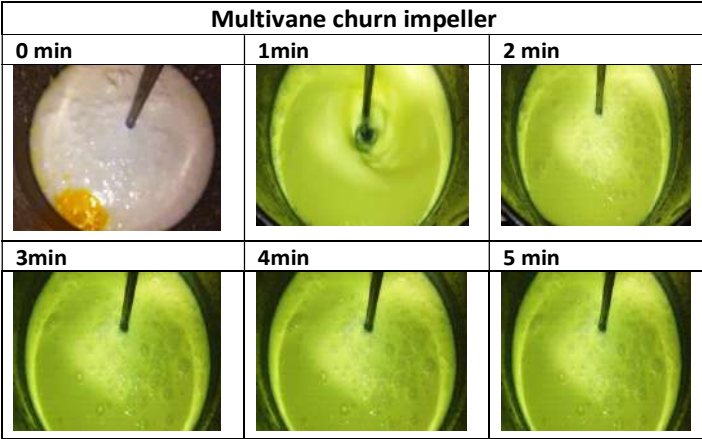
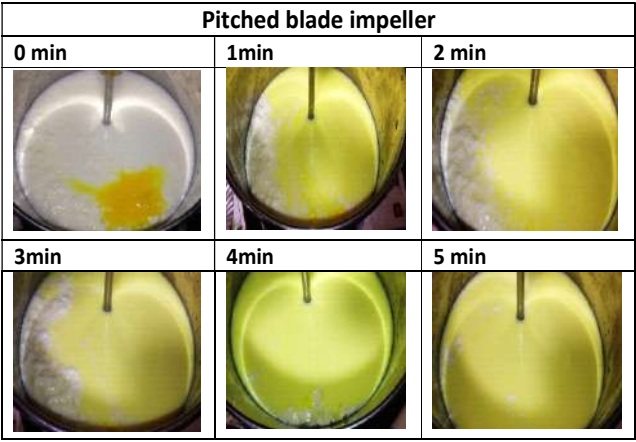


Fig 1 Mixing pattern created by the impellers a) saw tooth impeller b) pitched blade impeller c) multivane churn impeller

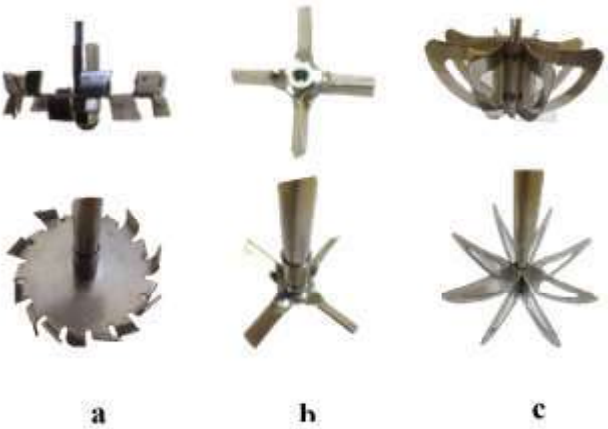


Fig. 2 Top and side view of a) Saw Tooth Impeller, b) Pitched Blade Impeller c) Multivane Churn Impeller

The viscosity of *lassi* was measured using Brookfield viscometer at a spindle speed of 200 rpm and temperature of 15 °C to elucidate the effect of various process conditions. From the analysis, it was found that both OBC and IS were deduced to exert significant

Table 1: Effect of processing parameters on various responses during preparation of *lassi*

Std Order	Factor 1 A: OBC(cm)	Factor 2 B: IS(rpm)	Mixing Time,(s)	Mixing Index	Power consumption(W)	Overall Acceptability
1	3	300	240	0.99	33.65	7.74
2	9	300	300	0.99	33.10	7.58
3	3	500	120	0.99	35.00	7.87
4	9	500	240	0.99	36.18	7.29
5	3	400	180	0.99	34.64	8.00
6	9	400	270	0.99	34.15	7.68
7	6	300	210	0.99	33.00	8.07
8	6	500	120	0.99	35.80	7.98
9	6	400	180	0.99	34.56	8.13
10	6	400	180	0.99	34.25	8.21
11	6	400	180	0.99	37.15	8.11
12	6	400	180	0.99	34.25	8.06
13	6	400	150	0.99	34.12	8.16

Table 2: Regression coefficients and ANOVA of quadratic model of MT, MI, PC and OA for different levels of OBC (A) and IS (B)

Factor	Mixing Time (MT)	Mixing Index (MI)	Power Consumption(PC)	Overall Acceptability(OA)
Intercept	172.75	0.99	34.60	8.14
A	45*	-0.0010 ^{NS}	0.023 ^{NS}	-0.176*
B	-45*	0.0003 ^{NS}	1.205**	-0.041 ^{NS}
AB	15**	0.00037 ^{NS}	0.4325 ^{NS}	-0.105*
A ²	55.34*	-0.000329 ^{NS}	-0.2037 ^{NS}	-0.347*
B ²	-4.65 ^{NS}	0.00087 ^{NS}	-0.1987 ^{NS}	-0.162*
R ²	0.97	0.13	0.57	0.97
Adj R ²	0.96	-0.48	0.27	0.95
Adq. Pre.	25.16	1.480	4.76	21.94
Model F-value	62.31*	0.22 ^{NS}	1.92 ^{NS}	54.80*
Lack of Fit	NS	NS	NS	NS

*Significant at $p < .01$, **Significant at $p < .05$, NS=Nonsignificant

Table 3: Comparison of predicted and observed values of responses to validate the optimized results

Attributes	Predicted value	Observed value	Calculated t ($\alpha=0.05$) value
Mixing Time, s	168	160±17.32	0.999 ^{NS}
Power Consumption, W	34.47	34.21±0.701	0.3716 ^{NS}
Overall Acceptability	8.16	8.16±0.153	0.2931

NS: Non-Significant, (Mean±SD, n=3)

influence on the viscosity of the product. The midpoint combination of 6 cm of OBC and 400 rpm was found to result in *lassi* samples of lowest viscosity, while the combination of the extreme points of the parameters resulted in more viscous product. This was in contrast to the subjective perception recorded for the product, where the judges remarked that higher rpm resulted in thinner *lassi*. Changes in viscosity of *lassi* processed at different OBC and IS are represented in Fig. 4.

Impeller speed or stirring speed also had a significant effect on viscosity of *lassi*. Within the same OBC, it was observed that as

the impeller speed increased, the product viscosity decreased. This may be attributed to the greater shearing action on curd leading to decrease in the viscosity. Lee and Lucey (2010) highlighted the effect of shearing on gel breakdown in fermented products such as yoghurt and its effect on the product rheology.

Regression analysis and model fitting

The selected responses i.e. mixing index, mixing time, power consumption and overall acceptability was modelled using quadratic models. The regression coefficients, p values and model fit statistics of the fitted quadratic models for the four responses

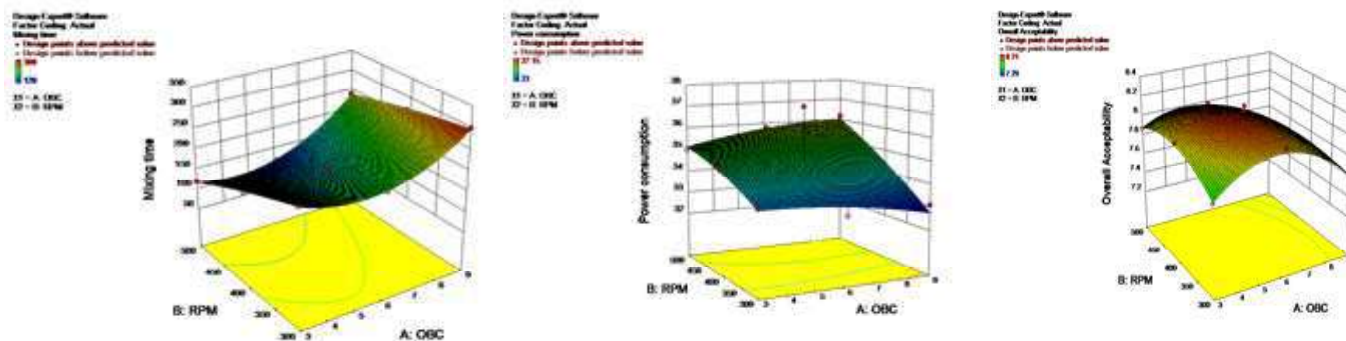


Fig. 3 Response surface graph relating to a) mixing time, b) power consumption and c) overall acceptability as influenced by OBC and IS

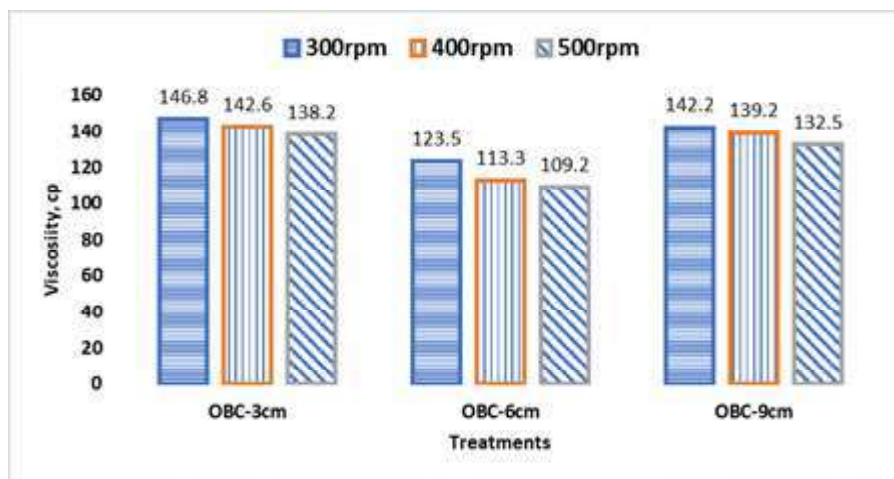


Fig. 4 Changes in viscosity of *lassi* processed at different OBC and IS

are presented in Table 2. It can be seen that the R^2 value for the responses mixing time, power consumption and overall acceptability were indicating a good fit to the model (Adepoju, 2014) while in case of mixing index, it was indicating no significant effect.

Optimization of process parameters for *lassi*

Numerical optimization was done to obtain the optimal conditions of the process parameters, namely off-bottom clearance and impeller speed for *lassi* preparation using Design Expert V. 10.0. The criteria for optimization was set to maximize the sensory score while minimizing the mixing time and power consumption. Though mixing index was not much influenced by the processing parameter, but it was used in the optimization process and set to maximize.

Parallel, constraints were set for the remaining parameters to remain within the range recorded for the experimental values. The solution with the most desirability factor (0.80) was chosen as the optimal point. Processing conditions of off-bottom clearance of 4.80 cm in combination with an impeller speed of 389

rpm was identified as the most optimal process conditions to prepare the *lassi* with multivane churn impeller.

Validation of the optimized formulation

The process conditions optimized in the study was validated against experimental values obtained by preparing the *lassi* under the recommended process conditions (OBC - 4.80cm, Speed - 389 rpm) and recording the values of all the responses, i.e. mixing time, mixing index, power consumption and sensory scores for overall acceptability.

The obtained values were compared with the predicted value and statistical difference between the experimental and predicted values were tested using the Students' t-test ($\alpha = 0.05$). The results of validation are presented in Table 3, no significant difference was observed between the experimental and predicted values, confirming the adequacy of the developed model.

Conclusions

Lassi is widely consumed fermented product and obtaining the product of uniform quality with optimized consistency at low processing cost is in high demand for the industry. Therefore, attempt was made to optimize the physical parameters with standard method of *lassi* preparation. Out of different types of impellers, multivane churn impeller was found to be more appropriate to obtain uniform consistency and quality product. The physical parameters like off-bottom clearance and impeller speed were optimized at 4.80 cm and 389 rpm, respectively to attain good quality product. With the optimized parameters, the power consumption and mixing time were at minimum during *lassi* preparation.

Acknowledgement

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Optimisation of the ingredients for the development of low-calorie *basundi*

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Abstract: In the present investigation the levels of sucralose (55–75 ppm), a mixture of maltodextrin and D-sorbitol (1:1) (4–6 %), and inulin (0.5–1.5 %) were optimised using a general factorial design. The combination resulted in maximum desirability of 0.851 with formulation *viz.*, sucralose (65 ppm), a mixture of maltodextrin and D-sorbitol (5 %), and inulin (1.5 %) was selected as an optimised solution based on the sensorial, rheological, and colour attributes of the product. The scores of quality attributes varied significantly when the level optimised factor changed to interact with the remaining two factors. An increase in flavour score was observed with an increase in inulin level and a mix of bulking agents while a decrease in varying sucralose from 55 to 65 ppm. The decrease was due to an undesirable medicinal flavour perceived in the product. The interaction among the factors gave the unique value for which no common trend was observed. The sensory scores *viz.*, flavour, colour and appearance, consistency, and overall acceptability were 7.70, 7.69, 7.54, 7.64, respectively, and the colour attributes *viz.*, L*, a*, b* value, whiteness, and brownness index were 78.21, 1.67, 17.79, 71.79, and 29.85, respectively. The calorie content in optimized *basundi* was reduced to 130.71 C/100 g on comparing with control *basundi* (189.55 C/100 g). The optimised *basundi* contained 65.30±0.01 % moisture, 1.11±0.01 % fat, 8.49±0.32 % protein, 9.22±0.61 %

lactose, 10.90±0.12 % carbohydrates (maltodextrin), 142.12 ppm sucralose, and 1.49±0.18 % ash. The fat content was reduced to 1.11 % with the complete eradication of sucrose. Thus, the total calorie value was reduced to 58.84 C/100 g. All samples had pseudoplastic flow behaviour with consistency coefficient 14.3±1.30 and 32.6±2.10 mPa.s, respectively.

Keywords: General factorial design, Low-calorie *basundi*, Sensory attributes, Rheology

Introduction

Traditional Indian Dairy Products (TIDPs) are highly valued in society due to their social, economic, religious, medicinal, and cultural significance (Rasane et al. 2015). The milk and milk products are so valued that the National Institute of Nutrition (NIN) of India (2011), the Advisory Committee of the United States Department of Agriculture (USDA, 2020), and the Food and Agriculture Organization of the United States (FAO, 2020), including many of the other countries, have recommended it in their dietary guidelines. In 2012, FAO and World Health Organization (WHO) issued worldwide published food-based dietary guidelines (FBDGs), which specifies the consumption of milk products, especially low-fat milk products (Montagenese et al. 2019). Presently, India ranks first among the world's milk-producing nations with a production of 187.7 million tonnes (2018–19) and per capita availability of 394 grams per day (DAHD, 2019). This increase in milk production represents sustained growth in TIDPs to meet the requirement of the growing population. The market for TIDPs is the second-highest after fluid milk, both in value and volume (Patil, 2013), and accounts for 95 % of all the milk-based products consumed (Rasane et al. 2015). Currently, there is a lack of reliable data about the exact quantity of milk used for the production of TIDPs (Sanyal, 2020, March 13)

Basundi is a traditional heat-desiccated thickened milk product, having white to light caramel colour, creamy consistency with soft textured flakes that are uniformly suspended throughout the product matrix. It has a sweetish caramel aroma and is consumed directly as a dessert. It contains all the solids of milk in an appropriate concentration in addition to sugar, dry fruits,

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and nuts. It is most prevalent in the west and south India and relished due to its rich, caramel, pleasant and nutty flavour, and thick consistency. The traditional product contains fat (12–15 %), protein (7–9 %), lactose (9–11 %), ash (1.3–1.5 %), and sugar (20–22 %). It may contain food additives like nuts, almonds, and pistachio (Gaikwad et al., 2015).

Traditional *basundi* contain a high amount of fat and sugar (Rao and Pagote, 2018). This high fat and sugar induce many types of health problems like cardiovascular diseases, hypertension, obesity (DiNicolantonio et al. 2015), myodegeneration (Suhail et al. 2018), hyperglycemia, and diabetes (Luukkonen et al. 2018). Also, in today's lifestyle, people are not only concerned about their appetite but also health benefits while choosing the eatables (Chatopadhyay, Raychaudhuri, and Chakraborty, 2014). A high fat and sugar content in traditional Indian dairy products has increased the calorie consciousness among the consumers and fear of becoming diabetic and hypertensive as well (Indrasinh et al. 2015; Lordan et al. 2018). WHO (2021) reported that the number of people suffering from diabetes worldwide rose from 108 million (1980) to 422 million (2014), particularly in low and middle-income countries. Among adults over 18 years of age diabetic patients rose from 4.7 % (1980) to 8.5 % (2014), and between the years 2000 and 2016, there was a 5 % increase in premature mortality from diabetes (Factsheet, WHO, 2021 April, 13). For this reason, there has always been a concern of possible cut back in the consumption of high fat and sugar-containing milk-based products. USDA (2020) limited the calories consumed from added sugars and saturated fats to less than 10 % of total calories consumed per day. Attempts have been made to reduce the calorie content of dairy products *e.g.*, *peda* (Indrasinh et al. 2015), low-calorie milk cake (Arvind et al. 2019), and others. To provide the body and texture usually provided by sugar; high-intensity sweeteners and low-caloric bulking agents such as maltodextrin, cellulose, and polyols are recommended in developing the new product. When designing a new product, the optimal proportions of ingredients can be decided by changing one ingredient at a time; however, this process is time-consuming, frequently fails to determine the best proportions and the cumulative effect of all the factors involved (Rathod and Khamrui, 2015). A randomised general factorial design (GFD) is appropriate for analysing the effects of the parameters, understanding the interactions of the parameters, and also optimising the desired responses (Sen, 2016). The purpose of this study was to optimise the level of ingredients for the production of low-calorie *basundi* and to determine the interrelationships between instrumental colour parameters, sensory and rheological attributes, and the ingredients sucralose, maltodextrin, sorbitol, and inulin using GFD. This would ensure the wholesomeness of the product with the same, or enhanced quality attributes and reduced fat and sugar content.

Materials and Methods

Raw materials

Raw buffalo milk had fat (7.1–9.3 %), solids-not-fat (SNF) (8.71–10.13 %), and acidity (0.14–0.16 % lactic acid), was procured from the Experimental Dairy plant of ICAR-National Dairy Research Institute (Karnal, Haryana, India). Food grade sucrose ($C_{12}H_{22}O_{11}$) used in the preparation of control *basundi* (sample) was purchased from the Dhampur Sugar Mills Ltd., located in Mansurpur, Muzaffar Nagar, Uttar Pradesh. Sucralose ($C_{12}H_{19}Cl_3O_8$), white to off-white solid powder, 600 times sweeter than sucrose had a pH of 6.30 (10 % solution) at 25 °C. The specific rotation and melting points were +87.29 ° and 130 °C, respectively. Maltodextrin ($C_{86}H_{145}O_{73}$), white to off-white colour hygroscopic powder, having dextrose equivalent (DE) (approximately) 14.33 was used in the formulation. It had a drying loss of 3.34 % when heated at 105 °C for 1 hour, and water solubility of 100 mg/mL.

D-sorbitol ($C_6H_{14}O_6$), used had an appearance of white hygroscopic crystals with water solubility and specific rotation of 1 g/mL and 108.60 °, respectively. Sucralose and sorbitol were purchased from M/s Hi-Media Laboratories Pvt. Ltd. supplied by Nu-Scientific Biotechnologies, Karnal. Long-chain inulin (Orafti® HPX) having a degree of polymerisation (DP) more than 23 and a sweetness level (0 %), suitable for fat replacement and high-temperature processing (above 105 °C) was procured from SFA Food and Pharma Ingredients Pvt. Ltd., Wagle Industrial Estate, Thane (W), Maharashtra. All the ingredients used were of HPLC grade except inulin which was food-grade.

Types of equipment

The centrifugal cream separator was used for separating cream from raw buffalo pre-heated to 60±1 °C. A shallow bottom pan and the milk-scrapper made up of stainless steel (SS 304) was used for the product preparation.

Refractometer

A hand-held refractometer ranged 28–62 ° Brix used for testing the total soluble solids (TSS)/°Brix of the concentrate during the manufacture of control and low-calorie *basundi*. The total solid (TS) of the concentrate was determined from TSS by the equation suggested by Moore (2009) mentioned below.

$$TS \% = 0.9984 (^\circ\text{Brix}) + 2.077$$

Flow curve analysis

Flow curve was determined using rheometer MCR-52, Anton Paar, Germany. The cone and plate (CP-75-1-SN23597) probe having an angle of inclination of 1° was used for measurement by maintaining the distance of 0.149 mm between the tip of the probe

and the base of the test platform. During testing, a hundred points were measured, and each at the interval of 2 s. The shear rate ($\dot{\gamma}$) was set in range 0–100 s⁻¹ and the apparent viscosity (η) was observed at 50 s⁻¹ from the flow curve obtained at 20 °C. The flakes were removed from the sample before testing.

Instrumental colour analysis

A Hunter Lab Colour Flex[®] reflectance meter supplied by (Hunter Associates Laboratory Inc., Reston, Virginia, U.S.A.) used for the colour determination by employing reflectance spectroscopy technique. The results were presented in tri-stimulus (International Commission on Illumination, CIE) L*, a*, b* values, at a standard illumination of D65 (6500 K, daylight) and 10 ° view angle, as suggested by CIE (1971). The L* value signifies the lightness index of the product which runs top to bottom and has values between 100 and 0. The a* value in CIE L* a* b* space runs from left to right. The left side has a negative value that indicates the green colour and the right side has a positive indicate red colour. The b* value in the same space indicates the colour between blue and yellow. It runs from negative to a positive value, which indicates the blue and yellow colour, respectively. The whiteness index (WI) and brownness index (BI) were calculated using equations 1 and 2 (Kadam et al. 2013).

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

equation (1)

$$BI = \frac{|100(x_1 - 0.31)|}{0.17}$$

equation (2)

$$x_1 = \frac{(a^* + 1.75 L^*)}{(5.645 L^* + a^* - 3.012 b^*)}$$

Preparation of control sample

The control sample was prepared according to Patel and Upadhyay (2003) with a slight adjustment related to the equipment used. The raw buffalo milk (4–7 °C) was first pre-heated to 45±1 °C and then filtered using a muslin cloth to remove extraneous particles. After filtration, the milk was heated to 60±1 °C for cream separation and then standardized to 5 % fat and 9 % SNF (w/w, wet basis). The product was prepared in a shallow bottom pan over a gentle fire with an addition of 5 % sugar after concentrating 2 times initial TMS at 90±1 °C to give the final concentration of 2.5 times (w/w, wet basis) initial total milk solids (TMS). The product *basundi* was collected in stainless steel (SS 304) tray

and was subsequently packed in hot condition (90±1 °C) in polypropylene (PP) containers sealed with aluminum lids. The containers were cooled and stored under refrigeration at 5±1 °C

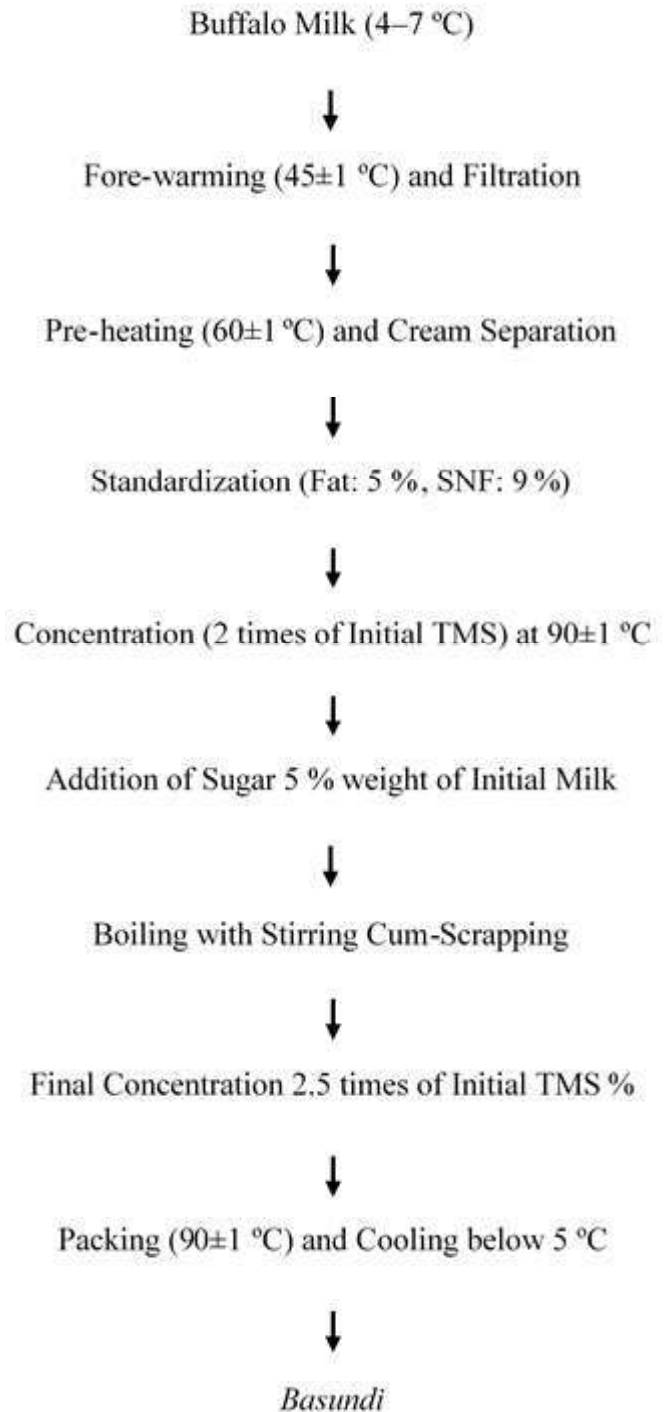


Fig. 1 Process flow chart for the manufacture of control *basundi*

for further study. The product prepared in a batch of 2.5 kg a Milk standardised to 0.5 % fat and 9.0 % SNF was taken in a shallow bottom pan and boiled with continuous string cum shown in Figure 1. scrapping till concentration reached about 20 % TMS. After

Preparation of low-calorie *basundi*

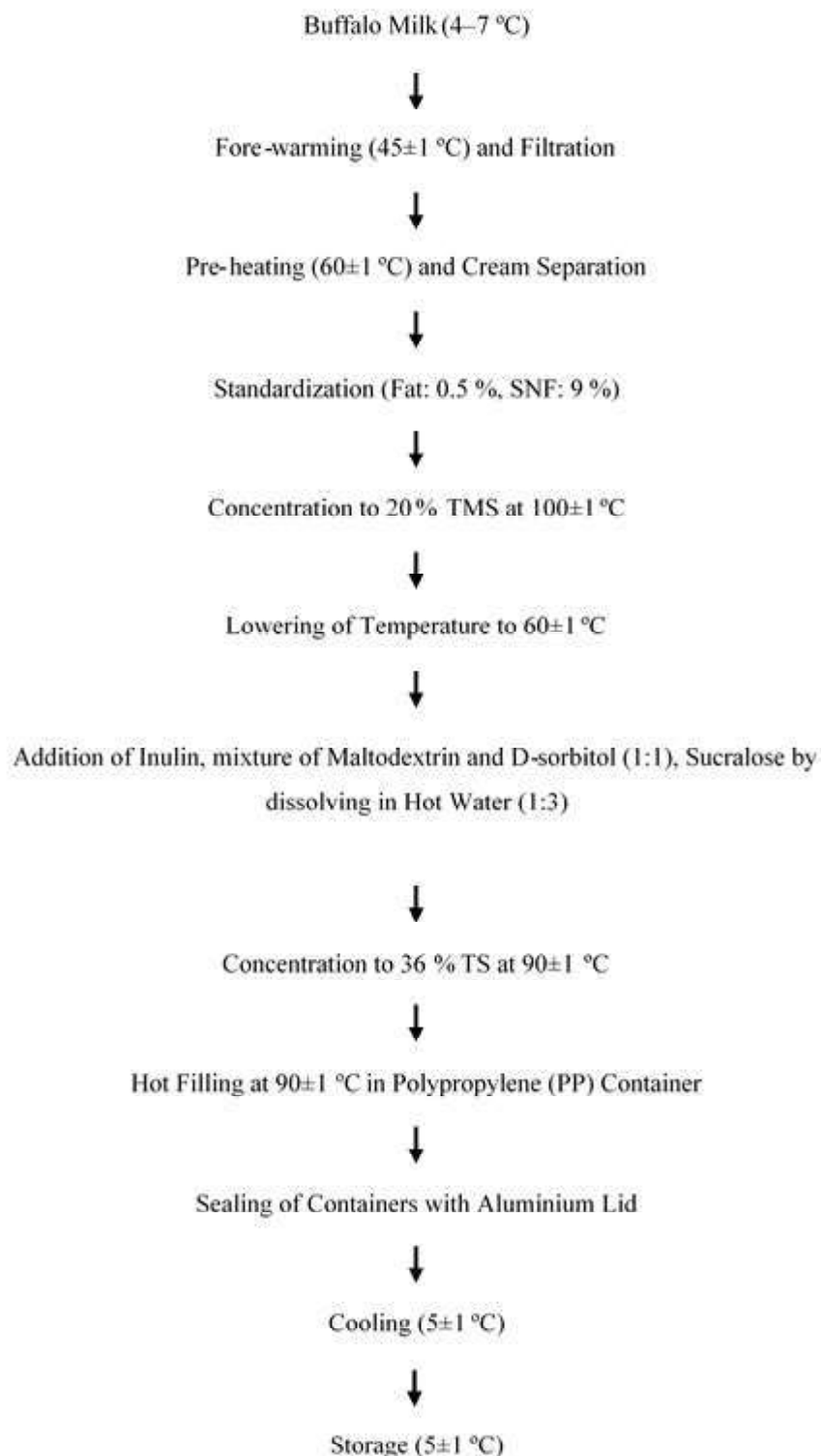


Fig. 2 Process flow chart for the manufacture of low-calorie *basundi*

concentrating to 20 %, the bulking agent's viz., a mixture of maltodextrin and D-sorbitol (1:1) was added in the form of a slurry prepared in water. The sucralose and inulin were also incorporated in the concentrate as per the run described by GFD. The content was further concentrated to 36 % (w/w, wet basis) TS and then packed in polypropylene (PP) containers sealed with aluminum lids. For further analysis, the containers were refrigerated and stored at 5 ± 1 °C. The low-calorie *basundi* was prepared in a batch of 2.5 kg. The detailed flow diagram for the manufacture of low-calorie *basundi* is shown in Figure 2.

Sensory analysis

The sensory quality of the *basundi* was analysed using the 9-point hedonic scale (Drake, 2009) by a semi-trained panellist selected from the faculty of Dairy Technology Division of NDRI, Karnal. The panellist of seven semi-trained members judged the sample based on colour, flavour, consistency, and overall acceptability. The samples were presented with random three-digit codes in closed PP containers.

Design of experiment

The optimisation of independent variables (factors) was performed using GFD, also called a multilevel categoric design (MCD). The design consisted of three levels of each of three independent variables with a total twenty-seven number of combinations which were conducted in replications (n) of three; thus, forming eighty-one experiments. The factors were selected as a true categorical factor and were sucralose (55, 65, and 75 ppm), a mixture of maltodextrin and D-sorbitol (4, 5, and 6 %) in an equal ratio (1:1) called bulking agents, and inulin (0.5, 1.0 and 1.5 %) with their levels. The experiments were conducted by incorporating the above ingredients for the manufacture of *basundi* following the methodology given by Patel and Upadhyay (2003). The effect of change in levels of independent variables was measured in terms of sensory attributes and apparent viscosity (i) called responses. The quality indicating response (R) is a second-order polynomial function of the experimental independent variables, i.e., $Y_n = f(A, B, C)$, and is shown in equation (3). The sensory attributes explained are flavour (R_1), colour and appearance (R_2), consistency (R_3), overall acceptability (R_4), apparent viscosity (R_5) measured at a 50 1/s shear rate, and the L^* (R_6), a^* (R_7), b^* (R_8) colour attributes. The general multiple regression equation is shown below.

$$R_n = \beta_0 + \beta_1 A + \beta_2 A^2 + \beta_3 B + \beta_4 B^2 + \beta_5 C + \beta_6 AC^2 + \beta_7 AB + \beta_8 A^2 B + \beta_9 AB^2 + \beta_{10} A^2 B^2 + \beta_{11} AC + \beta_{12} A^2 C + \beta_{13} AC^2 + \beta_{14} A^2 C^2 + \beta_{15} BC + \beta_{16} B^2 C + \beta_{17} BC^2 + \beta_{18} B^2 C^2 + \beta_{19} ABC - \beta_{20} A^2 BC + \beta_{21} AB^2 C - \beta_{22} A^2 B^2 C - \beta_{23} ABC^2 + \beta_{24} A^2 BC^2 + \beta_{25} AB^2 C^2 + \beta_{26} A^2 B^2 C^2$$

————— equation (3)

Where ' β_0 ' denotes the intercept and β_n denotes the regression coefficients of the rest of the interaction (cross point) of the

equation containing the three-independent variable interaction term.

Statistical analysis

The design-expert ver. 12.0.9.0 Stat-Ease Inc., Dulles, Washington, USA. was used for applying GFD and obtaining mathematical models in the form of multiple regression equations (Bingham et al. 2008). The two-way analysis of variance (ANOVA) determined the differences in the mean of the responses and interpreted experimental outcomes and the influence of independent variables on responses (Ostertagová and Ostertag, 2013). The comparison between the means for two groups was made using Student's t-test and for three or more groups Duncan's post-hoc test was applied using SPSS, IBM statistics (version 23) at the 5 % level of significance as described by Snedecor and Cochran (1994).

Results and Discussion

Preliminary study

Initially, the buffalo and the cow milk were used for the experiment. The cow milk was found unfit for *basundi* preparation due to the high perception of salty flavour contributed by chloride content. In 2016, Khedkar, Kalyankar, and Deosarkar reviewed both buffalo and cow milk for salt content and concluded high salt content in cow milk. After the selection of milk, the different types of high-intensity sweeteners were varied to get information about their effects on the product characteristics. The aspartame, sucralose, and sodium saccharin, stable for high-temperature processing were tried in different concentrations, viz., 55, 65, and 75 ppm. The aspartame was rejected due to loss of sweet taste after heat treatment to milk nearly 100 ± 1 °C at pH 6.38. Chattopadhyay, Raychaudhuri, and Chakraborty (2014) have reported the relationship between pH and stability of aspartame is a bell-shaped curve with the maximum stability at pH 4.3. The sodium saccharin was rejected due to its intensely sweet and the off bitter flavour provided by it in the product prepared. The bitter flavour by incorporation of sodium saccharin in milk products during high thermal treatment has been reported by Mahmood and Al-Juboori (2020). The bulking agents viz., maltodextrin, and D-sorbitol as filler were tried in (1:1) ratio at different levels, viz., 4, 5, 6, and 7 %. The inulin and psyllium husk were also tried at different levels, viz., 0.5, 1.0, 1.5, and 2.0 %, to observe the effect on body and texture, and consistency. Based on these observations, sucralose (55, 65, and 75 ppm), a mixture of maltodextrin and sorbitol (4, 5, and 6 %), and inulin (0.5, 1.0, and 1.5 %) were selected as ingredients with levels in the present investigation.

Criteria for the diagnostic check of the model

The mathematical models generated explained the relation between the independent variables and the responses. The models were evaluated for their fitness based on the significance of F-value and p-value. The F-test was performed by utilising an initial model, modifying it subsequently by adding suitable independent variables (terms). The ANOVA test was applied for each type of model, and the highest process order model with significant terms was chosen for the prediction. The quadratic model with three factors interaction (3FI) or process order was found significant ($p < 0.05$) for all the responses. The F-value higher than the F-statistic defines models to be significant. The coefficients of regression (R^2) value define the percentage of the total variation in the response that is accounted for by the independent variable or how well a regression model fits the data (Smith, 2015). According to Henika (1982), the R^2 value of the model more than 0.70 indicates the model is significant. The predicted R^2 value in agreement with adjusted the R^2 value shows more possibility of data to be fitted well (goodness-of-fit) into any other model to explain the effect even when the number of independent variables varies. The high predicted R^2 explains how

well a regression model makes predictions. The R^2 , adjusted R^2 , and predicted R^2 values for multivariate equation all the responses in the present investigation are depicted in Table 3 and the $\hat{\alpha}$ -coefficients in Table 5. The higher adequate precision value than 4.0 denotes that the effect on response was more due to independent variables than the errors. The coefficients with positive signs denote the direct relation of the dependent variable with the process order of independent variables and the negative, indirect relation. The effect of the levels of ingredients on various sensorial, colour and rheological attributes is discussed below.

Sensory attributes

Flavour score

The flavour scores of low-calorie *basundi* ranged between 5.9–7.8, estimated on the 9-point hedonic scale. The minimum and maximum scores were observed at 65 ppm sucralose, 4 % bulking agents, 1.5 % inulin, and 65 ppm sucralose, 5 % bulking agents, 1.5 % inulin, respectively. The scores changed significantly when

Table 1: Sensory scores and apparent viscosity at a shear rate (50 1/s) of low-calorie *basundi* at different combinations of levels of independent variables¹

A	B	C	Flavour	Colour and appearance	Consistency	Overall acceptability	Apparent viscosity (i, Pa.s)
55	4	0.5	7.52±0.009 ^{ijk}	7.38±0.017 ^{efg}	7.55±0.148 ^{gh}	7.54±0.060 ^{ijk}	0.0152±0.000 ^e
55	4	1.0	6.90±0.006 ^{de}	6.98±0.033 ^{bc}	6.29±0.000 ^a	6.80±0.150 ^{bcd}	0.0187±0.0003 ^{de}
55	4	1.5	7.08±0.336 ^{ef}	6.92±0.009 ^{bc}	6.64±0.14 ^{bc}	6.67±0.063 ^{ab}	0.0260±0.000 ^e
55	5	0.5	7.12±0.050 ^{efg}	7.46±0.043 ^{efgh}	7.55±0.024 ^{gh}	7.50±0.202 ^{hijk}	0.0293±0.0003 ^h
55	5	1.0	7.36±0.031 ^{ghij}	7.68±0.040 ^{hi}	7.33±0.083 ^{efg}	7.33±0.053 ^{efghijk}	0.0363±0.0003 ^{lmn}
55	5	1.5	7.30±0.006 ^{ghij}	7.30±0.100 ^{de}	6.94±0.037 ^{cd}	7.30±0.073 ^{efghijk}	0.0473±0.0003 ^o
55	6	0.5	6.77±0.053 ^{cd}	7.01±0.048 ^{bc}	6.46±0.100 ^{ab}	6.72±0.030 ^{abc}	0.0373±0.0003 ^{lmn}
55	6	1.0	7.39±0.012 ^{ghij}	7.77±0.032 ⁱ	7.53±0.078 ^{gh}	7.45±0.023 ^{ghijk}	0.0380±0.000 ⁿ
55	6	1.5	6.79±0.153 ^{cd}	7.33±0.088 ^{def}	7.01±0.052 ^{de}	7.00±0.001 ^{cde}	0.0570±0.000 ^p
65	4	0.5	7.14±0.037 ^{efg}	7.58±0.010 ^{ghi}	7.50±0.003 ^{gh}	7.10±0.200 ^{defg}	0.0113±0.0003 ^a
65	4	1.0	7.58±0.083 ^{jk}	7.46±0.069 ^{efgh}	7.56±0.063 ^{gh}	7.40±0.027 ^{efghijk}	0.0200±0.000 ^e
65	4	1.5	5.93±0.033 ^a	7.57±0.085 ^{fghi}	7.42±0.266 ^{gh}	7.27±0.193 ^{efghij}	0.0313±0.0009 ^{ji}
65	5	0.5	7.18±0.040 ^{fgh}	7.31±0.060 ^{de}	7.30±0.069 ^{efg}	7.07±0.033 ^{def}	0.0147±0.0003 ^c
65	5	1.0	6.62±0.019 ^e	7.56±0.046 ^{fghi}	7.31±0.153 ^{efg}	7.06±0.057 ^{def}	0.0303±0.0003 ^{hi}
65	5	1.5	7.70±0.006 ^k	7.69±0.015 ^{hi}	7.54±0.131 ^{gh}	7.64±0.124 ^k	0.0326±0.0003 ^{jk}
65	6	0.5	7.04±0.015 ^{ef}	7.34±0.013 ^{def}	7.06±0.060 ^{def}	7.32±0.123 ^{efghijk}	0.0157±0.0003 ^c
65	6	1.0	7.26±0.019 ^{fghi}	7.47±0.030 ^{efgh}	7.41±0.037 ^{fgh}	7.27±0.037 ^{efghij}	0.0323±0.0023 ^j
65	6	1.5	7.42±0.017 ^{hij}	7.61±0.018 ^{ghi}	7.25±0.203 ^{defg}	7.30±0.100 ^{efghijk}	0.0356±0.0003 ^l
75	4	0.5	7.47±0.062 ^{ijk}	7.79±0.041 ⁱ	7.61±0.074 ^{gh}	7.27±0.083 ^{efghij}	0.0183±0.0003 ^d
75	4	1.0	6.35±0.006 ^b	7.40±0.060 ^{efg}	7.40±0.190 ^{fgh}	7.23±0.133 ^{efghi}	0.0377±0.0003 ^{mn}
75	4	1.5	7.45±0.027 ^{hijk}	7.61±0.215 ^{ghi}	7.72±0.063 ^h	7.62±0.117 ^{jk}	0.0240±0.000 ^f
75	5	0.5	7.44±0.060 ^{hijk}	7.73±0.02 ⁱ	7.54±0.030 ^{gh}	7.41±0.003 ^{fghijk}	0.0130±0.000 ^b
75	5	1.0	6.59±0.132 ^e	6.88±0.095 ^{7b}	6.25±0.037 ^a	6.43±0.147 ^a	0.0340±0.000 ^k
75	5	1.5	7.05±0.006 ^{ef}	7.23±0.015 ^{de}	7.54±0.063 ^{gh}	7.23±0.133 ^{efghi}	0.0360±0.000 ^{lm}
75	6	0.5	7.41±0.006 ^{hij}	6.40±0.12 ^a	7.52±0.023 ^{gh}	7.33±0.103 ^{efghijk}	0.0143±0.0003 ^{bc}
75	6	1.0	7.36±0.030 ^{ghij}	7.72±0.086 ⁱ	7.29±0.140 ^{efg}	7.15±0.038 ^{efgh}	0.0363±0.0003 ^{lmn}
75	6	1.5	7.17±0.003 ^{fgh}	7.12±0.107 ^{cd}	6.99±0.080 ^{de}	6.79±0.001 ^{bcd}	0.0467±0.0003 ^o

¹The values depict mean±SE; replications (n) = 3; means with different superscripts within the column differ significantly ($p < 0.05$)

the level of anyone factor changed to interact with the remaining two factors as depicted in Table 1. At 0.5 % inulin level, the highest score (7.51) was observed at 55 ppm sucralose, and 4 % bulking agents, while the lowest score (6.77) at 55 ppm sucralose, and 6 % bulking agents. The change in inulin level to 1.0 % resulted in the highest value (7.41) for score at 55 ppm sucralose and 6 % bulking agents, and the lowest value (6.72) was observed at 55 ppm sucralose and 6 % bulking agents. However, at 1.5 % inulin, the highest score (7.41) was observed at 65 ppm sucralose and 5 % bulking agents, and the lowest score (6.72) at 55 ppm sucralose and 6 % bulking agents. Thus, an increase in score was observed with an increase in inulin content. When the bulking agent increased from 4 to 5 % the scores improved relatively, but at 6 % decreased significantly ($p < 0.05$). A decrease in score was also observed on increasing sucralose from 55 to 65 ppm at all levels of bulking agents keeping inulin at 0.5 and 1.0 % but increased when sucralose increased to 75 ppm. This decrease in scores was due to an undesirable medicinal flavour perceived at a higher percentage of the bulking agents during sensory analysis by experts. The high heat treatment given a mixture of bulking agents, inulin, and milk during the manufacturing process might have caused the formation of degraded products. The presence of bulking agents also hindered milk stability as larger flakes were formed in comparison to control *basundi*. Although the inulin provided a positive effect on flavour, yet no common trend was observed. The change in flavour was also reported by Goel (2008) who prepared a similar product using non-conventional sweeteners. The ANOVA and the fit statistics concerning R^2 (0.92), adjusted R^2 (0.88), and predicted R^2 (0.82) are tabulated in Table 3. The R^2 greater than 0.7 indicated model was found highly significant ($p < 0.05$).

Colour and appearance score

The colour and appearance are the sensory attributes of the product, which attract the consumer first. The score for the product prepared at all combinations considering replications varied in the range of 6.41–7.80 as shown in Table 1. At 0.5 % inulin, the scores increased non-linearly with an increase in bulking agents. The minimum score (6.41) was observed at 75 ppm sucralose, 6 % bulking agents while the maximum (7.80) at 75 ppm sucralose, 4 % bulking agents. Maintaining the same inulin level an increase in bulking agents to 5 % caused an increase in scores at all levels of sucralose but when the bulking agent was increased to 6 %, the scores first increased at 65 ppm sucralose then decreased as shown in Table 1. It was due to the different quantities of bulking agents which had changed the pH of the milk, finally affected casein micelles stabilization and formed irreversible small flakes. The decrease in pH might have caused the destabilization of casein with solubilization (the decrease in casein micelle size) of calcium phosphate from the micellar complex and increase in pH accompanied by an increase in casein micelle size which gets enhanced by high temperature. A similar report about the change in the size of casein micelle due to alteration in

pH has been presented by Sinaga, Bansal and Bhandari (2017). The effect got pronounced due to heating and resulted in the formation of bigger sizes flakes which scored low in appearance attribute. The best colour and appearance scores were observed at pH 6.42.

At 1.0 % inulin, the minimum (6.91) and maximum (7.73) scores were observed at the 75 ppm sucralose, 5% bulking agents, and 75 ppm sucralose, 6 % bulking agents, respectively, while at 1.5 % inulin, the minimum (6.91) and maximum (7.73) scores were observed at the 55 ppm sucralose, 4 % bulking agents, and 65 ppm sucralose, 5 % bulking agents, respectively. It was due to variation in the quantity of bulking agents used which overall changed the dextrose quantity and might have accelerated the Maillard browning at different rates; thus, the formation of brown pigment melanoidin. Gaikwad et al. (2015) reported the change in colour and appearance of *basundi* prepared using different ingredients. Indrasinh et al. (2015) also reported the change in the colour of *dietetic peda* when sugar was replaced with artificial sweetener and bulking agents. Furlán (2017) reported an improved sensory and physical properties when used inulin as fat mimetic and non-synthetic sweetener on the physicochemical properties of sugar-free reduced-fat dairy dessert. The ANOVA and fit statistics had shown the model F-value (20.79), R^2 value (0.909), predicted R^2 value (0.795) and adjusted R^2 value (0.865) was significantly ($p < 0.05$) high to accept the model.

Consistency score

The consistency scores ranged between 6.25–7.72. The minimum and maximum scores were observed at 75 ppm sucralose, 5 % bulking agents, 1.0 % inulin, and 75 ppm sucralose, 4 % bulking agents, 1.5 % inulin, respectively. At 0.5 % inulin, the minimum (6.46) and maximum (7.61) scores were observed at the 55 ppm sucralose, 6 % bulking agents, and 75 ppm sucralose, 4 % bulking agents, respectively. At 0.5 % inulin, the scores increased non-linearly with an increase in sucralose content at all levels of bulking agents. But when inulin increased to 1.0 % no specific trend was observed, and the interaction between bulking agents and sucralose lead to a unique value. On further increasing the inulin to 1.5 % and the bulking agents to 4 and 5 %, the scores increased at all levels of sucralose but at 6 % bulking agents, the scores first increased then decreased as depicted in Table 1. Rodríguez-García, Salvador and Hernando (2013) reported that fat and sugar replacement by inulin decreased batter apparent viscosity values. The reason for such variations in consistency scores is the interaction between the major and minor milk components with the added ingredients. The low scores at a low level of bulking agents were due to the high heat treatment provided to attain 36 % TS. The heating might have caused the denaturation of proteins for prolonged processing but at the high level of bulking agents, less heat treatment was required, which caused proper gel formation by proteins, thus increased consistency. A slight variation in quality scores provided by the

Fig. 3 Flow curve between viscosity (η) to shear rate ($\dot{\gamma}$) depicting pseudoplastic behaviour of low-calorie *basundi*

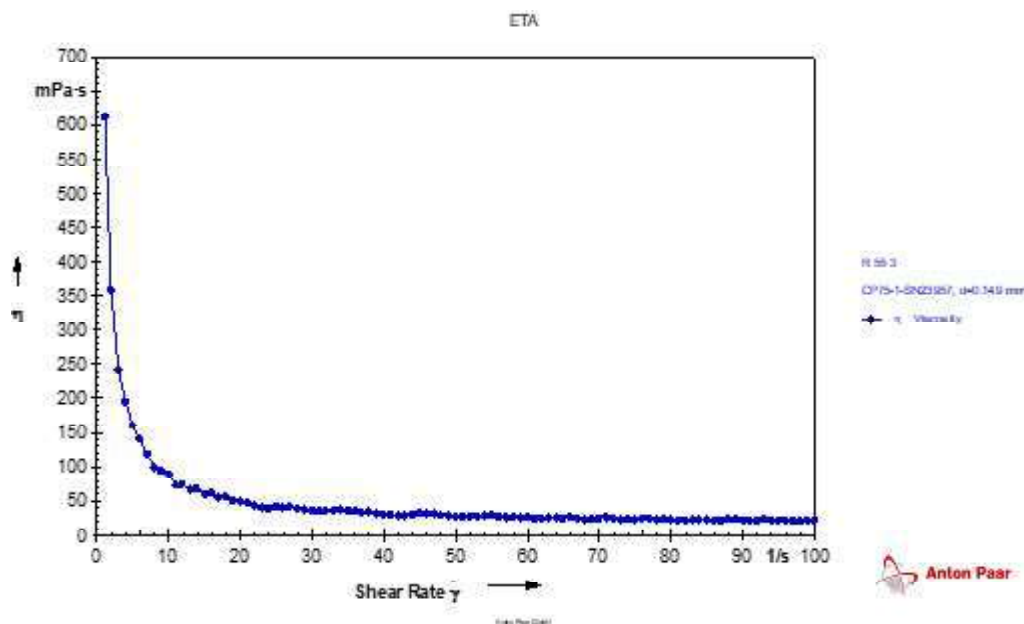


Table 2: Instrumental colour characteristics of low-calorie *basundi* at different combinations of levels of independent variables^s

A	B	C	L*	a*	b*	WI	BI
55	4	0.5	79.62±0.019 ^j	0.94±0.02 ^g	15.75±0.069 ^f	74.24±0.149 ^{efg}	23.51±0.244 ^f
55	4	1.0	77.66±0.021 ^d	1.25±0.069 ^h	16.85±0.035 ^h	71.91±0.091 ^d	22.41±0.117 ⁱ
55	4	1.5	81.07±0.003 ^q	“0.09±0.003 ^c	15.15±0.059 ^d	75.75±0.035 ⁱ	25.15±0.078 ^c
55	5	0.5	80.77±0.009 ^p	0.38±0.026 ^e	15.44±0.009 ^e	75.31±0.024 ^{ij}	20.13±0.096 ^d
55	5	1.0	79.00±0.03 ^g	0.93±0.021 ^g	15.61±0.017 ^{ef}	73.82±0.031 ^e	21.08±0.028 ^f
55	5	1.5	80.34±0.003 ⁿ	0.61±0.028 ^f	15.69±0.023 ^{ef}	74.81±0.022 ^{hi}	22.39±0.014 ^e
55	6	0.5	79.16±0.009 ^h	1.54±0.078 ⁱ	15.77±0.035 ^f	73.98±0.23 ^{ef}	21.81±0.047 ^{gh}
55	6	1.0	81.87±0.048 ^s	0.11±0.037 ^d	15.01±0.103 ^{cd}	76.28±0.204 ^k	23.11±0.201 ^c
55	6	1.5	79.58±0.020 ^{ij}	0.95±0.007 ^g	16.28±0.058 ^g	73.87±0.05 ^e	19.94±0.151 ^h
65	4	0.5	81.49±0.003 ^r	“0.54±0.02 ^b	14.17±0.245 ^b	76.76±0.272 ^k	23.26±0.09 ^b
65	4	1.0	79.51±0.031 ⁱ	0.61±0.043 ^f	16.04±0.072 ^g	73.92±0.131 ^{ef}	18.19±0.327 ^{fg}
65	4	1.5	78.29±0.006 ^f	1.85±0.015 ^{lm}	17.75±0.013 ^j	71.83±0.036 ^d	22.61±0.103 ⁱ
65	5	0.5	82.50±0.024 ^t	“0.96±0.059 ^a	13.37±0.071 ^a	77.88±0.032 ^l	26.95±0.007 ^a
65	5	1.0	76.80±0.038 ^a	2.54±0.029 ^o	18.84±0.292 ^m	70.26±0.074 ^a	16.64±0.208 ⁿ
65	5	1.5	78.21±0.015 ^f	1.67±0.086 ^{jk}	17.79±0.084 ^j	71.79±0.084 ^d	29.85±0.36 ^j
65	6	0.5	80.83±0.043 ^p	0.10±0.022 ^d	15.11±0.116 ^{cd}	75.46±0.039 ^l	26.85±0.075 ^c
65	6	1.0	77.08±0.014 ^b	2.12±0.037 ⁿ	17.82±0.012 ^j	70.83±0.057 ^b	20.35±0.128 ^k
65	6	1.5	77.32±0.006 ^c	1.54±0.017 ⁱ	17.48±0.029 ⁱ	71.22±0.049 ^{bc}	27.8±0.067 ⁱ
75	4	0.5	80.63±0.018 ^o	0.41±0.003 ^e	14.84±0.023 ^c	75.52±0.057 ^j	26.6±0.007 ^c
75	4	1.0	77.04±0.003 ^b	1.76±0.023 ^{kl}	18.46±0.091 ^l	69.88±0.769 ^a	20.24±0.042 ^m
75	4	1.5	80.12±0.111 ^m	0.20±0.003 ^d	15.70±0.012 ^{ef}	74.67±0.093 ^{gh}	28.83±0.515 ^{de}
75	5	0.5	77.72±0.025 ^d	1.96±0.067 ^m	18.42±0.029 ^{kl}	71.03±0.027 ^{bc}	21.49±0.047 ^l
75	5	1.0	78.00±0.025 ^e	1.58±0.088 ^{ij}	18.17±0.112 ^k	71.4±0.085 ^{cd}	28.33±0.066 ^k
75	5	1.5	79.91±0.044 ^l	1.06±0.006 ^g	16.13±0.023 ^g	74.16±0.074 ^{efg}	27.44±0.186 ^{gh}
75	6	0.5	76.98±0.023 ^b	2.18±0.029 ⁿ	19.05±0.057 ^m	69.97±0.029 ^a	23.05±0.039 ⁿ
75	6	1.0	79.76±0.007 ^k	0.40±0.062 ^c	15.66±0.040 ^{ef}	74.46±0.075 ^{gh}	29.96±0.042 ^e
75	6	1.5	79.55±0.006 ^{ij}	1.03±0.041 ^g	16.08±0.036 ^g	73.81±0.067 ^e	21.7±0.057 ^{gh}

^sThe values depict mean±SE; replications (n) = 3; means with different superscripts within the column differ significantly (p<0.05)

semi-trained panel members was observed which might be due to the different regions of the country to which they belong. The ANOVA for the consistency attribute had shown the quadratic

mathematical model with 3FI. The model F-value (14.44), R² value (0.87), adjusted R² (0.81) and adequate precision (13.68) were

significantly ($p < 0.05$) high to accept the model statistically.

Overall acceptability

The overall acceptability score for low-calorie *basundi* prepared at all combinations varied in the range of 6.43–7.64. The minimum (6.43) overall acceptability scores were observed at 75 ppm sucralose, 5 % bulking agents, 1.0 % inulin, while the maximum (7.64) at 65 ppm sucralose, 5 % bulking agents, 1.5 % inulin. The vast variation in overall acceptability scores of the runs was due to the difference in likeness judged by experts. The scores for optimized and control *basundi* are depicted in Table 4. The quadratic mathematical model having 3FI process order was found highly significant ($p < 0.05$) for describing the overall effect (Table 3).

Apparent viscosity

The apparent viscosity (i) for low-calorie *basundi* was in the range of 0.011–0.057 Pa.s determined at a shear rate of 50 s^{-1} . The minimum (0.011) and maximum (0.057) viscosity were observed at 65 ppm sucralose, 4 % bulking agents, 0.5 % inulin, and 55 ppm sucralose, 6 % bulking agents, 1.5 % inulin, respectively. At 0.5 % inulin and 4 % bulking agents when the sucralose level

increased to 65 from 55 ppm the viscosity first decreased and then increased. But at the same inulin level when the bulking agent changed to 5 % a decrease in viscosity was observed. At 1.0 % inulin and 4 % bulking agents, a sharp increase in viscosity was observed at all levels of sucralose while at 5 and 6 % bulking agents the viscosity first decreased at 65 ppm and then increased at 75 ppm sucralose. The continuous increase in viscosity was found at all levels of sucralose when bulking agents increased to 1.5 % inulin, as shown in Table 1. The ANOVA and fit statistics as tabulated in Table 3 had shown the mathematical model was highly significant ($P < 0.05$) to explain the effect on viscosity.

The flow curve had shown shear thinning behaviour for both low-calorie and control *basundi*. A decrease of viscosity with an increase in the shear rate is called shear thinning. The typical flow curve between viscosity to the varying shear rate obtained during experimentation is shown in Figure 3. A similar result had been reported by Parmar et al. (2018) during the study of the rheological behaviour of *basundi*.

Tri-stimulus colour characteristics

L^* value represents the light- reflected by the product at the surface. It increases from the down to the upside of the three-

Table 3: ANOVA results for the dependent variables

		Flavour	Colour and appearance	Consistency	Overall acceptability	L^* value	a^* value	b^* value	Apparent viscosity (i, Pa.s)
F-value	Model	24.44	24.44	20.79	14.44	8.21	2695.83	410.67	559.74
	A: Sucralose	0.82	0.82	21.40	25.64	3.72	2712.52	236.06	741.64
	B: Bulking agents	6.60	6.60	7.30	3.39	1.23	319.37	243.00	1431.40
	C: Inulin	12.23	12.23	4.75	7.13	3.32	4848.69	424.74	3259.88
	AB	14.05	14.05	28.04	14.15	9.80	998.41	83.96	385.49
	AC	22.79	22.79	7.52	12.36	8.74	6172.02	1251.21	237.64
	BC		32.07	45.85	18.16	9.80	2626.13	308.00	126.53
	ABC	32.07	40.06	18.49	15.55	10.44	1893.04	287.14	86.10
	Co-efficient of regression (R^2)	0.92	0.92	0.91	0.87	0.80	1.00	1.00	0.99
	Adjusted (R^2)	0.88	0.88	0.87	0.81	0.70	1.00	0.99	0.98
	Predicted (R^2)	0.82	0.82	0.80	0.72	0.55	1.00	0.99	0.99
	Adequate Precision	21.5	21.54	19.24	13.68	11.59	182.23	81.43	91.68
	Model p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Significance					Significant				

Table 4: Verification of predicted values against actual values of the low-calorie *basundi*

Responses	Predicted values	Actual values [#]	Significance (2-tailed)
Flavour	7.70	7.75±0.29	0.73 ^{ns}
Colour and appearance	7.69	7.65±0.05	0.66 ^{ns}
Consistency	7.54	7.53±0.32	0.96 ^{ns}
Overall acceptability	7.64	7.68±0.15	0.84 ^{ns}

[#] The values depict mean±SE; replications (n) = 3; means are compared within the row using *students t-test* at 5 % level of significance

Table 5: β -coefficient of the dependent variable's multivariate equation

Co-efficient	Flavour	Colour and appearance	Consistency score	Overall acceptability	L* value	a* value	b* value
β_0	7.130	7.380	7.240	7.190	79.290	0.967	16.390
β_1	0.011	-0.066	-0.208	-0.047	0.609	-0.232	-0.658
β_2	-0.028	0.128	0.131	0.077	-0.176	0.025	0.100
β_3	-0.080	0.029	0.058	0.018	0.203	-0.258	-0.307
β_4	0.026	0.045	0.014	0.027	-0.039	0.117	0.221
β_5	0.106	-0.049	0.103	0.058	0.678	-0.302	-0.618
β_6	-0.080	0.055	-0.088	-0.068	-0.765	0.289	0.552
β_7	0.108	-0.250	-0.263	-0.161	-0.651	0.222	0.498
β_8	-0.134	-0.001	0.063	-0.031	0.446	-0.095	-0.192
β_9	0.098	0.120	0.225	0.205	0.180	-0.215	-0.368
β_{10}	0.045	-0.035	-0.002	-0.041	0.094	-0.026	-0.040
β_{11}	H0.106	0.018	0.051	0.049	H0.723	0.518	0.544
β_{12}	-0.085	-0.052	-0.188	"0.164	1.810	-1.160	-1.650
β_{13}	0.158	0.108	0.106	0.118	0.378	-0.259	-0.458
β_{14}	0.139	-0.067	0.145	0.038	-0.553	0.477	0.528
β_{15}	0.222	0.222	0.150	0.034	0.410	-0.140	-0.542
β_{16}	-0.011	0.122	0.107	0.046	0.401	-0.325	-0.246
β_{17}	-0.022	-0.184	-0.127	0.001	-0.656	0.211	0.484
β_{18}	-0.214	-0.109	-0.203	"0.210	-0.551	0.309	0.379
β_{19}	0.129	0.098	0.421	0.397	-0.191	0.164	0.447
β_{20}	0.009	-0.079	-0.061	"0.085	-1.180	0.420	0.995
β_{21}	-0.129	-0.109	0.014	"0.034	0.378	-0.153	0.182
β_{22}	0.002	-0.231	-0.102	"0.129	0.435	-0.259	-0.784
β_{23}	-0.322	-0.091	-0.429	"0.253	-0.747	0.312	0.356
β_{24}	0.662	0.121	0.140	0.169	1.720	-1.000	-1.510
β_{25}	0.235	0.144	0.246	0.116	-0.100	-0.046	-0.447
β_{26}	-0.390	0.161	0.075	0.041	-0.498	0.382	0.715

Table 6: Proximate composition and estimated calorie content of low-calorie and control *basundi*

Composition	Low-calorie <i>basundi</i>		Control <i>basundi</i>	
	Quantity (%) (mean $\pm$ SE)	Calorie content (C/100 g)	Quantity (%) (mean $\pm$ SE)	Calorie content (C/100 g)
Moisture	65.30 $\pm$ 0.013	0	63.42 $\pm$ 0.142	0
Fat	1.11 $\pm$ 0.010	10.11	9.38 $\pm$ 0.492	85.37
Protein	8.49 $\pm$ 0.322	33.11	7.78 $\pm$ 0.428	30.34
Sucrose	—	0	9.04 $\pm$ 0.111	37.06
*Other carbohydrates	10.90 $\pm$ 0.120	36.58	—	0
Lactose	9.22 $\pm$ 0.061	37.80	8.97 $\pm$ 0.032	36.78
Sucralose	0.0142 $\pm$ 0.021	0	—	0
Inulin	3.28 $\pm$ 0.014	13.11	—	0
Ash	1.49 $\pm$ 0.180	0	1.22 $\pm$ 0.121	0
Calorie/100g		130.71		189.55
Total calorie reduction (%)			31.04	

The values depict mean $\pm$ SE; replications (n) = 3; *Other carbohydrates contributed by maltodextrin

dimensional *CIE* chromaticity diagram. The L* value of the product manufactured at all combinations ranged from 76.80–82.50. The minimum and maximum values were observed at 65

ppm sucralose, 5 % bulking agents, 0.5 % inulin, and 65 ppm sucralose, 5 % bulking agents, 1 % inulin, respectively. At 55 and 65 ppm sucralose, the L^* value increased to 5 % bulking agents after that it decreased significantly ($p < 0.05$). This might be due to the change in the amount of ingredients which caused a change in absorbance of light as every material possesses a specific amount of light-absorbing capacity. At 1.5 % inulin, the decrease in L^* value was observed at all levels of sucralose with an increase in the bulking agents as depicted in Table 2, and the fit statistics in Table 3.

The axis for the a^* value extends denoting the green to red colour and the value for the product ranged “0.96–2.59 manufactured at all combinations. The minimum and maximum values were observed at 65 ppm sucralose, 5 % bulking agent, 0.5 % inulin, and 65 ppm sucralose, 5 % bulking agent, 1.0 % inulin, respectively. On varying the ingredients no specific trend was observed. This was due to the change in processing time effected a change in the composition of ingredients to achieve final total solids of 36 %. The interactions and change in the ingredient's composition caused a change in wavelength when the light passed through the product which caused the colour change.

The b^* value axis extends from blue to yellow for the same varied in the range 13.37–19.05. The bluish tinge was observed at 65 ppm sucralose, 5 % bulking agent, and 0.5 % inulin while the yellowish tinge was observed at 75 ppm sucralose, 6 % bulking agent, and 0.5 % inulin. An increase in yellowness was found at 75 ppm sucralose with an increase in bulking agents while at 55 and 65 ppm sucralose the colour intensity first decreased and then increased due to variation in heating time to attain 36 % TS. When the inulin level changed to 1.0 and 1.5 %, the b^* value yellowness again increased with sucralose up to 65 ppm and then decreased at all levels of bulking agents. The trend here and in all attributes strictly denoted the interaction effect (3FI) was highly dominating, and each combination of the level of ingredients had a unique value as depicted in Table 2. Singh et al. (2018) reported an increase in L^* value and change in a^* and b^* value of rice *kheer* with an increase in the total solid concentration of milk. The F-values and fit statistics for CIE L^* , a^* and b^* values of low-calorie *basundi* have shown the model was highly significant (Table 3). The whiteness (WI) and brownness (BI) indexes for the samples are depicted in Table 2.

Optimisation of independent variables

The GFD with three categorical factors and three replications (n) was used for the optimisation of levels of ingredients for the manufacturing of low-calorie *basundi*. The sensory attributes, apparent viscosity at a shear rate of 50 s^{-1} , and colour characteristics CIE L^* , a^* and b^* values were the quality determining responses. The goals assigned were; (a) all three independent variables were in range while among dependent variables all sensory attributes were maximized. (b) The apparent

viscosity and instrumental colour parameters were in range with exception of the b^* value which was maximized. (c) The dependent and independent variables were subjected to the equal importance of 3 stars. The solution with the highest desirability value (0.851) was selected as the optimised solution. The suggested solution had sucralose (65 ppm), a mixture of maltodextrin and D-sorbitol (5 %), inulin (1.5 %), and the responses were flavour (7.70), colour and appearance (7.69), consistency (7.54), overall acceptability (7.64), L^* value (78.207), a^* value (1.673), b^* value (17.793), WI (71.79), BI (29.85) and apparent viscosity (0.033 Pa.s).

Validation of formulation

The verification of formulation for the optimized product was done by comparing the predicted sensory scores with the actual scores of low-calorie *basundi*. Non-significant ($p > 0.05$) differences in the sensory scores were obtained when compared using 2 tailed t-tests as depicted in Table 4. Therefore, the predicted formulation with desirability (0.851) was verified successfully.

Reduction in calorie content

The chemical composition of low-calorie and control *basundi* was estimated, and calorie content was calculated empirically as shown in Table 6. The total calorie reduction was 58.84 C/100 g (31.04 %) as compared to its full-fat counterpart.

Conclusions

The general factorial experimental design approach was successfully applied in the optimisation of the levels of ingredients for the development of low-calorie *basundi*. The variables investigated in this study, sucralose, a mixture of maltodextrin and sorbitol, and inulin significantly influenced product quality when used as single and in combinations. The quadratic mathematical models consisting of three process orders (3FI) were developed for the sensory attributes (flavour, colour and appearance, consistency, overall acceptability), colour attributes (L^* , a^* , and b^* values), and apparent viscosity (i). The optimised solution had sucralose (65 ppm), a mixture of maltodextrin and D-sorbitol (5 %), inulin (1.5 %), flavour (7.70), colour and appearance (7.69), consistency (7.54), overall acceptability (7.64), L^* value (78.207), a^* value (1.673), b^* value (17.793), WI (71.79), BI (29.85), apparent viscosity (0.033, Pa.s) and desirability (0.851). The actual experimental results at optimum conditions presented a good agreement with the predicted value. The fat and total calorie content in low-calorie *basundi* was reduced to 1.11 from 9.38 %, and 130.71 from 189.55 C/100 g, respectively, as compared to its full-fat counterpart leading to a 31.04 % reduction in total calorie content.

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Detection and quantification of anionic detergent in milk using rhodamine 6g dye and toluene solvent

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Abstract: In the present study different proportion of synthetic milk (2, 4, 8, 10 and 15 %) was blended with natural milk and tested for detergent qualitatively as well as quantitatively successfully using Rhodamine 6G dye and Toluene solvent. In positive milk samples, colour of the solvent changed from transparent to pink, and the intensity of colour increased with an increase in detergent concentration. In contrast, colour of solvent layer in control milk sample remained transparent. Quantity of detergent determined by uptake of benzethonium chloride. This new method was sensitive enough to detect as low as 16 mg of detergent per 100 ml of adulterated milk.

Keywords: Detergent, Rhodamine 6G dye, Synthetic milk, Toluene

Introduction

Milk is an essential commodity and essential part of our diet. It is a highly nutritious secretion of mammary gland and primary source of nutrition for the infant. It is the nature's most nearly perfect food and consists of all the required nutrients. Consumption of milk and dairy products is growing in Asia, Latin America, and the Caribbean region (Adesogan and Dahl, 2020).

India is the world's largest milk producer (NDDB, 2018). India is a second largest populated country in the world, and growth rate is 1.2 % every year (WHO 2016) that leads to more demand for milk which may result in adulteration to meet the demand supply and also for personal benefits. (Handford et al. 2015). Milk production is observed throughout the year in India. However, its production decline during summer, combined effect of both scarcity of fodder and heat stress during this season influence metabolism of milking animals leading to decrease milk production. Shortage of milk during festive and summer seasons provides temptation for its adulteration to increase its bulk. Milk adulteration in developing countries like India is a socioeconomic problem. Milk fraud is a serious concern and in recent years it has come under increased scrutiny. Typically, milk adulteration is linked to financial gain, but unhygienic conditions during handling, transportation, processing, storage and marketing also add unintentionally to adulteration process. In Indian dairy sector, large share of marketable milk goes through informal channel where quality is a big concern and sometimes this is common in formal channel also (Adesogan and Dahl, 2020). Quality of Indian milk and milk products is a big concern and it act as an entry barrier to export market (FAO, 2009). Synthetic milk is an excellent imitation of natural milk (Mudgil and Barak, 2013). As the composition of milk is well known, there are increasing foul practices in a different parts of the country for the preparation of synthetic milk using harmful chemicals (Bansal and Bansal 1997; Mudgil and Barak 2013). Fat is important and most expensive component of milk (Parodi 2004). Fat in milk occurs in microscopic globules form an oil-in-water emulsion. To financial gain, unscrupulous suppliers remove this high-value natural fat from milk and replace it with cheap vegetable oils (Handford et al. 2015). Milk fat usually substituted with vegetable oil and detergent to emulsify vegetable oil in milk (Mudgil and Barak 2013; Tohidi et al. 2018). Detergents are not natural components of milk, but it is the main and basic ingredient of synthetic milk (Bansal and Bansal 1997; Paradkar et al. 2000; Sadat et al. 2006; Mudgil and Barak 2013). Detergents are amphipathic molecules that possess both a hydrophobic and a hydrophilic groups (Seddon et al. 2004). Anionic detergents are the most commonly used emulsifying agents for synthetic milk preparation due to their low cost, well emulsification capability, solubility in soft as well as in hard water, make much foam when agitated (Paradkar et

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al. 2000; Barui et al. 2013). Commonly used detergents in households are anionic in nature. Linear alkylbenzene sulfonate (LABS) is the anionic surfactant which is most commonly used in making synthetic detergent throughout the world (NIIR, 2006). Synthetic detergent has been reported to be used in preparation of synthetic milk used for adulteration. Detergents are added in synthetic milk to emulsify and dissolve the oil in water system resulting in a frothy and characteristic white colour of milk. Detergents impair male reproductive ability and deform sperm, apart from giving food poisoning, problems in the gastrointestinal system, such as vomiting, diarrhea, and abdominal pain (Handford et al. 2015). Around 68.7% of milk and milk products sold in India are not as per standards laid down by food safety standards authority of India (Theweek, 2018). It is a grave issue, where the entire population of India is on the risk.

Several techniques used for qualitative detection of anionic detergent in milk are based upon spectrophotometric (Jaiswal et al. 2017), photometric (Acevedo et al. 2018), nanotechnology (Kumar et al. 2016) and fourier transform infrared (FTIR) spectroscopy-chemometric approaches (Nascimento et al. 2017). However, these techniques used for quantitative detection of anionic detergents in milk are very expensive such as spectroscopic techniques, requires complicated sample preparation and sophisticated instruments and time consuming. So, there is a need of simple, accurate, fast and non-destructive method for quality testing of milk products.

Hence the objective of this study was to develop a simple, fast and accurate method for detection and quantification of anionic detergent in milk.

Materials and Methods

Reagents

- I. Dye solution: Five mg of Rhodamine 6G dye (Sigma-Aldrich©) dissolved in 100 ml of distilled water.

- II. Toluene (Sulphur Free, Sigma-Aldrich©)

- III. Benzethonium chloride (Sigma-Aldrich©) 0.004 M solution

Sample preparation

Cow milk samples collected in sterilized jars from a local dairy farm and synthetic milk was prepared in a laboratory as it is legally not available. The ingredients in the making synthetic milk were calculated in such a way that the fat and solid nonfat (SNF) percentage is similar to natural milk. The synthetic milk was prepared by adding refined oil (Soyabean oil, Fortune®, India), detergent (sodium laureth sulfate), salt, baking powder urea (all ingredients procured from local market) and tap water. Milk samples brought to room temperature ($27 \pm 1^\circ\text{C}$). Samples were prepared by adding synthetic milk to the natural milk at known concentrations (2%, 4%, 8%, 10% and 15%). One liter of synthetic milk contains 4.103 g of anionic detergent. One ml of synthetic milk contains 4 mg of detergent added at 10 ml to 990 ml natural milk equivalent to 1% of adulteration. Similarly, the other samples were prepared at the rate of 2 %, 4 %, 8 %, 10 % and 15 %.

Sensory analysis

To check the effect of heat treatment on detergent, both natural and synthetic milk samples were subjected to sensory analysis at different levels of adulteration. Samples were evaluated for sensory attributes such as flavour, body & touch, odour and colour & appearance, on a 100-point grading score (Flavor-40, Body & touch-30, Odour-20 and Colour & appearance-10) by a panel of 4 semi-trained (discriminative and communicative) judges from the faculty of Department of Food Technology, Guru Jambheshwar University of Science and Technology, Hisar, India. The samples were heated (near to boiling) and cooled desirably at a temperature of about 40°C for evaluation. Colour and Appearance: Milk samples, containing different concentration

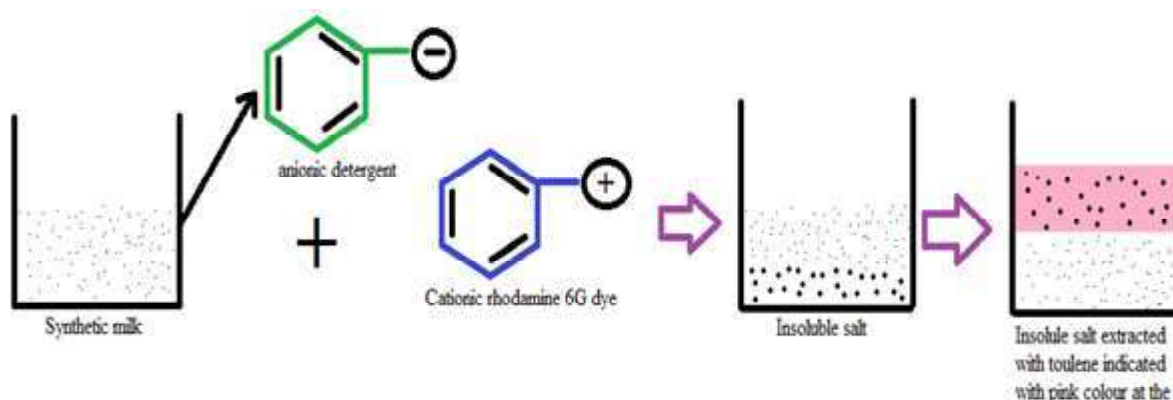


Fig.1 Working principle of anionic detergent detection in milk using rhodamine 6G dye and toluene solvent

of detergent were put into different test tubes and colour was observed in presence of natural light. **Flavour and Odour:** Milk samples were poured into clean, odourless glass containers and heated at 40°C. Containers of milk sample were opened and smelt immediately. Milk was tasted but does not swallowed and spited. **Touch/ Body:** Few drops of milk was taken and rubbed between fingers. The panelists were professionals having adequate knowledge about sensory evaluation methods and product attributes.

Chemical Analysis

Qualitative analysis of detergent in milk

Milk sample (2 mL) of each natural milk and adulterated milk were taken in tubes, added with 1 ml of dye solution (rhodamine 6G dye) and shaken. Further, toluene (5 mL) was added and again shaken for 30 s then keep undisturbed for 1 min. The anions of anionic detergent in adulterated milk reacted with cations of cationic dye (rhodamine 6G dye) result in the formation of an insoluble salt, that can easily extracted by organic solvent (toluene). The intensity of colour in the toluene layer of each sample observed. The illustration of the principle shown in Fig. 1. The milk samples with different concentrations of adulterations treated with dye solution observed for colour change compared to the natural milk (control sample).

Quantitative Estimation of Detergent in Milk by Titration

Quantity of detergent determined by uptake of benzethonium chloride. The samples were titrated against 0.004 M benzethonium chloride solution until the toluene layer in adulterated samples (Fig 2, B, C, and D) turns colourless. The final volume reading of the benzethonium chloride solution in the burette was taken. Benzethonium chloride forms a bond with anionic detergent and displaces Rhodamine 6G dye from Toluene solvent layer to the milk layer by imbalance their charge. When benzethonium chloride completely bounded to anionic detergents, it replaces cationic dye from the organic solvent layer to the milk layer. As benzethonium chloride is a colourless solution on titration, the

Toluene layer becomes transparent and endpoint of the titration. The amount of detergent calculated from the regression equation obtained from the standard curve (Fig 4). The rate of benzethonium chloride uptake affected by adulteration calculated by using $Y=0.3622x-0.379$, where Y is mL of benzethonium chloride used for titration and x is the percentage of synthetic milk.

MBRT Test

Methylene blue dye reduction test performed according to IS SP 18 (Part XI) (1981) to check the effect of detergent on the microbiological quality of milk. Ten ml of pure milk and detergent adulterated samples mixed with 1 ml of MBRT dye solution in sterilized test tubes, capped and incubated at 37°C. The beginning time of incubation and time after decolorization recorded.

Results and Discussion

Sensory analysis of adulterated milk

The sensory analysis of milk samples at different levels of detergent was given in Table 1. At zero level of addition (natural milk) there was no observed difference in sensory characteristics of heated and unheated natural milk. Similarly, results shown that there were no observed difference in sensory characteristics i.e., colour, odour, body, flavour, taste of between samples up to 15 % level of adulteration (Table 1). At a 15 % level of adulteration, heated synthetic milk shown higher whiteness than natural milk due to foam formation by detergent and turned into slight yellow colour compared to unheated synthetic milk. It is also observed mild soapy odor, flavour, and bitter taste in unheated synthetic milk due to its highly alkaline nature imparted by detergent as compared to natural milk. However, by heating synthetic milk (at 15% detergent) the mild soapy odour and flavour disappeared. The bitterness in taste was unaltered between both heated and unheated synthetic milk. Results clearly showed that platform tests are unable to detect adulteration of synthetic milk in natural milk up to a level of 10 %. Jaiswal et al. (2017) had taken heated synthetic milk in their study and found no significant difference in organoleptic tests.

Table 1 Sensory analysis of adulterated milk samples

Percent of adulteration	Colour and appearance		Odour		Body/ Consistency		Flavour		Taste	
	Raw milk	Heated milk	Raw milk	Heated milk	Raw milk	Heated milk	Raw milk	Heated milk	Raw milk	Heated milk
Zero	CW	CW	ND	ND	NS	NS	ND	ND	P	P
2	CW	CW	ND	ND	NS	NS	ND	ND	P	P
4	CW	CW	ND	ND	NS	NS	ND	ND	P	P
8	CW	CW	ND	ND	NS	NS	ND	ND	P	P
10	CW	CW	ND	ND	NS	NS	ND	ND	P	P
15	White	Yellow	MS	No odour	NS	NS	MS	ND	MB	MB

CW= Cream white, ND= Not distinctive, MS= Mild soapy, NS= Not soapy, P= Palatable, MB= Mild bitter



Fig. 2 Qualitative detection of detergent in milk samples using Rhodamine 6G and Toluene solvent



Fig. 3 Quantitative detection of detergent in milk samples using Rhodamine 6G and Toluene

Table 2 Chemical and microbial analysis of adulterated milk samples using rhodamine 6G and toluene

Percent of adulteration	Qualitative analysis (using 1 ml rhodamine 6G and 5ml toluene)		Quantitative analysis		Microbial analysis
	Colour indication	Amount of titrant used (ml)	Amount of detergent present (mg/100 ml milk)	Methylene blue	Quality reduction time (h) of milk
Zero	Negative	0	0	2	Fair
2	Negative	0	0	2	Fair
4	Positive	1.33±0.05	14.91±0.12	2	Fair
8	Positive	2.76±0.11	32.54±0.27	3	Good
10	Positive	3.23±0.05	38.83±0.31	3	Good
15	Positive	4.91±0.05	60.24±0.22	3	Good

Values are mean ± standard deviation of three independent determination

Chemical Analysis of Adulterated Milk Samples

Qualitative Detection of Detergent in Given Milk Samples

Milk samples with added synthetic milk above four percent (Table 2) showed positive (pink colour) in the toluene layer (Fig 2, B) and intensity of colour increased with an increase in detergent concentration (Fig 2, C and D). In contrast, in the control milk sample (Fig 2, A), colour of toluene layer was negative (transparent) as there was no anionic detergent that can form a salt with cationic dye. The developed method can detect adulteration of synthetic milk in natural milk at a level as low as 4 %. Jaiswal et al. (2017) reported that anionic detergent (lissapol) in synthetic milk can be detected as low as 0.2% using total reflectance fourier transform infrared spectroscopy. Paradkar et al. (2000) reported a quantitative approach for anionic detergent detection in synthetic milk (up to 6%) using methylene blue (dye)

through spectrophotometric method. Similarly, Barui et al. (2013) reported that presence of anionic detergent (labolene) in synthetic milk can be detected as low as 0.1% using methylene blue (dye) through paper chromatographic method. However, in the present study as an alternate to methylene blue dye, rhodamine 6G was used to detect anionic detergent in synthetic milk and able to detect as low as 4% that could be a possible rapid method.

Quantitative Estimation of Detergent in Milk by Titration

The dye started reacting with anionic detergent at 4% level while unreactive at zero and two percent (Table 2). This may be the dye was insufficient to react with anionic detergent. This may be because benzethonium chloride as cationic as and more reactive than Rhodamine 6G, which binds with anionic detergent during titration. It displaces the Rhodamine 6G dye from coloured toluene layer (Fig 3) to the milk layer by imbalance their charge. Results

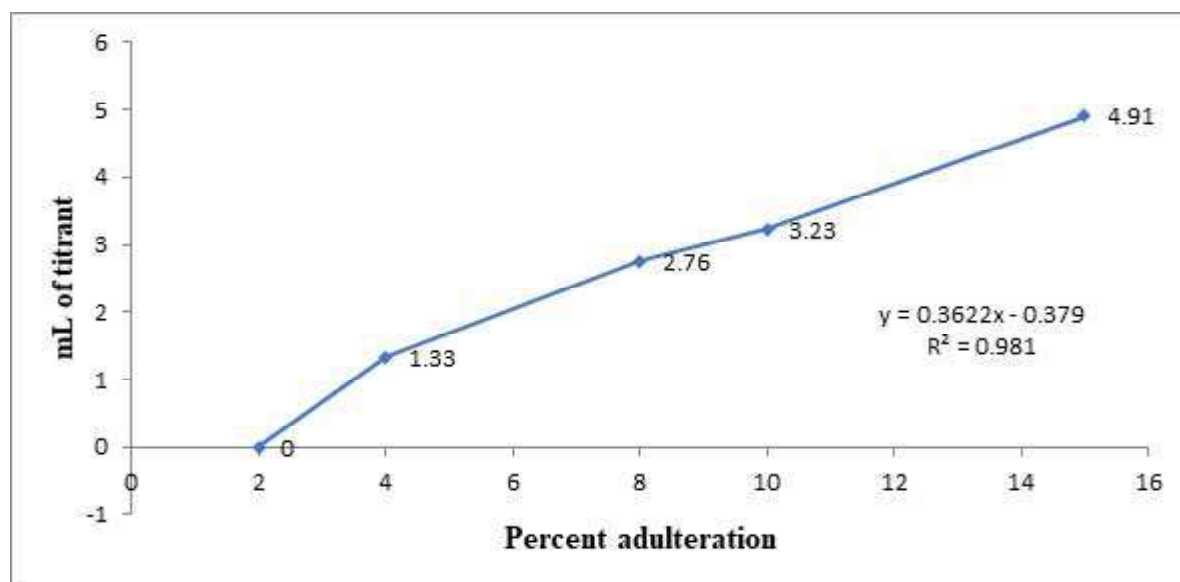


Fig. 4 Rate of benzethonium chloride uptake as affected by adulteration percentage

showed that the amount of benzethonium chloride in titration increased linearly with an increase in the detergent concentration of adulterated milk samples (Fig 4). Based on the obtained linear relationship between titrant level and detergent concentration, the quantity of detergent present in adulterated milk calculated from the graph.

Effect of Detergent on MBRT Test

Results showed that the dye reduction time taken by microbial load present in milk samples increases with an increase in concentration (above 8%) of detergent adulterated to milk (Table 2). The removal of the oxygen from milk and the formation of reducing substances during bacterial metabolism causes the color to disappear. In contrast, detergents slow down the total metabolic reactions of the microorganism present in milk due to the antibacterial and alkaline nature of detergent, so colour imparted by methylene blue dye in adulterated milk be stable for a longer duration than natural milk. According to results, the microbial quality of adulterated milk was better than natural milk, which is a negative response toward the consumer health point of view.

Conclusions

Tests performed at the milk receiving center to check the authenticity of milk are unable to detect the presence of harmful chemicals in milk. This new approach for detection of anionic detergent in adulterated milk sample was developed based on the principle that anions of anionic detergents react with cations of cationic dye and result in the formation of an insoluble salt which quickly extracted in an organic solvent. The working of the method was checked by the preparation of different concentrations of detergent adulterated milk samples and mixing with dye and solvent. In the positive milk sample, colour of the

solvent layer changes from transparent to pink, and the intensity of colour increases with an increase in detergent concentration. The method was so sensitive that it can detect as low as 16 mg of detergent per 100 ml of adulterated milk and intensity of endpoint colour increases with an increase in the concentration of detergent in milk.

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Ethical statements and Conflict of Interest

Sensory evaluation (only physical examination like colour, flavour and appearance) of synthetic milk done by informed consent was obtained for experimentation with human subjects.

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RESEARCH ARTICLE

Studies on the organoleptic perception of different flavours and consumer acceptance of desiccated *Chhana-murki*

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Abstract: *Chhana-murki* is a traditional Indian nutritious dairy product manufactured by heat desiccation of *paneer* cubes in sugar syrup. The flavour of milk products is a critical aspect that promotes its marketability. Therefore, an attempt was made to add different flavours viz., Rose, Pineapple, Butterscotch, and *Elaichi*, at various levels to enhance the organoleptic preference of the formulated product. The scores for different flavours and their levels (Rose and Pineapple) have differed significantly ($p \leq 0.05$), with Rose-flavoured *Chhana-murki* rated maximum flavour score (8.00). On the other hand, the physico-chemical parameters of desiccated *Chhana-murki* were nearer to the most favoured market sample; the values for moisture content, fat, protein, lactose, sucrose, and ash content being 15.77, 15.10, 14.12, 1.41, 52.42, and 1.10 %, respectively. Further, a consumer acceptance study was conducted with 180 randomly selected probable respondents, and 50% of consumers rated the product excellent, and 51.1% of consumers were willing to purchase even at a cost higher than the market price. This specifies the tremendous market potential of the developed flavoured *Chhana-murki*.

Keywords: *Chhana-murki*; Consumer study, Flavour; Physico-chemical parameters

Introduction

Milk and milk products have always contributed significantly to uplift the socio-economic prominence of human beings. Its significance has been known since *vedic* times. India is the largest milk producer in the world. The total milk production share corresponds to 20.17 percent (Arora, 2019) and the current milk production is 185.5 (2019-2020, DAH&D) million metric tonnes (Arora et al. 2021b). To avoid spoilage of milk and to increase its consumption and shelf life, wide ranges of milk products are being prepared from surplus milk such as traditional sweets (Arora et al. 2021) that constitutes the significant portion. About 50-55% of milk in India is converted to value-added dairy products (Banerjee, 1997). The broad ranges of sweets like *Dodha-burfi*, *Milk-cake*, *Kheer*, *Chhana-podo*, *Cham-cham*, *Gulabjamun*, *Rasogulla*, *Kalakand*, *Milk cake* etc are manufactured and consumed in various parts of the country combine luscious taste, flavours with nourishment and health (Aneja et al. 2002). In addition, multiple fruits and nutraceuticals have also been added such as in milk shakes (Kumar et al. 2021) and fermented dairy products (Arora et al. 2021a and Sharanagouda et al. 2019) wherein milk could act as a delivery vehicle for providing essential nutrients. Therefore, milk products are the country's significant selling, lucrative, and potential sector after fluid milk (Patil, 2011). With low capital investment, high profits could be achieved in this sector. The estimated volume of indigenous sweets in the Indian market was about 3 million tons valuing about Rs 7,00,000 million (Khanna and Gupta, 2011; Gurditta et al. 2019).

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Chhana-murki is a well demanded sweet in northern and eastern parts of India. It is prepared from *Chhana/Paneer* by open pan heat desiccation. The characteristics of *Chhana-murki* are its unique, cohesive texture, firm body, white to creamy appearance, and sweet, crunchy taste (Arora et al. 2021b). It contains almost all the milk nutrients (except the whey component) with a comparatively longer shelf life than the other milk-based sweets. Further, *Chhana-murki* has a typical milk solid flavour with cooked flavour notes (Arora et al. 2019). Various novel alternative ingredients have been tried in the milk to increase milk consumption and the marketability of dairy products (Gurditta et al. 2014).

Consequently, there is a tremendous opportunity to add multiple components/ flavours in milk and milk products to increase their consumption and marketing. The product may have high nutritional quality, but the consumer must like it to make it a great success in the market than it would be of commercial significance

(Land, 1988). Further, only the consumer can tell the intensity by which the product is liked or needs any further improvement (Choi et al. 2007). Thus, consumers' likability reflects their quality and market appeal (Munoz and Chambers, 1993). Therefore, the present research work was conceptualized to find out the effect of different added synthetic flavours on the sensory flavour perception of *Chhana-murki*, the physic-chemical sensory and texture quality of the developed product *vis a vis* market product, and the consumer acceptance studies to evaluate the commercial significance of the developed product.

Materials and Methods

Materials

Freshly pooled buffalo milk was brought from the Experimental Dairy Plant, ICAR-National Dairy Research Institute, Karnal, India. The milk was standardized to 5.5 % Fat and 8.5% SNF and

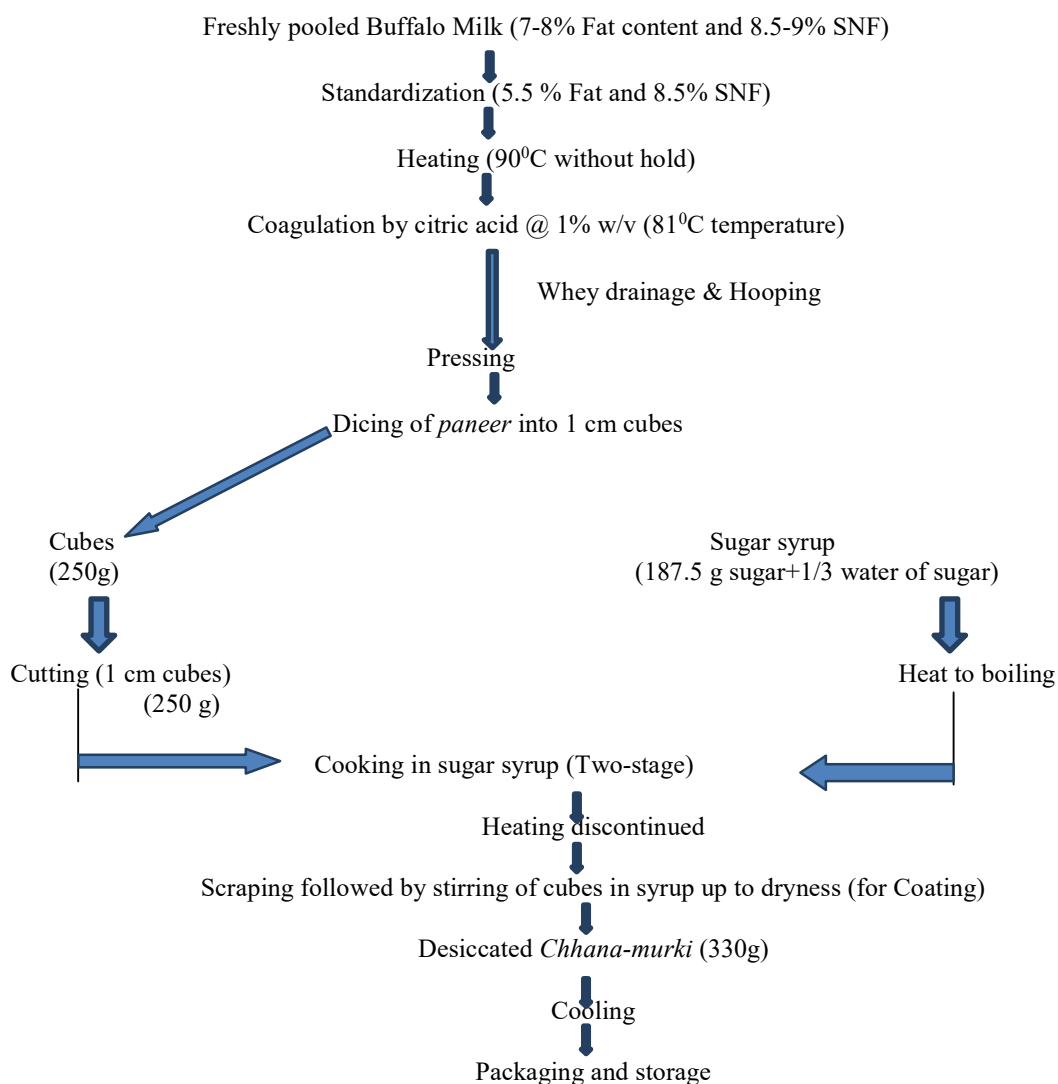


Fig. 1 Standardized procedure for the production of desiccated *Chhana-murki*

coagulated using commercial grade citric acid, monohydrate at 81°C. Cane sugar of commercial-grade was brought from the Experimental Dairy, NDRI, Karnal. The two best market samples of *Chhana-murki* were again procured aseptically and placed at low temperatures to avoid contamination and evaluated and compared with the lab-developed *Chhana-murki* for physico-chemical and sensory attributes.

Production protocol of the developed *Chhana-murki*

Preparation of *Paneer* and *Chhana-murki* was carried out as per the method given by Arora et al. (2019). The flow diagram of the formulated product is shown in Fig. 1.

Flavourings of developed *Chhana-murki*

Different Flavourings were tried to improve the organoleptic quality of the developed desiccated *Chhana-murki*. Rose, Pineapple, Butterscotch, and *Elaichi* flavour of Bush brand (IFF: International Flavours and Fragrances Inc.) was used at a concentration of 0.1, 0.15, and 0.2% to identify the most rated concentration of one particular flavour (one flavour was tried at a time at different concentration). The highest score obtained by different flavour at specific concentrations were again compared with the control.

Physical-chemical analysis of developed *Chhana-murki*

Physical analysis

The instrument colour characteristics in terms of Hunter L*, a*, and b* value was evaluated by Hunter Colour Flex (Hunter Associates Laboratory, Inc., Reston, VA, USA) colorimeter using inbuilt Universal software. The textural quality of the product was measured by TA- IXT2i Texture Analyzer (Stable Micro Systems, U.K.). The Texture Profiling was carried out using 25 kg load cell with 70% double compression cycle with pre-test, test, and post-test speed of 2 mm/sec. The measurement temperature was $25 \pm 1^\circ\text{C}$. The water activity of *Chhana-murki* was measured by a water activity meter (CXT-2, Aqua Lab, WA, USA). The percent yield of *Chhana-murki* was worked on per kg of *panner*. Bulk density was calculated as an increase in water level in 500 ml measuring cylinder by adding 50 gm of *Chhana-murki*, and it is denoted by ratio of weight to volume. The *Sugar-coat to Paneer-cube* ratio was calculated by scraping the sugar from the surface of *Chhana-murki*. The difference in the initial and final weight of the ten pieces of *Chhana-murki* was worked out as a *Paneer-cube* ratio.

Chemical Analysis

The Rose-Gottlieb's process explained for fat evaluation in *Chhana* (BIS, 1981) was employed for determining the fat content in prepared and market samples of *Chhana-murki*.

Moisture content in the prepared *Chhana-murki* and the market samples were evaluated by the Gravimetric method (BIS, 1981). The total nitrogen content was determined by Micro *Kjeldahl* method (AOAC, 1995). Reducing sugar (Lactose content) and total sugars of *Chhana-murki* were found as per condensed milk method (BIS, 1981). Ash content in *Chhana-murki* was determined as per the method described by AOAC (1995).

Sensory evaluation

Control and flavoured samples of desiccated *Chhana-murki* were evaluated organoleptically for flavour attributes on the 9-point Hedonic scale (Amerine et al. 1965) by ten sensory panelists from Dairy Technology, NDRI, Karnal. The primary aim of conducting sensory evaluation was to collect feedback about the acceptability of developed desiccated *Chhana-murki* with different flavours. Lukewarm water (25°C) was provided to cleanse the palate before and in between samples evaluation.

Consumer acceptance evaluation

The developed desiccated Rose-flavoured *Chhana-murki* was distributed among arbitrarily chosen probable respondents. The 60-70 g bags of packaged *Chhana-murki* were provided to each of the 180 respondents. The panellist was requested to fill in their opinion regarding the product in the given proforma. Consumers willing to participate in the evaluation were of different ages, incomes, sex, and no sign of allergy from dairy ingredients. The responses were obtained, and data generated from the survey was analysed (Table 4 and 5). The serving was done at two different timing in a day: morning 11.00 a.m. or evening 4.00 p.m. They had to respond to their degree of liking of the product and willingness to purchase at a relatively higher cost than the market.

Statistical analysis

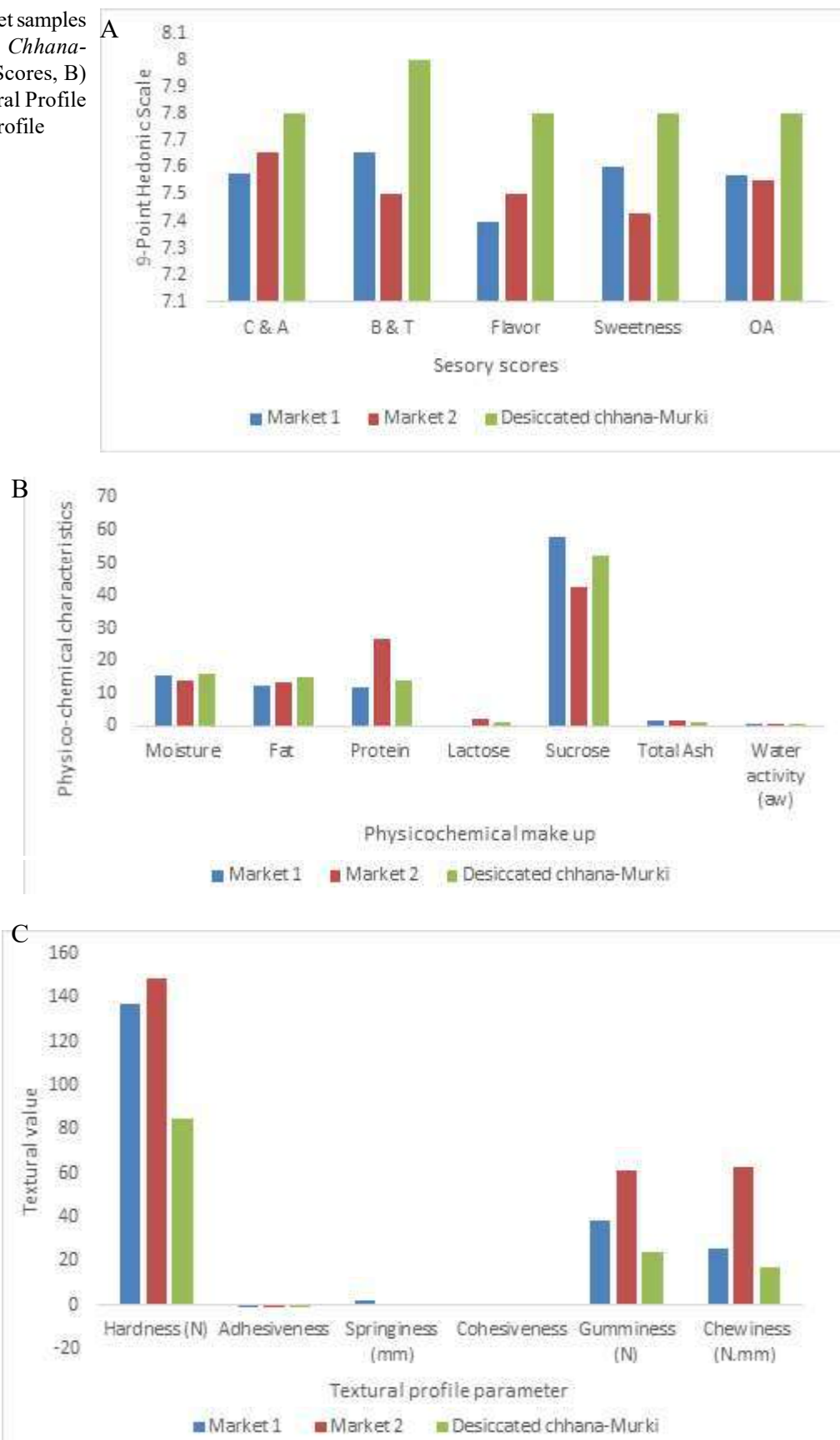
All trials were carried out in triplicate, and analysis of variance (ANOVA) was used to evaluate the difference in flavour and comparison of the prepared product with the market samples. Chi-square test was used to find out the difference between the degree of liking of the product

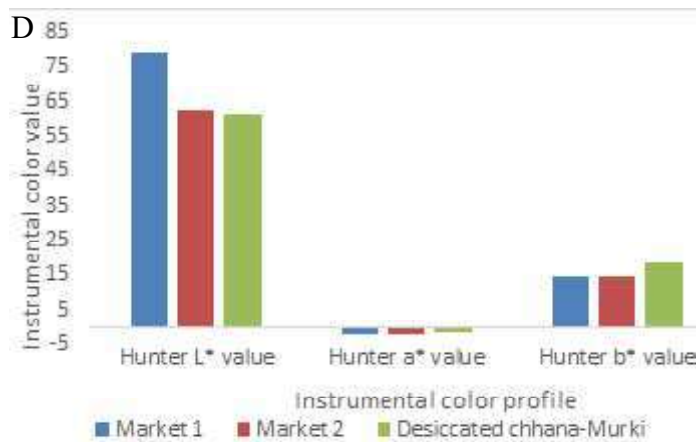
Results and Discussion

Effect of addition of flavours on sensory perception

The synthetic flavours were added to the product to enhance the organoleptic quality of lab formulated *Chhana-murki*. Initially, the effect of different flavours viz., Rose, Pineapple, Butterscotch, and *Elaichi* on organoleptic flavour perception and identification of most desirable flavour and their levels were studied as shown in Table 1. It can be observed that the scores for different flavours and their levels (Rose and Pineapple) differ significantly, while the Rose-flavoured product scored higher (score, 7.2-8.0) compared to the other flavours. The addition of Rose-flavour

Fig. 2 Characterization of best market samples (2) and the developed desiccated *Chhana-murki* on the basis of A) Sensory Scores, B) Physicochemical Profile, C) Textural Profile Parameter, D) Instrumental Color Profile





greatly enhanced the flavour perception rating (8.00) at 0.1% level while the three different flavours, when used at 0.15 %, scored maximum flavour scores of 7.64, 7.57, and 7.96 for Pineapple, Butterscotch, and Cardamom flavours, respectively. No significant effect was observed by the addition of Butterscotch and Cardamom flavour though the highest flavour perception scores were observed when the two Flavours were used at 0.15% level.

All flavours at their best flavour scores were evaluated with the control sample to find the most preferred flavours among them. As shown in Table 2, the overall acceptability scores for flavour addition were significantly ($p \leq 0.05$) higher for rose at 0.1% and *Elaichi* at 0.15 %. Similarly, Pineapple flavour also received the highest sensory score at 0.15%, and the effect was significant ($p \leq 0.05$). The statistical analysis of the data revealed that no significant difference in the flavour scores for control, Pineapple, and Butterscotch flavour. However, incorporating different flavours increased the flavour perception compared to control

samples. Rose and Cardamom (*Elaichi*) flavoured *Chhana-murki* were appreciably superior compared to the control sample.

Physico-chemical profile of the market and developed desiccated *Chhana-murki*

The developed product and the two best market samples of desiccated *Chhana-murki* were evaluated for various physico-chemical, sensory (Hedonic rating), colour, and instrumental texture characteristics (Fig 2). The consumption of any product relies upon its sensory quality. Therefore, the primary feature of any food product development is to evaluate it sensorially. After the addition of flavour, the product becomes even more likable (Table 1 & 2). It can be seen that all the sensory parameters received a maximum sensory score of 7.75 (for flavour) and above. In addition, the scores of 7.79 for colour and appearance, 7.96 for texture, 7.83 for sweetness, and 8.00 for overall acceptability were obtained. It can be seen from fig. 2a that the organoleptic rating (hedonic scale) of the two most favoured market samples was recorded as 7.57 and 7.50, respectively, which is significantly ($p \leq 0.05$) lower than the developed product. The lab formulated product had moderate glossiness cuboid shape and dry surface, a reasonably thick sugar coating, a moderately tough texture, and chewiness. The product's flavour was a blend of milk solid, cooked, and perception of sweetness and rose.

The textural characteristics of the developed products are shown in Table 3. The desiccated *Chhana-murki* had moderate hardness (148.34N), almost insignificant adhesiveness (-0.18N.s), springiness (0.6 mm) and cohesiveness (0.41) but moderate gumminess (60.99 N) chewiness (62.63 N.mm). The two best market samples of desiccated *Chhana-murki* had 136.90 and 84.40 N hardness, -0.02 and -0.15 N.s adhesiveness, 1.64 and 0.67 mm for springiness, 0.28 and 0.29, cohesiveness, 38.44 and 24.28 N gumminess, and 25.20 and 17.48 N.mm chewiness (Fig. 2b).

Table 1 Flavour score of optimized desiccated *Chhana-murki* flavoured with different synthetic flavourings

Sensory attributes	Percentage of flavour addition			CD _{0.01}
	0.1	0.15	0.2	
Rose	8.00 ^a ±0.11	7.71 ^a ±0.13	7.21 ^b ±0.13	0.37
Pineapple	7.14 ^b ±0.09	7.64 ^a ±0.11	7.50 ^a ±0.09	0.19
Butterscotch	7.46 ^a ±0.13	7.57 ^a ±0.15	7.43 ^a ±0.10	ns
Cardamom (<i>Elaichi</i>)	7.74 ^a ±0.14	7.96 ^a ±0.12	7.89 ^a ±0.13	ns

Values are Mean ± S.D. for $n=3$. Means within same row not sharing common superscript letters (a and b) differ significantly ($P \leq 0.05$). ns-not significant

Table 2 Comparative acceptability of different flavourings in desiccated *Chhana-murki*

		Percentage of flavour addition based on maximum acceptability			
Control	Rose	Pineapple	Butterscotch	Cardamom	CD _{0.01}
Nil	0.1%	0.15 %	0.15 %	0.15%	
7.35 ^b ±0.12	7.67 ^a ±0.17	7.38 ^b ±0.22	7.40 ^b ±0.20	7.56 ^a ±0.20	0.16

Values are Mean ± S.D. for $n=3$. Means within same row not sharing common superscript letters (a and b) differ significantly ($P \leq 0.05$).

Table 3 Physico-chemical, sensory, textural and colour characteristic of the formulated desiccated *Chhana-murki*

Property	Attribute	Value
Hedonic rating*	Colour and appearance score	7.8±0.1
	Body and texture score	8.0±0.1
	Flavour score	7.8±0.2
	sweetness score	7.8±0.1
	Overall acceptability score	8.0±0.1
Instrumental texture profile analysis (TPA)	TPA Hardness (N)	118.34±3.79
	TPA Adhesiveness	-0.18±0.04
	TPA Springiness(mm)	0.60±0.05
	TPA Cohesiveness	0.41±0.03
	TPA Gumminess (N)	60.99±3.47
	TPA Chewiness (N.mm)	62.63±2.46
Physico-chemical properties*	Moisture content (% by wt.)	15.77±0.9
	Fat content (% by wt.)	15.10±0.10
	Protein content (% by wt.)	14.12±0.06
	Lactose content (% by wt.)	1.41±0.06
	Sucrose content (% by wt.)	52.42±0.50
	Total Ash content (% by wt.)	1.10±0.04
	Water activity (a _w)	0.84±0.06
	Sugar-coat-to-paneer cube ratio	0.59±0.01
	Yield (g)	132%

*Mean ± SE from 3 replications

Table 4 Consumer rating of desiccated *Chhana-murki* by 180 consumers^a

Rating	No. of respondents(n=180) Gender group of consumers		
	Males	Females	Total
Excellent 9	52 (52.5)	38 (46.9)	90(50)
Very good 8	36(36.4)	29 (35.8)	65(36.2)
Good 7	11 (11.1)	14 (17.3)	25(13.8)
Fair 6		nil	
Just acceptable		nil	
Mean weighted score	8.33 ^a	8.30 ^a	8.31 ^a
Total	99 (100)	81 (100)	180(100)

Figures in parentheses indicate per cent of consumers

^a Chi-square test significant at 5 per cent

Milk and milk products are rich in lactose and proteins, and during heating or processing of these products, the colour may change due to Maillard browning. Desiccated *Chhana-murki* contained a high amount of protein and lactose; therefore, it is expected that slight browning may occur due to heat processing during preparation. Consequently, the instrument color profile as Hunter L*, a*, and b* values of the developed product was found as 61.01, -1.64, and 18.97, respectively (Table 3). The desiccated *Chhana-murki* thus had relatively high lightness, moderate yellowness, and a slight greenish tinge. However, Hunter L*, a*, and b* values were 79.27 and 62.39, -1.74 and -2.06 and 14.82 and 14.66, respectively, for the two most favoured market samples (Fig. 2c).

The compositional profile of any food product talks about the physico-chemical parameters and nutritional information. The physico-chemical description of desiccated *Chhana-murki* was nearer to the most favoured market sample (Fig.2d); the values for moisture content, fat, protein, lactose, sucrose, and ash content were 15.77, 15.10, 14.12, 1.41, 52.42, and 1.10 %, respectively (Table 3). The yield of the product was 132% / kg *paneer*. Previously also many researchers have characterized the market sample of dairy products like, *Paneer* (Ranjan Naik et al. 2016), *Khoa-Peda* (Singh et al. 2018), *Rasogolla* (Srinivasa et al. 2017), *Sandesh* (Singh et al. 2019) and other sweets (Nawadkar et al. 2010) etc. based on physico-chemical, sensory and textural attributes. The target is to know the quality of the existing product

Table 5 Consumers' willingness to buy the desiccated *Chhana-murki* offered at a price higher than the normal market price

Willing to buy at	Respondents		
	Males	Females	Total
The average market price	99(55)	81(45)	180(100)
A higher cost then the market average	56(28)	36(24)	92(51.1)

Figures in the parentheses () indicate percentile of the total

and do further research and development work to upgrade the technology at an industrial scale.

Consumer likability of desiccated *Chhana-murki*

The consumer acceptance of the laboratory-developed *Chhana-murki* was assessed by offering it to a group of consumers belonging to different classes of the society and based on socio-economic structure for seeking their opinion about the product. The results analysed in the present study for quality rating by consumers are shown in Table 4. It was found that responses given by the consumers for the prepared product were quite encouraging, with 52.9% (58) males and 46.9% (38) females respondents rated as 'excellent', 36.4% (36) and 11.1% (11) males and 35.8% (29) and 17.3% (14) females had rated it 'very good' and 'good', respectively. The mean weighted scores of the product were 8.31 by males and 8.30 by females, the overall average being 8.31 (i.e., between 'very good' and 'excellent'). The developed product was uniformly liked by both males and females, as indicated by the non-significant chi-square test. No unpleasant comments or disapproval was received for the product. So, it could be concluded that desiccated flavoured *Chhana-murki* in the current work received extremely satisfactory responses by the consumers. Concerning the consumer's eagerness to procure the product, 51.1% were curious to purchase the desiccated *Chhana-murki* even at a price higher than the market (Table 5). This specifies that the excellent market potential of the developed flavoured *Chhana-murki*.

Conclusions

The masses always enjoy traditional dairy products, and consumer acceptance will reveal the market potential of the developed product. The addition of flavours to milk and milk products is a smart move to add nutrition to the diet. *Chhana-murki* flavoured with rose flavour was liked by the sensory panellist and rated highest compared to the other flavours. The formulated product had desirable physico-chemical and sensory attributes compared to the market samples. The lab-developed product received a high degree of liking by consumers of all age and income groups. A highly encouraging consumer response evidenced the potential for commercial marketing as a bite-size yet nutrient-dense product.

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Evaluation of synergistic preservative effect of cinnamaldehyde and citral against yeast in milk and *Lassi*

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Abstract: The aim of present study was to assess the potentials of enhancing the inhibitory effect of combined application of cinnamaldehyde, eugenol and citral on the growth of food spoilage yeasts using fractional inhibitory concentration (FIC) index in laboratory media and time-kill assay in a food model. The minimum inhibitory concentrations (MIC) of cinnamaldehyde, eugenol and citral were 0.125, 0.5 and 0.5 µL/mL, respectively. The FIC indices of combinations assessed showed that cinnamaldehyde and citral combination exhibit synergistic interaction and no difference were found among other combinations tested. The combined effect observed was validated in skimmed milk and *Lassi* against the most common yeast *Saccharomyces cerevisiae*. The tested combination demonstrated stronger anti-yeast activity by completely inhibiting of the growth when compared to control. These results demonstrated potential of green chemistry as an alternate to chemical preservative to prevent yeast spoilage of milk and fermented milk products could be used as valuable additives to improve microbiological stability of dairy products and meet expectations of consumers.

Keywords: Antiyeast, Essential oil, *Lassi*, Milk, Natural preservation

Introduction

Yeasts are known for their both beneficial and problematic role in food processing. They are utilized as starter cultures to initiate

fermentation during preparation of fermented foods. However, yeasts are also established to cause spoilages of many foods including salad, fruit juices, milk products, meat, and beverages etc. (Jakobsen and Narvhus, 1996). Dairy products such as cream, butter, cheese and yoghurt are more likely to deteriorate by the yeasts, causing financial losses to the milk processing sector in several countries (Fleet, 2011). It has been estimated that worldwide almost 11-22% of dairy products is lost as a result of fungal spoilages (FAO, 2011). The growth of yeast species in these products is attributed to their ability to grow at low temperature, acidic pH, ferment lactose and produce enzymes to hydrolyze fat and protein (Mayoral et al. 2005). The spoilage caused by yeast is characterized by undesirable flavor and swelling of product packages due to gas production. The most prevalent yeasts implicated in spoilage of yoghurt and fermented milks are *Candida* sp, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Saccharomyces exiguus*, *Debaromyces hansenii*, and *Rhodotorula* sp. (Mayoral et al. 2005; Fleet, 2011; Nielsen et al. 2021). *Saccharomyces cerevisiae* is frequently isolated from the food and beverages containing high sugar content (Viljoen et al. 2003). The yeast contamination to fermented dairy products can occur due to exposure to air, aerial transmission of surfaces, processing environment and from raw ingredients added after fermentation step (Temelli et al. 2006).

Essential oils derived from plant sources have a long history of their use in food preservation and food flavor enhancement. Many essential oils are identified as safe food additives by the US-FDA (Anbarasu and Vijayalakshmi, 2007; Hsouna et al. 2011). These features make it an interesting alternative to chemical additives for food preservation applications. While choosing the dose to be added into food to achieve effective inhibition attention should be paid to the sensory changes that might arouse in the food product. To enhance the efficacy of essential oils several studies have been focused on exploiting synergistic activity with other antimicrobials to minimize the negative effect on sensory quality (Belletti et al. 2010).

Essential oil from a chemical composition perspective is a complex mixture made of several individual compounds which are responsible for biological activities of the whole essential oil. Although several scientific studies have acknowledged that some

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plant essential oils exhibit antibacterial activity only few studies have investigated the inhibitory activity of purified compounds of essential oils against spoilage yeast (Wang et al. 2005). Some studies have concluded that combination of whole essential oils have greater antimicrobial activity than the major constituents combined. However, Ouedrhiri et al. (2017) suggested that the mixing of major constituents with minor constituents of other essential oils which have weaker antimicrobial activity may give synergistic or additive effect. Moreover, combining the major constituents enables standardization of doses for applications in food by avoiding the change in activity caused due to compositional variation found in whole essential oil owing of geographical area, stage of harvesting, extraction procedure etc (Ait-Ouazzou et al. 2011).

Cinnamaldehyde, eugenol and citral are the most commonly used compounds of essential oils from cinnamon, clove and lemon grass, respectively (Friedman et al. 2002; Liang et al. 2015; Ju et al. 2020). Therefore, in this study we have investigated the dynamics of anti-yeast effects of cinnamaldehyde, eugenol and citral alone and also combined application both *in vitro* and in skimmed milk and lassi as model foods.

Materials and Methods

Antimicrobial agents

The antimicrobials used in this study were cinnamaldehyde (99%), eugenol (99%) and citral (99%). They were purchased from Sigma Aldrich Pvt Ltd, Bangalore, India.

Microorganisms

Yeast cultures of *Saccharomyces cerevisiae* NCDC 42 and NCDC 49, *Candida guilliermondii* NCDC 44 and *Kluyveromyces marxianus* NCDC 46 were obtained from National Collection of Dairy Cultures, ICAR-NDRI, Haryana, India on agar slants. They were cultured at 30 °C in yeast extract potato dextrose (YPD) medium (20 g glucose, 20 g bacteriological peptone, 10 g yeast extract per liter, pH 4.0: Hi-media laboratories, Mumbai, India) and were maintained on slants of YPD agar at 4 °C.

Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations of essential oil compounds were determined by microdilution method by monitoring the growth by measuring the optical density (OD) at 595 nm. Briefly, 100 µL of YPD broth containing 0.1% Tween 80 (to enhance solubility) was dispensed in to the wells of 96-well flat bottom plate. A 100 µL of YPD broth containing essential oil compounds (8 µL/mL) was loaded into the first well and subsequent double dilutions were carried out to obtain concentrations ranging between 4 - 0.03 µL/mL. Each well was then added with 100 µL of broth containing 10⁶ CFU/mL of yeast cells. In similar way three wells of control were prepared including

broth with essential oil compounds, broth with test organism and only YPD broth. After the incubation of plates at 30 °C for 48 h, absorbance was measured using a spectrophotometer (OD_{595nm}; iMark™ Microplate Reader). MIC is the lowest concentration of the essential oil that inhibits the growth of organism as evident from absence of increase in OD. In the case of milk the concentration showing no formation of yeast colony on plate count assay was taken as MIC for that essential oil compound.

Combined effect testing

Fractional inhibitory concentration (FIC) index is cited by most researchers as a measure of the interactive antimicrobial effect of essential oil compounds *in vitro*. The FIC index was determined in YPD broth by the checkerboard method in a 96-well microtiter plate by microdilution method as described by Tserennadmid et al. (2011). Briefly, essential oil compound A was diluted two fold along x-axis and compound B was diluted two fold along the y-axis. The concentrations of them were prepared corresponding to 1/2, 1/4 and 1/8 of the MIC values, respectively. The final volume in each well was 100 µL comprising 50 µL of each dilution. Then 100 µL of broth containing 10⁶ cfu/mL of yeast cell suspension was added to each well. After the incubation of plates at 30 °C for 48 h, absorbance was measured using a spectrophotometer. The interaction between two essential oil compounds was determined by calculating the FIC index (FICI) as below:

$$FICI = FIC_A + FIC_B = (C_A^{Comb} / MIC_A) + (C_B^{Comb} / MIC_B)$$

Where MIC_A and MIC_B are MIC of agents A and B acting alone and C_A^{Comb} and C_B^{Comb} are the MICs of agents A and B when in combination respectively. A or B represent single agent: cinnamaldehyde or eugenol or citral. Interpretation criteria of FIC index was followed, if, FICI <1 indicate synergy, FICI of =1, indicate additive, FICI of between 1 and 2 indicate indifference and > 2 indicate antagonism (Pei et al. 2009).

The EC index of the combination of essential oil compounds was determined in skimmed milk as described by Zhou et al. (2007). EC was calculated as follows:

$$EC = [\log DP_I - \log DP_{II}]$$

Where, DP_I and DP_{II} represents the difference in population of single agent or combination system, respectively. For this 1/2 MIC and 1/4 MIC of MIC value obtained by microdilution method in skimmed milk was calculated for each essential oil compound and when three compounds when combined 9 different combinations were obtained. In 50 mL of skimmed milk the combinations were added and all samples were inoculated to obtain yeast population of 10⁶ cfu/mL. At time 0 h and after 24 h incubation at 30 °C the viable counts were determined by plate count method after serial dilution.

Time kill assays in milk and *Lassi*

UHT processed *Lassi* (50 mL) or skimmed milk from the local market was dispensed into the test tubes and then essential oil compound at desired concentrations were added. According to label declarations the fat, protein and sugar content in *Lassi* was 2.1, 2.3 and 12%, respectively and in skimmed milk was 0.1, 3.5 and 0% respectively. The pH of *lassi* was 3.8 and milk was 6.8 analyzed using pH meter (Cyberscan, Eutech instruments, Singapore). After vortex mixing the tubes for 1 min, 10^6 CFU/mL yeast suspension was inoculated and tubes were incubated at 30 °C for 24 h without shaking. At different time intervals (0, 1, 3, 5, 8 and 24h) samples were drawn and viable count was determined by plate count method.

Sensory analysis

The organoleptic evaluation of the essential oil added *lassi* samples was carried out by five judges by giving a simple description of the odour and taste (Tserennadmid et al. 2011).

Statistical analysis

The one way ANOVA was conducted using SAS software and the significance was determined at confidence levels of 95% or 99%. The results of triplicate analysis were used for statistical analysis.

Results and Discussion

The use of essential oils in food preservation technology can help to reduce application of chemical preservatives offering natural alternative to meet consumer expectations. The present study examined three active compounds of well-known essential oils from clove, eugenol and lemon grass for the control of yeast growth. The final purpose is to compare activity of essential oil compounds acting alone or in combination that allow us to lessen the dose required to achieve the desired efficacy, thereby avoid undesirable changes of sensorial properties of food.

The MICs of essential oil compounds tested against yeast by broth microdilution method are presented in Table 1.

The essential oil compound with greatest anti-yeast property in YPD broth was cinnamaldehyde with MIC of 0.25 μ L/mL. All the yeasts tested showed susceptibility to eugenol and citral at MIC of 0.5 μ L/mL. The data on susceptibility of yeast to essential oil

compounds in food was obtained through determination of MIC in skimmed milk as food constituents offer resistance towards antimicrobial compounds. As shown in Table 1 the MIC values of three essential oil compounds in skimmed milk confirmed the results of strong antiyeast activity observed in broth medium. In skimmed milk cinnamaldehyde had the lowest MIC of 0.5 μ L/mL and eugenol had MIC of 1 μ L/mL against *S. cerevisiae*, *C. guilliermondi* and *K. marxianus*. Whereas the MIC observed for citral was 1 μ L/mL against all the tested yeasts except *K. marxianus* at 0.5 μ L/mL. Cinnamaldehyde has been reported previously to have a strong antimicrobial activity against *S. Typhimurium*, *E. coli*, *Aspergillus flavus* and *S. cerevisiae* (Zhou et al. 2007; Sanla-Ead et al. 2012; Liang et al. 2015). The higher MIC in skimmed milk reveals a strong protection of the milk constituent, probably protein, against the effect of the essential oil compound (Ait-Ouazzou et al. 2011; Tserennadmid et al. 2011; Ju et al. 2020). Therefore, slightly higher concentration of essential oils to be added to food to obtain a useful inhibitory effect as compared to the concentrations observed in broth.

To explore the possibility of reducing the required antimicrobial doses we extended our study to understand the interactive effect of essential oil compounds. The FIC indices observed ranging from 0.75-1 for binary combinations of essential oil compounds tested are presented in Table 2. According to interpretation criteria established for interaction between the compounds only synergistic effect was found between cinnamaldehyde and citral (FIC=0.75) on the growth of yeasts. Only one group showed an increase in inhibitory effect against all the yeast used in this study and the rest combinations revealed indifference (FIC>1). As compared to MIC of cinnamaldehyde or citral alone for all the yeast, the MIC of both in combination decreased by 50%. In a previous study Tserennadmid et al. (2011) assessed the combined effects of essential oils and its major compounds against *S. cerevisiae* in synthetic media by the FIC index method. The authors reported similar effect for combinations of juniper with marjoram and α -pinene with linalool from clary sage and indifference for juniper with the other essential oils (Zhou et al. 2007).

With respect to standardization of useful combinations for practical applications it is important to evaluate efficacy in food models since food components may reduce their antimicrobial effect (Gutierrez et al. 2009). The effects of binary combinations of cinnamaldehyde and citral were evaluated in the case of *Saccharomyces cerevisiae* being frequent spoilage yeast in sugar

Table 1 Minimum inhibitory concentrations of essential oil compounds against yeasts

Essential oil	<i>S. cerevisiae</i>		<i>C. guilliermondi</i>		<i>K. marxianus</i>	
	Broth	Skimmed milk	Broth	Skimmed milk	Broth	Skimmed milk
Cinnamaldehyde (μ L/mL)	0.25	0.5	0.25	0.5	0.25	0.5
Citral (μ L/mL)	0.5	1	0.5	1	0.5	1
Eugenol (μ L/mL)	0.5	1	0.5	1	0.5	0.5

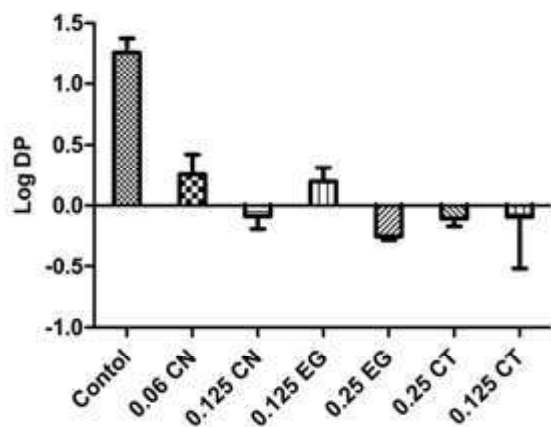


Fig. 1 Effect of cinnamaldehyde (CN), eugenol (EG) and citral (CT) at below MIC concentrations in $\mu\text{L}/\text{mL}$ on *S. cerevisiae* in skimmed milk after 24 h at 30 °C.

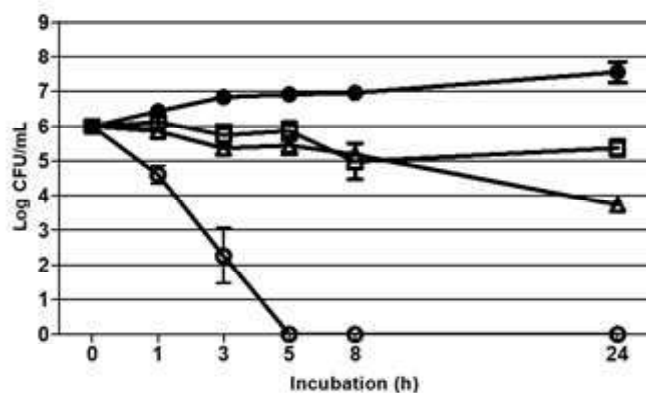


Fig. 2 Sensitivity of *S. cerevisiae* towards combinations of essential oil compounds in skimmed milk at 30 °C. (●): control (0 $\mu\text{L}/\text{mL}$); (□): cinnamaldehyde (0.125 $\mu\text{L}/\text{mL}$); (▲): citral (0.25 $\mu\text{L}/\text{mL}$); (○): cinnamaldehyde (0.125 $\mu\text{L}/\text{mL}$) + citral (0.25 $\mu\text{L}/\text{mL}$).

Table 2 FIC indices of combination of cinnamaldehyde, eugenol and citral against the yeast

Essential oil combination		<i>S. cerevisiae</i>	<i>K. marxianus</i>	<i>C. guilliermondii</i>
Cinnamaldehyde (CN)/Eugenol (EG)	MIC of CN in CN+EG	0.25	0.25	0.25
	MIC of EG in CN+EG	0.125	0.25	0.125
	FIC Index	1.25 (I)	1.5 (I)	1.25 (I)
Cinnamaldehyde (CN)/Citral (EG)	MIC of CN in CN+CT	0.125	0.125	0.125
	MIC of CT in CN+CT	0.125	0.125	0.125
	FIC Index	0.75 (S)	0.75 (S)	0.75 (S)
Eugenol (EG)/Citral (CT)	MIC of EG in EG+CT	0.5	0.5	0.5
	MIC of CT in EG+CT	0.25	0.5	0.125
	FIC Index	1.5 (I)	1.5 (I)	1.25 (I)

The FICI interpreted as S: synergistic (FICI <1); I: indifference (FICI >1).

Table 3 Effect of combination of essential oil compounds on the growth of *S. cerevisiae* according to Log DP and EC index.

Cinnamaldehyde	Eugenol	Citral	LogDP	EC	
				A vs A+B	B vs A+B
0.125	0.25	0	-0.63	-0.54	-0.38
0.125	0.125	0	-0.31	-0.22	-0.51
0.06	0.125	0	-0.61	-0.86	-0.80
0.125	0	0.25	-4.55	-4.47***	-4.45***
0.125	0	0.125	-2.00	-1.91**	-1.91**
0.06	0	0.125	-0.69	-0.94	-0.59
0	0.25	0.25	-1.46	-1.21	-1.36
0	0.125	0.25	-1.28	-1.48	-1.18
0	0.125	0.125	-0.57	-0.77	-0.48

** $P \leq 0.01$; *** $P \leq 0.05$; Log DP = $\text{Log}(N/N_0)$; where Log DP is the logarithm of the difference in population, N and N_0 are population of yeast at time 't' and zero, respectively. A or B is single agent: cinnamaldehyde or eugenol or citral; A+B is combination of single agents: cinnamaldehyde + eugenol; cinnamaldehyde + citral; eugenol + citral.

rich beverage through comparing the log DP results of paired combinations against *S. cerevisiae* in skimmed milk.

As shown in Figure 1 the log DP of single agents was less effective in reducing the yeast populations. Samples treated at below MIC concentrations observed in milk revealed similar effect with less

than 0.5 log DP ($P > 0.05$). This was similar to findings of Pei et al. (2009) that cinnamaldehyde, carvacrol and thymol have shown almost equal the log DP of *E. coli*.

The effect of binary combination of cinnamaldehyde, eugenol and citral on *S. cerevisiae* in skim milk is listed in Table 3. At concentrations shown to be less active when paired combinations with each other cinnamaldehyde/citral showed stronger effect. Only cinnamaldehyde (0.125 $\mu\text{L/mL}$) and citral (0.125 and 0.25 $\mu\text{L/mL}$) showed the higher activity with log DP values >2 in combination was greater than those of used alone which revealed synergistic effects on *S. cerevisiae*. The effect of combinations was further judged using the EC index. According to EC results (Table 3) combination of citral and cinnamaldehyde had significant activity and combination was more effective than separate application ($P < 0.01$). These findings are strongly supported by the work of Pei et al. (2009) as they also found that mixture of cinnamaldehyde (200 mg/L) and carvacrol (200 mg/L) had additive interaction by FIC method whereas synergistic interaction by log DP method. By combining cinnamaldehyde and citral the effective concentrations required can be reduced. But no synergistic effect was observed in our investigation with the combinations of cinnamaldehyde-eugenol and eugenol-citral.

The synergistic activity of cinnamaldehyde and citral was also verified in skimmed milk and *Lassi* challenged with *S. cerevisiae* NCDC 42 and was monitored over 24 h incubated at 30 °C. As shown in Figure 2 cinnamaldehyde (0.125 $\mu\text{L/mL}$) and citral (0.25 $\mu\text{L/mL}$) combination in skimmed milk decreased the viable count of *S. cerevisiae* to below detection limit at 5 h and maintained complete inhibition over the monitored 24 h storage period indicative of its efficacy to inhibit the yeast. Whereas the addition of cinnamaldehyde and citral alone reduced the cell count numbers about 5.36 log CFU/mL and 3.72 log CFU/mL, respectively after 24 h of storage.

As for the results represented in Figure 3 for the combined effect of cinnamaldehyde (0.125 $\mu\text{L/mL}$) and citral (0.25 $\mu\text{L/mL}$) in *Lassi*, the combination treatment showed reduction in the viability after 8 h of exposure time and completely inhibited the growth of *S. cerevisiae* after 24 h of storage.

When the results in skimmed milk and *Lassi* were compared, slightly delayed inactivation was found in *Lassi* w.r.t. combination of compounds. The slight delayed inactivation might be due to presence of sugar and low pH of *Lassi*. A study by Tserennadmid et al. (2011) also noted the maximum inhibitory effect of essential oils at pH 7 rather than the pH 4. Presence of sugar and low pH favor the growth of yeast such as *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* even under low water activity (Bennis et al. 2004). In addition, fat offers resistance to microorganism by forming a protective film around the microorganism or prevents the dispersion of essential oil in the water phase by favoring the migration of antimicrobial to

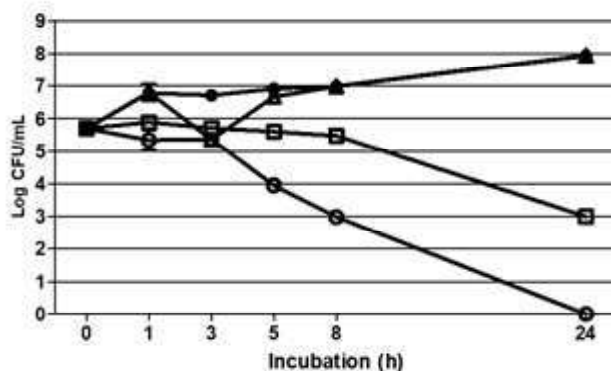


Fig. 3 Sensitivity of *S. cerevisiae* towards combinations of essential oil compounds in lassi at 30 °C. (●): control (0 $\mu\text{L/mL}$); (□): cinnamaldehyde (0.125 $\mu\text{L/mL}$); (▲): citral (0.25 $\mu\text{L/mL}$); (○): cinnamaldehyde (0.125 $\mu\text{L/mL}$) + citral (0.25 $\mu\text{L/mL}$).

hydrophobic phase of food fat, thus leading to the loss of their efficacy (Belletti et al. 2010; Ma et al. 2013; Chen et al. 2014). However, from the results of our study it is evident that enhanced activity of the combination was sustained both in *Lassi* and skimmed milk.

The mechanism behind enhanced activity of cinnamaldehyde and citral combination is still subject of research. The crucial factor in achieving additive or synergistic inhibitory effect is to interference at multiple target sites of a cell with respective mechanisms of action simultaneously so as to disrupt the homeostasis (Zhou et al. 2007; Ganière and Denuault, 2009). Citral being a member of α , β unsaturated aldehydes which act as alkylating agent towards nucleophilic groups of essential cellular constituents alters both membrane and cell wall of *Zygosaccharomyces bailii* (Rivera-Carriles et al. 2005) and cinnamaldehyde inhibit the cell wall synthesizing enzymes of *Saccharomyces cerevisiae* as a non-competitive inhibitor (Bennis et al. 2004). This might make the microorganism more susceptible for combination of cinnamaldehyde and citral causing difference in kinetics of killing when they are in combination. Lv et al. (2011) suggested that the damage caused to cell membrane might explain the synergistic effect of oregano and perilla essential oils on *S. cerevisiae*.

The sensory analysis for the organoleptic acceptability of essential oil compounds addition in lassi followed the order: citral > cinnamaldehyde > eugenol. *Lassi* with only cinnamaldehyde or eugenol the odour was dominant. However, the flavor of citral combination with cinnamaldehyde or eugenol was acceptable and had a pleasant, lemon like and refreshing odour in *Lassi*. There are examples in the literature of application of essential oils and their compounds to improve the shelf life of fruit based beverages and fermented milk products (Tserennadmid et al. 2011; Badola et al. 2018; Keshavarzi et al. 2020). Essential oil extracted from citrus and cinnamon has been successfully used against

food spoilage yeast and molds. Essential oil compounds citral, linalool, *p*-cymene combined with mild heat treatment enhanced the inhibition of *S. cerevisiae* in beverages (Belletti et al. 2010). In summary, the effective concentrations of cinnamaldehyde and citral can be reduced when combined due to synergistic effect. Nonetheless, these results support the use of combinations of natural antimicrobials as alternative to traditional chemical preservatives to control the yeast related spoilages in food matrices.

Conclusions

In this work, the results revealed that cinnamaldehyde and citral together synergistically and significantly inhibited the growth of *S. cerevisiae* in milk and *Lassi*. Therefore, such combination of cinnamaldehyde and citral possess valuable potential for practical applicability in fermented milks and beverages to control the yeast related spoilages by reducing undesirable influence on sensory attributes. However, the quantity to be added and the mode of dispersion are to be addressed before advancing to commercial application.

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Antibiotic spectrum of characterized Lactic Acid Bacteria obtained from domestic *Dahi* samples

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Abstract: From forty lactic acid bacterial isolates obtained from fifteen domestic dahi samples of dahi, thirteen were found to possess probiotic characteristics such as acid and bile tolerance. These acid and bile tolerant lactobacilli isolates further, were identified phenotypically and genotypically as *Lb.rhamnosus* (5 Nos), *Lb. fermentum* (3 Nos), *Lb. plantarum* (3 Nos) and *Lb. delbrueckii* ssp. *bulgaricus* (2 Nos). The identified cultures were subjected for the antibiotic test using the disc diffusion technique against Penicillin (10ig), Gentamycin (10ig), Streptomycin (10ig), Chloramphenicol (10ig), Kanamycin (30ig) Erythromycin (15ig) and Bacitracin (10 ig). All the five strains of *Lb.rhamnosus* strains were susceptible to all the seven antibiotics tested as they showed more than 10 mm diameter inhibitory zone. Similarly all the three strains of *Lb.plantarum*, *Lb.fermentum* and *Lb.delbrueckii* ssp. *bulgaricus* were susceptible to all the antibiotics to varying degrees. In this study the results clearly demonstrate the susceptible nature of the probiotic *Lactobacilli* isolates against the commonly used antibiotics.

Keywords: Antibiotics, Genotype, Lactic Acid Bacteria, Probiotic, Spectrum

Introduction

Lactic acid bacteria (LAB) are a group of microaerophilic, Gram positive, nonspore forming organisms that ferment lactose to produce primarily lactic acid. LAB include a variety of industrially important genera such as *Lactococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus*. LAB are known to common man as starter bacteria or starter cultures. LAB are naturally present in raw food material such as milk, vegetables and also in the human gastro-intestinal tract. LAB play an important role as starter cultures for fermentation in dairy and food industries (Coskun et al. 2019). Starters are selected strains of microorganisms deliberately added to milk during conversion to dahi, yoghurt, cheese and other fermented dairy products to bring about specific changes in the appearance, body, texture and flavour characteristics of desirable final product (Annachiara et al., 2017). LAB take part in fermentation of milk, certain starters also possess health promoting benefits which are called as probiotics.

Antibiotic resistance is the most debated topic of today among the health benefiting bacteria used in functional food formulations. The antibiotic resistance genes if transferable among the common and pathogenic gut flora, there is a serious concern for using them in the food formulations. The capacity of probiotics to transfer antibiotic resistance genes is one of the most important parameters for their selection. The safety of these probiotic strains is becoming prerequisite with antibiotic resistance as an emerging issue and their potential to transfer antibiotic resistance genes to pathogenic/commensal bacteria cannot be neglected (Poonam et al. 2014). It is a well established fact that the resistance to antibiotics is plasmid coded and genes present in these plasmids can be transferred to other microbes. The probiotics normally do not carry transferable antibiotic resistance genes, since they are chromosomally encoded. Nevertheless, certain reports revealed that probiotics used in certain foods do carry the transferable antibiotic resistance genes, this resistance may be related to transposon or plasmid located genes (Mari et al. 2013). The LAB have shown resistance, intermediate and susceptibility to various antibiotics and the microbiological break point for the antibiotics have been recommended by the international organization such as European

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Food Safety Authority (EFSA) for regulating the use of probiotic bacteria in the food applications. There is a lot of interest on the antibiotic resistance of probiotic cultures. An attempt is made in the present article to detect the antibiotic spectrum of isolated probiotic strains of LAB from domestic dahi samples.

Materials and Methods

Dahi samples

Fifteen Domestic dahi samples (100 g) were collected from the locations of Atturu, Chowdeshwarinagar, Byatarayanpura, Jalahalli, Jekkur, Kempegowda nagar, Kodigehalli, Mathikere, Vidyananyapura and Yelahanka new town of Bengaluru. A total of fifteen dahi samples, three from each location were collected for further study.

Acid tolerance and Bile tolerance

MRS broth of 10 ml was adjusted pH to 2.0 using 1M HCL. The broth was inoculated with the overnight lactobacilli isolates and incubated at 37 °C anaerobically. Samples were drawn immediately and after 2 h of incubation and tested for the number of survivors by plating using MRS agar medium (Ana Carulina et al. 2018).

MRS broth containing 0.3% ox bile, were inoculated with 1% of lactobacilli isolates and incubated 37 °C anaerobically. The broth samples were immediately plated to determine the initial count at 0 h. Then the remaining broth was incubated further for 6 h and enumerated for survivors using MRS agar (Ana Carulina et al. 2018).

The selected colonies of lactobacilli from MRS agar plates showing acid and bile resistance were transferred to sterile MRS broth and incubated anaerobically at 37°C for 48 -72 h. Single and discrete colonies on the MRS agar were selected, purified thrice and maintained in soft MRS agar having 0.75 % agar-agar, appropriately numbered and stored in refrigerator as stock cultures. Once in a month, the isolates were subcultured.

Identification of acid and bile tolerant lactobacilli isolates to species level

The lactobacilli isolates obtained were identified to their species level phenotypically as per standard procedures and genotypically (Paul De Vos et al. 2011)

Screening for antibiotic susceptibility

The solidified MRS agar plate was inoculated with the isolates having density of cell 10^8 cells/ml. The inoculum was spread evenly on the entire surface of the plate by swabbing in three directions. Disc diffusion method was used to screen for the antibiotic susceptibility of isolates using discs of penicillin (10ig),

gentamycin (10ig), streptomycin (10ig), chloramphenicol (10ig), kanamycin (30ig), erythromycin (15ig) and bacitracin (10 ig) procured from Himedia, Mumbai were used in this study. Antibiotic discs were firmly applied on the surface of the dried MRS agar plates. Four antibiotic discs were placed in one plate, plates were incubated at 37 °C for 48-72 h anaerobically. The diameters of the inhibitory zones formed were measured using the calibrated scale. If the diameter of the zone formed including the disc is more than 10 mm, such isolates were considered as susceptible, while isolates which showed less than 10 mm zone noted as resistant (Fatinal et al. 2019 and Charteris et al. 2008).

Results and Discussion

All the thirteen lactobacilli acid and bile tolerant isolates (Table 1) obtained from the domestic dahi samples, when subjected for phenotypic and genotypic identification tests revealed the identity of five lactobacilli isolates - LB1, LB2, LB3, LB4 & LB5 as *Lb.rhamnosus*; three isolates - LB8, LB11 and LB12 as *Lb.fermentum* and LB9, LB14 and LB15 as *Lb. plantarum* and LB20 & LB23 as *Lb. delbrueckii* ssp. *bulgaricus* (Table 2). All the phenotypically identified lactobacilli isolates were Rods, Gram positive, Catalase negative, Non spore formers, and ARC positive in litmus milk except LB20 and LB 23 all isolated produced CO₂ from glucose, these isolates again were confirmed by the 16S rRNA gene sequencing. Nagyzbekkyzy et al. (2016) The *Lactobacillus casei* isolate 11LB obtained from the cottage cheese had highest acid tolerance of 63% at pH 3.0 after 1 at 37°C. Generally the probiotic cultures are ingested with a product containing protein, fat and prebiotic may coat the probiotic culture and action of both acid and bile on probiotics may be lesser, where as in the laboratory experiments for acid and bile, the lactic isolates are used directly without any other ingredients and hence the effect may be harsh on the isolates than when eaten with a product. Probiotic products also may contain natural prebiotics that support the viability of probiotic cultures.

It was found in the present study that *Lb. rhamnosus* LB3 and *Lb.plantarum* LB9 were found to be the most resistant strain to acid and bile conditions (2 log reductions). The significant ($P<0.05$) reduction in survival of the lactobacilli strains observed after subjecting for bile treatment, even though all the strains showed reduction in viable counts. *Lb. rhamnosus* LB3 and *Lb. plantarum* LB9 were less affected during the bile exposure after 6 h. The variability in sensitivity/resistivity to bile conditions among strains may be attributed to differences in their Bile Salt Hydrolase (BSH) activity (an enzyme which de-conjugates and decreases the digestive capability of bile) as reported (Singh et al. 2012). The findings of the study in this regard confirm the observations of several other researchers (Mishra and Prasad, 2005) who also reported sizeable variations in the bile tolerance among their probiotic strains. Maryam et al. (2009) observed that *Lb. plantarum* A7 and *Lb. rhamnosus* GG showed superior growth rates when compared to the other strains tested, but after

the addition of 0.3% (w/v) Oxgall, only *Lb. plantarum* A7 displayed the best growth ability. *Lb. acidophilus* H26 and *Lb. rhamnosus* L5K1 exhibited nearly the same bile tolerance as that of *Lb. plantarum* A7. Similarly, other researchers have reported the ability of *Lactobacillus* strains to survive simulated gastric-intestinal conditions (Pisano et al. 2014). Mari et al. (2013) have reported that the isolates obtained from commercial dairy and pharmaceutical products, 34 strains declared as probiotics, belonging to the genera *Bifidobacterium* and *Lactobacillus* and 21 strains belongs to streptococci. The identity of isolates of lactobacilli were *Lb. acidophilus*, *Lb. casei*, and *Lb. delbrueckii* ssp. *bulgaricus*. All the isolates of streptococci were confirmed as *Str. thermophilus*,

Antibiotic spectrum of *Lactobacillus* isolates

The need to assess the antibiotic spectrum of the cultures isolated in this study in order to know whether the isolates can be incorporated in products. It may be seen in Table 3 that all the five strains of *Lb. rhamnosus* LB1, LB2, LB3, LB4 & LB5 strains were susceptible to all the seven antibiotics tested as they

showed more than 10 mm diameter inhibitory zone. Similarly LB9, LB14, LB15 three strains of *Lb. plantarum*; LB8, LB11 and LB12 strains of *Lb. fermentum* and LB20, LB23 strains of *Lb. delbrueckii* ssp. *bulgaricus* were susceptible to all the antibiotics to varying degrees. These results clearly demonstrate the susceptible nature of the probiotic *Lactobacillus* starters isolated in this study towards commonly used antibiotics. From the results obtained it can be inferred that antibiotic resistant is strain specific, species specific and antibiotic specific.

Klein et al. (2000) reported that *Lactobacillus* sp. are generally susceptible to chloramphenicol, erythromycin and tetracycline. Chang Liu et al. (2009) found that 35 strains out of 41 LAB were resistant to antibiotic agents, some of which resisted multiple drugs and all isolates were susceptible to chloramphenicol, tetracycline, erythromycin, and β -lactams.

According to Mari et al. (2013), among 34 probiotic lactic strains, all the strains were susceptible to ampicillin, bacitracin, clindamycin, dicloxacillin, erythromycin, novobiocin, penicillin G, rifampicin (MIC₉₀ ranging from 0.01 to 4 μ g/ml); resistant to

Table 1: Acid and bile tolerance of the *Lactobacillus* isolates

Group	No. of Isolates	Isolate code	Acid Tolerance, Viable count log ₁₀ cfu/g		CD (P<0.05)	Bile Tolerance, Viable count log ₁₀ cfu/g		CD (P<0.05)
			Incubation time (h) 0	2		Incubation time (h) 0	6	
<i>Lactobacillus</i>	13	LB1	7.32±0.28 ^a	4.42±0.63 ^b	0.52	6.78±0.53 ^a	4.34±0.20 ^b	0.09
		LB2	7.5±0.14 ^a	2.7±0.36 ^b	0.76	7.56±0.04 ^a	4.33±0.22 ^b	0.44
		LB3	7.21±0.09 ^a	5.14±0.49 ^b	0.43	7.21±0.09 ^a	5.59±0.15 ^b	0.28
		LB4	7.43±0.40 ^a	2.37±0.55 ^b	0.91	6.87±0.11 ^a	4.87±0.32 ^b	0.55
		LB5	7.13±0.06 ^a	2.42±0.15 ^b	0.42	7.10±0.10 ^a	4.10±0.10 ^b	0.23
		LB8	8.31±0.02 ^a	3.17±0.29 ^b	0.25	7.31±0.02 ^a	4.31±0.02 ^b	0.52
		LB9	6.23±0.06 ^a	5.07±0.00 ^b	0.29	7.25±0.13 ^a	5.27±0.15 ^b	0.07
		LB11	6.72±0.11 ^a	4.13±0.08 ^b	0.51	7.05±0.13 ^a	4.13±0.35 ^b	0.6
		LB12	6.35±0.00 ^a	3.87±0.32 ^b	0.38	6.82±0.16 ^a	4.87±0.21 ^b	0.42
		LB14	6.73±0.36 ^a	3.17±0.30 ^b	0.32	6.52±0.03 ^a	4.43±0.11 ^b	0.19
		LB15	6.01±0.22 ^a	3.93±0.15 ^b	0.56	6.09±0.08 ^a	5.14±0.00 ^b	0.16
<i>Lactobacillus</i>	13	LB20	5.80±0.55 ^a	3.14±0.15 ^b	0.56	5.51±0.02 ^a	2.43±0.00 ^b	0.26
		LB23	5.60±0.00 ^a	2.77±0.00 ^b	0.38	5.60±0.06 ^a	2.40±0.00 ^b	0.38

Results are expressed as means $\pm$ standard deviation of means,

abcd means with in a column without a common superscript are statistically significantly different (P<0.05)

Table 2: Phenotypic and genotypic identification of *Lactobacillus* isolates

Sl. No.	Group	Isolate code	Gas from glucose	Growth at 45°C	Growth at 15°C	Ammonia from arginine	Fermentation Salicin Ribose	Identity
1	Lactobacilli	LB1, LB2 LB3, LB4, LB5	-	+	+	-	+	<i>Lab. rhamnosus</i>
2		LB8, LB11 LB12	+	+	-	+	-	<i>Lab. fermentum</i>
3		LB9, LB14 LB15	+	-	+	-	-	<i>Lab. plantarum</i>
4		LB20, LB23	-	+	-	+	+	<i>Lab. delbrueckii</i> <i>ssp. bulgaricus</i>

Note: All these isolates were Gram positive, rods, catalase negative, ARC in Litmus milk. Except LB20, LB23 all isolates produce CO₂ from glucose

Table 3: Antibiotic spectrum of strains of *Lactobacillus* sp.

Name of <i>Lactobacillus</i>	Name of Antibiotic				
	Pen	Str	Gen	Kan	Chl
			Antibiotic Inhibitory Zone, mm		
<i>Lab. rhamnosus</i> LB1	24.67	12.67	20.33	14.00	22.33
<i>Lab. rhamnosus</i> LB2	22.00	12.00	19.00	14.33	19.33
<i>Lab. rhamnosus</i> LB3	18.67	11.67	18.00	10.67	16.33
<i>Lab. rhamnosus</i> LB4	21.00	11.67	22.00	10.67	21.67
<i>Lab. rhamnosus</i> LB5	24.33	13.67	17.33	12.33	21.00
<i>Lab. fermentum</i> LB8	22.00	13.33	17.67	13.33	19.67
<i>Lab. plantarum</i> LB9	24.33	12.67	16.00	14.67	30.67
<i>Lab. fermentum</i> LB11	21.67	12.33	18.00	14.67	26.33
<i>Lab. fermentum</i> LB12	22.00	14.00	20.67	14.33	23.00
<i>Lab. plantarum</i> LB14	31.00	18.67	21.33	17.67	25.00
<i>Lab. plantarum</i> LB15	35.33	10.67	18.00	13.67	23.00
<i>Lab. delbrueckii</i> ssp. <i>bulgaricus</i> LB20	22.33	12.33	22.33	13.00	20.67
<i>Lab. delbrueckii</i> ssp. <i>bulgaricus</i> LB23	23.67	11.33	18.67	12.00	19.33

Pen-Penicillin (10µg), Str-Streptomycin (10µg), Gen-Gentamycin (10µg), Kan-Kanamycin (30µg),

Chl-Chlroamphenicol (10 µg), Ery- Erythromycin (15µg), Bac-Bacitracin (10 µg)

All the values are mean of three trials

aztreonam, cycloserin, kanamycin, nalidixic acid, polymyxin B and spectinomycin (MIC₉₀ ranging from 64 to 1000 ig/ml) further, susceptibility to cephalothin, chloramphenicol, gentamicin, lincomycin, metronidazole, neomycin, paromomycin, streptomycin, tetracycline and vancomycin was variable and depending on the species.

In view of the commercialization of probiotic dairy products to meet growing demands of the consumers due to their claimed health benefits, the manufacturers are using the addition of probiotics through the food carriers as the food adjuvant. However, some of the countries, especially under developed countries, are not following strict probiotic regulation. Hence, relatively there could be a chance of undefined probiotic cultures entering into the foods and making situation still adverse. One such threat may be of transferable antibiotic resistance genes through the use of undefined probiotics (Courvalin, 2006). In fact, some probiotic strains with intrinsic antibiotic resistance could be useful for restoring the gut microbiota after antibiotic treatment. However, specific antibiotic resistance determinants carried on mobile genetic elements, such as tetracycline resistance genes, are often detected in the typical probiotic genera, and constitute a reservoir of resistance for potential food or gut pathogens, thus representing a serious safety issue (Tochukwu et al. 2020). This indicates that the isolated lactic strains require not only determination of probiotic nature (acid and bile resistance) and most importantly the antibiotic spectrum also.

Conclusion

All the identified *Lactobacillus* species from the domestic *dahi* samples had varying probiotic nature and revealed varied resistance and sensitivity against the antibiotics used in this study. The antibiotics breakpoints for all the LAB is still under the developing stage and lot of research work need to be done to establish the breakpoint for each antibiotic against LAB and probiotics. The results indicated that the antibiotic resistance is species specific and also antibiotic specific. However, the genetic characteristics of antibiotic resistant and sensitivity need to be established in the further studies as may be these characters are gene specific also.

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RESEARCH ARTICLE

Association of breed and non-genetic factors with freezing point and milk attributes of Zebu cattle

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Abstract: The present experiment was conducted to study the milk quality parameters of indigenous cattle which are important for farmers, manufactures and consumers. For this study, we have collected 150 milk samples of three different indigenous cattle breeds viz. Kankrej, Sahiwal, and Rathi from semi-arid region of Rajasthan, India. The samples were evaluated for the milk fat, protein, lactose, solid not fat (SNF), somatic cell count (SCC), pH, electric conductivity and freezing point, and observed the relationship of breed, stage of lactation, parity and body condition score (BCS) with their milk constituents and freezing point. Total Protein, freezing point and electric conductivity of milk differed significantly ($P < 0.01$) among the breeds. Freezing point, fat and protein contents of milk were significantly affected by parity and body condition score of the cattle. From this study it can be concluded that the milk constituents differ among the breeds of indigenous cattle and are affected by many non genetic factors, therefore these are the factors viz. breed, parity and body condition score which influences the quality of milk in different Zebu cattle breeds.

Keywords: Body condition score, Indigenous cattle, Milk constituents, Somatic cell count, Freezing point

Introduction

Livestock play important role in national economy and livelihood in India. India is bestowed with the quality indigenous germplasm and regarded for heat tolerance and inherent resistance to diseases, and ability to sustain under extreme climatic conditions and are cost-effectively well suited to the areas where they exist (Sharma et al. 2016). An experimental facts show that zebu cattle have lower metabolic heat production which suits them well in hot climates and makes them a fairly better utilize of low quality roughages (Hansen, 2004). Milk is an essential source of nutrients such as protein, minerals and vitamins in the human diet. In dairy industries, composition of milk is important which also affect the product quality, quantity and price of the finished product. The quality of dairy products largely depends on the composition of raw milk viz. fat, protein, solid not fat (SNF), milk freezing point (MFP), pH and somatic cell count (SCC) (Kedzierska-matysek et al. 2011). Technological parameters are closely associated with texture and stability of milk products, which are primarily governing by composition of milk. Concentration of milk protein is important parameter for cheese production. The concentration of water in milk affects the MFP that is the important technological character for production of good quality ice cream well as important indicator for detection of water adulteration in milk (Kedzierska-matysek et al. 2011). Therefore, MFP is used as important parameter for milk quality assurance and it is essential to have a good estimated legislative MFP discrimination limit for the purpose of quality control (Kedzierska-matysek et al. 2011).

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Breed and non genetic factors *viz.*, environmental conditions, parity, stage of lactation, body condition score (BCS), SCC nutritional status etc. of cattle plays a significant role in production of quality milk.

The present study was to explore the variation of milk constituents in different indigenous cattle breeds and the effect of non-genetic factors on MFP and other milk constituents in semi-arid region of India.

Materials and Methods

The present study was performed at different cattle farms of Rajasthan University of Veterinary and Animal Sciences (RAJUVAS), Bikaner, India. The Bikaner city situated in middle of the Thar Desert and hot semi-arid region of Rajasthan. During summer, the temperatures can rise up to 48°C and in winter comes below the freezing point of water.

Experimental Animals

For this study, 150 indigenous lactating cattle of 3 registered breeds (50 from each breed) *viz.*, Kankrej, Sahiwal, and Rath were selected randomly and all cattle were kept under loose housing system with standard space of accommodation. Cattle were fed according to standard feeding schedule along with *ad lib* seasonally available green fodder and free access to fresh drinking water round-the-clock. Daily milking time was fixed and done at 4.00 to 5.00 AM in the morning hours and 4.00 to 5.00 PM in the evening hours. Full hand method of milking was followed for all the experimental animals and due consideration was given for clean milk production. The body condition scoring of cattle was one on 5 points scales using 0.25 unit's increments (Ferguson et al. 1994). Body condition score was measured one time at time of sample collection. The parity of all experimental animals was recorded from available farm records. All the parameters were recorded single time. Parity of animal was classified in 1,2,3 4, above 4.

Collection of milk sample

The representative milk samples were collected from all the four quarters of lactating cows as pooled sample in single time. About 100 ml of pooled milk samples of all individual cows have been collected aseptically in the sterilized sampling bottle after discarding the first 4-5 streaks of foremilk. The milk composition, pH, EC (Electrical Conductivity) was tested by an electrically operated automatic milk analyzer (Lactoscan SL30, Rajasthan Electronic and Instruments Limited, Jaipur Rajasthan). Entire study has been done using a single analytical

instrument. Milk analyzer was calibrated with raw milk using known high fat value and low fat value milk (saras dairy milk). The smear for SCC was prepared within one hour of collection. The

SCC in milk samples was done as per the method reported by Schalm et al. (1971).

Statistical Analysis

The data with respect to breeds, parity, BCS and milk constituents such as Fat, Protein, SNF, Lactose and Freezing Point were analyzed by using one-way analysis of variance. The general linear model procedure of SPSS software statistical package (version 24.0) and the means were compared by Duncan's multiple range tests. The significance level was set at 95%.

Results and Discussion

Milk attributes

Milk composition is the key indicator for the evaluation of quality and it is not constant in dairy animals and is influenced by various factors *viz.* breed, species, parity and BCS (Sarkar et al. 2006; Mushtag et al. 2012). The breed is a genetic factor which affects the production, composition and quality of milk. The data on milk composition of three breeds of cattle are presented in the table 1.

Milk Fat (%)

The total milk fat percent was statistically similar among the Kankrej, Rath and Sahiwal breeds of cattle with average milk fat percent 3.55 ± 0.15 , 3.46 ± 0.38 and 3.70 ± 0.16 , respectively. The present study results are in general agreement with the findings of Talukder et al. (2013); Pintic et al. (2007) and Ivanov et al. (2017).

Protein

The average percent milk protein was significantly ($P < 0.05$) higher in Sahiwal breed than those of Kankrej and Rath cows. Whereas, percent milk protein level was statistically similar between the Kankrej and Rath cows (Table 1). In present study, the variations in milk protein may be due to different genetic and physiological status of the dairy cattle breeds. The present study results are in line with Kebede (2018) and Falta et al. (2014).

Lactose

Lactose is the main carbohydrate component of milk. It is formed by the union of one molecule of each D-galactose (engaged by its semi-acetyl function) and D-glucose (committed by its hydroxyl 4 positions). Its concentration varies slightly in milk (4.5 to 5.2 g/100 g) contrary to the concentration of fat. Lactose percent is the least variable component of milk. The present study revealed that the breed had a non-significant difference in milk lactose content (Table 1). Adesina (2012), also found similar results among three cattle breeds such as White Fulani, Red Bororo and Muturu. Kebede (2018) observed similar results in

Holstein Friesian, Ethiopian ogaden, Jersey x Horro crosses and Holstein Friesian x Jersey x Horro crosses. Kuczyńska et al. (2012) reported non-significant effect of breeds on lactose content in Montbeliarde and Polish Holstein-Friesia. Whereas, Myburgh et al. (2012) observed contradicted finding as observed significant effect of breed on milk lactose content.

SNF

In present study, the total SNF content was significantly varied ($P<0.05$) among cattle breeds. It was highest in Sahiwal and lowest in Rathi (Table 1). The significant difference in SNF among the breeds was also observed by Bobbo et al. (2014); Falta et al. (2014); Kedzierska-Matysek et al. (2011); Kalac and Samkova (2010) and De Marchi et al. (2007).

Freezing point

The Mean $\pm$ SE values of the freezing point were -0.565 ± 0.004 , -0.556 ± 0.007 and -0.580 ± 0.004 °C in Kankrej, Rathi and Sahiwal cattle breeds, respectively. The freezing points varied significantly ($P<0.05$) among different breeds (Table 1). The present study results are in general agreement with the results of Gencurova et al. (2008) and Henno et al. (2008) who had reported that freezing point significantly influenced by breed and further they have suggested that freezing point depression was influenced by genetic (species and breed) as well as non-genetic factors such as feed composition, water intake, milking time, milk yield, lactation stage, season of the year and herd size. In present study, the lowest freezing point was observed in milk from Sahiwal and highest in Rathi. Similar results were obtained by Kedzierska-matysek et al. (2011) and observed that the MFP was significantly affected by genetic factor (breed). Brzozowski and Zdziarski (2006) observed that the significantly higher MFP in cows with high HF gene frequency (over 75%) as compared to Black-White cows or crossbreeds with a lower HF gene frequency. Hanus et al. (2008) and Gencurova et al. (2008) were found significant differences of MFP among the different breeds and it's concluded that the breed influence on freezing point might be due to the variation of milk constituents, mainly protein content. The freezing point depression is an important indicator of the milk quality,

adulteration of exogenous water and it's directly related to quality of dairy products specially ice cream preparation.

Milk pH

The mean values of milk pH were 6.52 ± 0.02 , 6.54 ± 0.01 and 6.49 ± 0.02 in Kankrej, Rathi and Sahiwal cattle breeds, respectively. The milk pH significantly ($P<0.05$) varied among breeds and found highest in Rathi and lowest in Sahiwal milk. Sahiwal significantly ($P<0.05$) differed with Rathi but Kankrej has not significantly differed with Sahiwal and Rathi (table 1). Milk pH governs by alkali and concentration of alkali varies in different breeds. Bobbo et al. (2014) also observed a significant difference in the milk pH of different cattle breeds.

Milk Electric Conductivity

The Mean $\pm$ SE of EC was 3.60 ± 0.06 , 4.17 ± 0.08 and 3.50 ± 0.07 μ S/cm in Kankrej, Rathi and Sahiwal cattle breeds, respectively and found significantly ($P<0.05$) higher in Rathi as compared to Kankrej and Sahiwal. However non-significant differences were observed between Kankrej and Sahiwal breeds (Table 1). EC measures the ability of a solution to conduct an electric current between two electrodes, and it is measured in microsemen/cm (μ S/cm). The concentration of anions and cations (Na^+ , K^+ , and Cl^-) determines the EC of milk. When a cow is exposed to an intramammary infection, EC of the milk increases due to an increased concentration of Na^+ and Cl^- in the milk. This increase is caused by the destruction of tight junctions and the active ion-pumping system (Kitchen et al. 1980). As a result of the cell damage, Na^+ and Cl^- leak into the lumen of the alveolus, and K^+ and lactose move together out of the milk. However, other factors except mastitis, like breed, parity, stage of lactation, milking interval, and milk composition may affect the EC of milk (Norberg, 2005).

Milk Somatic Cell Count

The data on mean milk somatic cell count (Log_{10} SCC) are presented in the Table 1. The SCC was statistically similar among three breeds.

Table 1: Milk Attributes of Different Cow Breeds (Means $\pm$ SE) (50 cows of each breed)

Parameters	Kankrej	Rathi	Sahiwal	Level of significance
Fat(g/100g)	3.55 ± 0.15	3.46 ± 0.38	3.70 ± 0.16	NS
Protein(g/100g)	3.38 ± 0.02^a	3.33 ± 0.03^a	3.45 ± 0.02^b	**
Lactose(g/100g)	4.65 ± 0.04	4.60 ± 0.07	4.74 ± 0.03	NS
SNF(g/100g)	8.98 ± 0.06^a	8.86 ± 0.07^a	9.19 ± 0.06^b	*
Freezing point(°C)	-0.565 ± 0.004^{ab}	-0.556 ± 0.007^a	-0.580 ± 0.004^b	**
Log_{10} SCC	5.22 ± 0.07	5.26 ± 0.07	5.27 ± 0.06	NS
pH	6.52 ± 0.02^{ab}	6.54 ± 0.01^a	6.49 ± 0.02^b	**
Electrical Conductivity (μ S/cm)	3.60 ± 0.06^a	4.17 ± 0.08^b	3.50 ± 0.07^a	**

NS NS=Non-significant;*=Significant ($p<0.05$);**=highly-significant ($p<0.01$); Superscript a, b, c differed significantly between each others

Fig. 1 Pearson correlation between Freezing point with milk Protein($r=-0.879$)

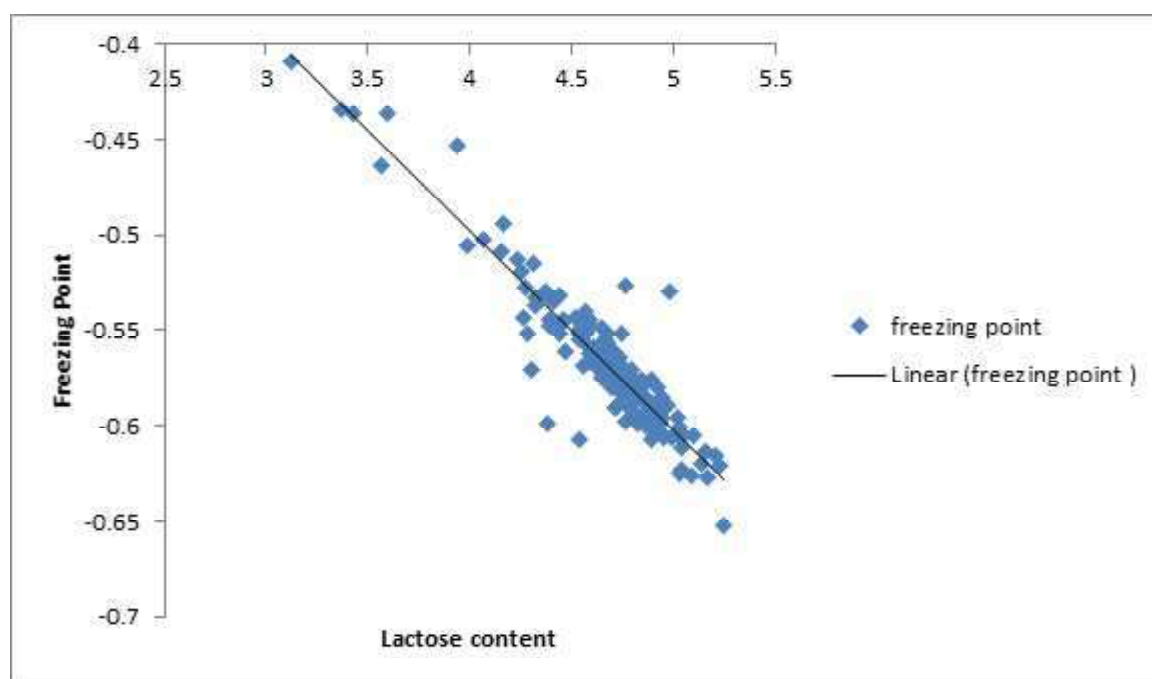
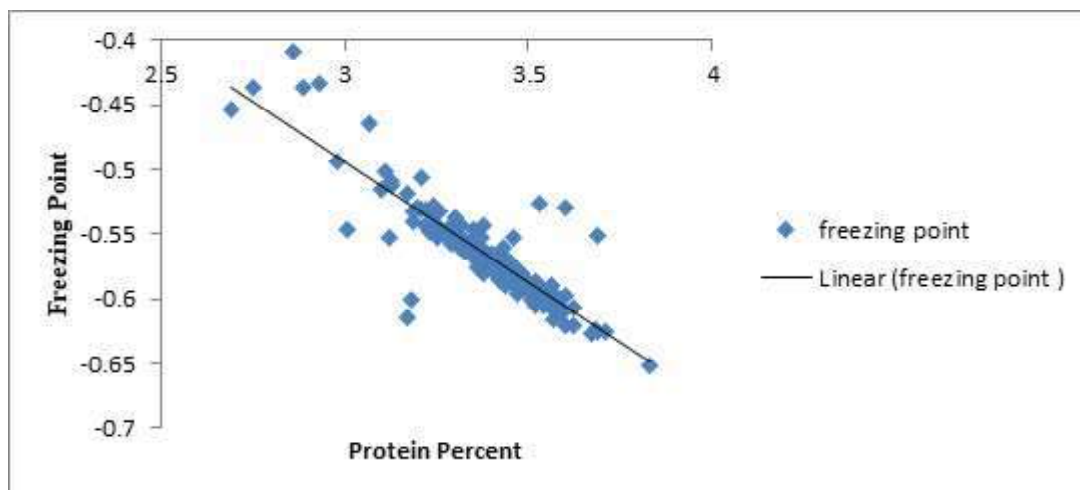


Fig. 2 Pearson correlation between Freezing point with milk Lactose($r=-0.929$)

Effect of parity on milk attributes

Freezing point

The Mean $\pm$ SE of MFP varied significantly ($P<0.05$) among lactations and increased with increase in lactation number (Table 2). MFP was lowest in the first lactation (-0.584°C) and highest in above fourth lactations (-0.555°C). MFP was significantly ($P<0.05$) differed in first, second, third, fourth and above fourth lactations. Similar results were reported by Otwinowska-Mindur et al. (2017); Kedzierska-matysek et al. (2011); Brzozowski and Zdziarski (2005) and observed that MFP increased with lactation number. Sawa and Oler (1999) found that the aging of animal was associated with an increase in freezing point depression values.

This might be due to with increase in number of lactation, milk yield increases up to fourth lactation. The water content in milk would be higher in advanced lactations which cause increasing MFP. So, milk would freeze faster. Kedzierska-Matysek et al. (2011) also observed that the MFP increased with number of lactation.

Milk constituents

Protein and SNF differed significantly ($P<0.05$) between parities and subsequently decrease with advanced parities (Table 2). Protein and SNF were found lowest ($P<0.05$) from fourth lactation. The SCC was significantly ($P<0.05$) increased with parity (Table 2). Yang et al. (2013) also observed milk protein percent and milk

Fig.3 Pearson correlation between Freezing point with BCS($r=-0.294$)

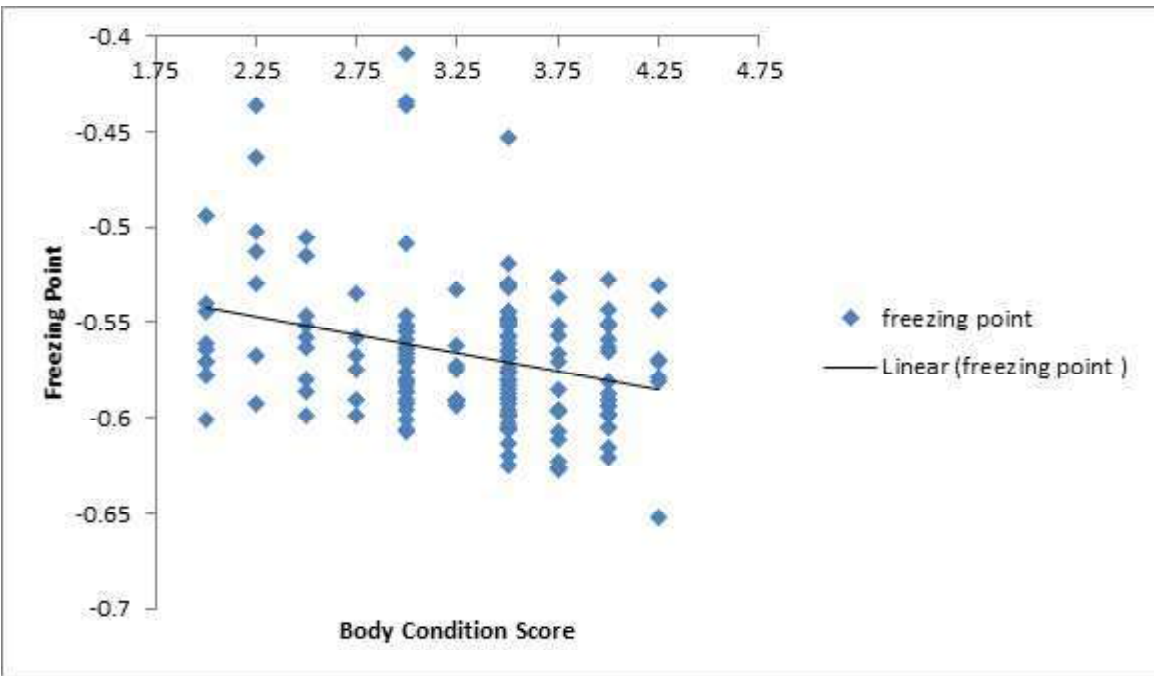


Table 2: Freezing point, milk composition and log₁₀ SCC in successive lactations of cows (50 cows of each breed)

Parity	Freezing Point	Protein	SNF	Lactose	Log ₁₀ SCC
1	-0.584±.005	3.49±.022	9.27±.060	4.76±.059	5.089±.093
2	-0.571±.007	3.42±.028	9.09±.078	4.70±.066	5.140±.070
3	-0.579±.008	3.41±.049	9.03±.124	4.76±.068	5.493±.104
4	-0.565±.007	3.38±.035	8.99±.093	4.63±.058	5.296±.081
Above 4	-0.555±.005	3.33±.025	8.87±.066	4.58±.051	5.245±.068
	P<0.05	P<0.05	P<0.05	NS	P<0.05

Table 3: Pearson’s correlation coefficients among freezing point, body condition score and milk constituents in cows

		BCS	Fat	Protein	Lactose	SNF	Freezing point	Log ₁₀ SCC
Freezing point	Pearson correlation	-0.294**	0.487**	-0.879**	-0.929**	-0.891**	1	0.332**
	Sig. (2- tailed)	.000	.000	.000	.000	.000		.000
	N	150	150	150	150	150	150	150
	Pearson correlation	1	0.047	0.381**	0.277**	0.344**	0.294**	-0.023
BCS	Sig. (2- tailed)		.565	.000	.001	.000	.000	.782
	N	150	150	150	150	150	150	150

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

solid percent decrease with parity and SCC increase with parity in Chinese Holstein cows.

Relationship between milk freezing point and milk constituents

The MFP was significantly ($P<0.01$) affected by milk constituents mainly lactose, Protein and SNF (Table: 3. Figure 2 and 3). Protein, Lactose, and SNF were significant ($P<0.01$) negatively correlated with freezing point. The lowest freezing point of milk is characterized by the highest concentrations of lactose, protein

and SNF. Kedzierska-matysek et al. (2011); Henno et al. (2008); Brzozowski and Zdziarski (2005) also found that the MFP was increase with decreasing of protein and lactose concentration in milk.

Relationship between milk freezing point and somatic cell count

The milk SCC significantly ($P<0.01$) correlated with freezing point and the milk with higher log₁₀ SCC had a higher freezing point (table 3). Kedzierska-matysek et al. (2011); Barowska et al. (2009) and Sawa and Oler (1999) also observed increased MFP with

SCC and said that it was due to changing of chemical composition of milk. Brzozowski and Zdziarski (2006) were observed that the MFP increased with increase in SCC and it was a main indicator of mastitis.

Relationship between milk freezing point and body condition score

BCS is a widely used for evaluating the nutritional status of dairy animals. Milk quality was influenced by BCS of animals. The BCS of animals was significantly ($P < 0.01$) correlated with MFP and milk constituents (table: 3). Figure 4 illustrates the MFP was influenced by the BCS of animals. Animal having higher BCS produced milk of lower freezing point because it was having higher protein, lactose, and SNF. BCS was significant ($P < 0.01$) positively correlated with protein, lactose, and SNF (table: 3). Hossain et al. (2015) also found a positive relationship of BCS with protein; lactose and SNF in Holstein Friesian crossbred dairy cows. However, with increase in milk production the energy requirements exceed its intake and cow goes in negative energy balance and initiates to mobilize lipid reserves by losing their BCS. Therefore, the milk production increased linearly and BCS tended to decrease gradually at the expense of reserved body fat.

Conclusions

The present study was demonstrated by the comparison of milk composition and technological properties of different indigenous cattle breeds viz. Kankrej, Sahiwal and Rathi. The breeds have significant effect on milk composition and technological properties. The milk composition and EC significantly differed among the different cattle breeds. The MFP was affected by breed, parity, SCC, BCS and milk constituents. Very scanty information is available for indigenous cattle milk. Therefore, much research is needed to develop some quality standards for global promotion of milk from indigenous cattle.

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Comparative study on the expression profile of Aquaporin5 (AQP5) gene in skin fibroblast cells of native goats enabling their water utilization efficiency

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Abstract: Erratic rainfall and climate change has resulted in the water crisis leading to deleterious effects in the agricultural sector especially on livestock population. In this scenario, native breeds adapted to sustain their life in water deficit areas without compromising their productivity come to play a role. Breeds native to arid and semiarid regions have developed genetic adaptations for the efficient water use by making alterations in osmoregulatory genes like Aquaporin (AQP). While most of the expression studies of AQP genes have been limited to humans, mice and rats. Hence, the present study was conducted on the native goat breeds-Barbari and Sirohi, to know the variations in the relative expression profile of AQP5 mRNA on goat skin fibroblasts during different temperature and osmotic concentration. The relative expression of AQP5 mRNA was decreased during low temperature in both the breeds. Whereas, the relative mRNA expression of AQP5 gene in Sirohi breed was found to be increased significantly ($P<0.05$) than Barbari at high temperature. The skin fibroblast cells of both the breeds exposed to hyperosmotic medium at 37°C showed increase in the relative expression of AQP5 mRNA and mRNA expression value of Barbari was found to be significantly higher ($P<0.05$) than Sirohi breed. The skin fibroblast

cells of Sirohi and Barbari breed subjected to hyperosmotic medium at higher temperature demonstrated a decrease in the relative expression of AQP5 mRNA showed the potential of survival in both the breeds during extreme climatic conditions.

Keywords: AQP5, Barbari, Hyperosmotic, Sirohi, Skin fibroblasts

Introduction

A multidisciplinary change in global climate along with regional climatic patterns impose a serious threat to the livestock population (Ali et al. 2020). At present, due to climate change water has become a very limiting resource affecting the sustainability of livestock and agriculture (Malley et al. 2009; Tarawali et al. 2011). Therefore, in future days the water availability will become more limited due to the intensified effects of climate change and unpredictable weather resulting in irregular rainfalls (Jaber et al. 2013). The livestock especially the small ruminants that are adapted to the arid regions have the ability to select high quality forages from the scanty vegetation prevailing in these regions and thus able to maintain a relatively similar basal diet quality in water deficit areas. Small ruminants, especially tropical breeds are more tolerant to feed and water scarcity and demonstrate several adaptive responses to cope up with adverse environmental conditions (Aziz, 2010). Small ruminants surviving in the arid and semi-arid regions, especially goats can remain in a state of dehydration for several days when they face water scarcity in summer season (Shkolnik and Choshniak, 1985; Francis et al. 2020). Breeds native to the arid and semi-arid region have developed several adaptive mechanisms over years that helps them to conserve water during water stress conditions (McNab, 2002). Fitness and adaptation are influenced by genetic make-up and it determines an animal's tolerance to adverse conditions such as high temperature, water scarcity and drought (Naskar et al. 2012).

Most of the studies on the genetic adaptations of animals to arid regions are limited to the expression studies of genes like HSP, ACT, ACE, etc. Very few studies are undertaken on the expression pattern studies of AQP (Aquaporin) gene. Aquaporins are the family of membrane bound proteins that are extensively

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distributed in microorganisms (Calamita, 2000), animals (Agre et al. 2002; Agre and Kozono, 2003; King et al. 2004) and plants (Johansson et al. 2000; Schaffner, 1998) that functions in the transport of water and small solutes across the cell. These 13 aquaporins identified so far are divided into two main groups: Orthodox aquaporins (AQP1,2,4,5 and 8) that selectively transports water and Aquaglyceroporins (AQP3,7,9 and 10) that are pervious to water and small solutes like glycerol and urea (Campos et al. 2011; Boury-Jamot et al. 2006). Aquaporin (AQP) water channels are distributed throughout different tissues in the body and regulate transcellular water transport (Verkman et al. 1996; King and Agre, 1996; Francis et al. 2020). The distribution and expression pattern of AQP genes vary between tissues, breed and species. Our earlier in vitro study on AQP 3 gene expression in the skin fibroblast cells of native goats revealed higher expression of AQP3 gene in Sirohi breed adding to its survivability in water stressed arid regions (Francis et al. 2020).

Though several studies have been piloted on the tissue distribution, cellular localization, regulation, structure and function of mammalian AQPs, scanty studies have been found in animals on the expression of AQP 5 and its osmoregulatory role in animal skin. A full-fledged knowledge about the variation in the expression of AQP gene and its osmoregulatory role is required, so as to exploit full genetic potential of native breeds that are well adapted to water scarce regions. Therefore the present study was conducted in-vitro in the skin fibroblast cells of native Barbari and Sirohi breeds of goat to have an insight on expression profile of AQP5 gene under different temperature and osmotic concentrations.

Materials and Methods

Fibroblast cell culture and treatment

Skin fibroblast culture of ear skin explants were established in the laboratory. Skin explants used for the establishment of fibroblast culture were collected from the adult native Barbari and Sirohi breeds of goat. The culture media used for the skin fibroblast growth contained Dulbecco's Modified Eagle's medium (Sigma Aldrich, USA), 1% Penicillin- Streptomycin antibiotic solution (Himedia, Mumbai) and 20% Fetal Bovine Serum (Sigma Aldrich, USA). The skin fibroblasts were then sub-cultured in the culture media at 37 °C and 5 % CO₂. When the cell culture attained confluency, the fibroblasts cells were passaged using 0.25% trypsin – EDTA solution (Thermofischer scientific, USA) at a split ratio of 1:3. The obtained confluent culture of skin fibroblasts after the 4th passage was used for carrying out the 3 different temperatures and 2 hyperosmotic concentrations treatments. The cultured skin fibroblast cells of Barbari and Sirohi breed was divided into 5 groups and treated as follows:

Treatment 1: The cell culture flask containing confluent skin fibroblast cells was incubated at 25°C in the CO₂ incubator for 3

hours. These fibroblast cells were grown in cell culture media supplemented with 20% FBS (Sigma Aldrich, USA) and 1 % penicillin- streptomycin- amphotericin B solution (Himedia, Mumbai).

Treatment 2: The cell culture flask containing confluent skin fibroblast cells was incubated at 37°C in the CO₂ incubator for 3 hours. These fibroblast cells were grown in cell culture media supplemented with 20% FBS (Sigma Aldrich, USA) and 1 % penicillin- streptomycin- amphotericin B solution (Himedia, Mumbai).

Treatment 3: The cell culture flask containing confluent skin fibroblast cells was incubated at 42°C in the CO₂ incubator for 3 hours. These fibroblast cells were grown in cell culture media supplemented with 20% FBS (Sigma Aldrich, USA) and 1 % penicillin- streptomycin- amphotericin B solution (Himedia, Mumbai).

Treatment 4: The cell culture flask containing confluent skin fibroblast cells was incubated at 37°C in the CO₂ incubator for 3 hours. These fibroblast cells were grown in hyperosmotic cell culture media supplemented with 20% FBS (Sigma Aldrich, USA) and 1 % penicillin- streptomycin- amphotericin B solution (Himedia, Mumbai).

Treatment 5: The cell culture flask containing confluent skin fibroblast cells was incubated at 42°C in the CO₂ incubator for 3 hours. These fibroblast cells were grown in hyperosmotic cell culture media supplanted with 20% FBS (Sigma Aldrich, USA) and 1 % penicillin- streptomycin- amphotericin B solution (Himedia, Mumbai).

Hyperosmotic cell culture media was prepared by the addition of 100mM NaCl to the normal cell culture medium so as to mimic dehydration conditions in cells. The samples for two breed were treated in triplicate at each treatment. The skin fibroblast cells in normal culture medium at 37°C were taken as the control sample.

RNA isolation and RT- PCR

Total RNA was isolated from the treated skin fibroblast cell by miRNeasy kit (Qiagen, U.S). Total RNA concentrations obtained were measured by Nanoquant and those RNA samples isolated having purity between 1.9 and 2.0 were only used further for cDNA synthesis so as to avoid protein contamination. The quality and integrity of the obtained RNA was analysed by 1.5% agarose gel electrophoresis and illustrated two distinct bands, one for 18S and other for 28S of RNA. First strand cDNA was prepared by using Thermoscientific RevertAid First Strand cDNA Synthesis kit

Relative mRNA expression of AQP5 gene was studied by quantitative real time-PCR (qPCR) using SYBR® Green qPCR kit. A master mix for qPCR was prepared as per the following

components: Maxima SYBR Green qPCR Master Mix(2X) - 5 µl, reverse primer - 0.5 µl, forward primer - 0.5 µl, cDNA sample - 2 µl and nuclease free water - 2 µl. The optimum annealing temperatures (T_m) for AQP5 gene and GAPDH (housekeeping gene) were determined by performing gradient PCR of 55°C to 65°C temperature range. The sequence information of AQP5 gene was retrieved from the NCBI database and suitable primers were designed using primer 3 web interfaces. The primer sequence and annealing temperature of the genes are given in Table 1.

PCR reactions were carried out using following protocol: (50°C X 2 min; 95°C X 10 min; 95°C X 30 s; T_m of gene X 30 s; 72°C X 30 s; 95°C X 1 min; 55°C X 30 s; 95°C X 30 s) X 25 cycles. Amplification plots and dissociation curves for all reactions were analyzed for the presence of primer dimers or secondary structures or non-specific amplification. The relative expression of AQP3 was analyzed using $\Delta\Delta C_t$ method by keeping GAPDH as the reference gene and 37°C in the normal culture medium as the control sample.

Statistical analysis

The statistical significance of variations in relative expression of the AQP3 mRNA in Barbari and Sirohi breed during different treatments was assessed by two-way ANOVA. A difference with value $P < 0.05$ was considered statistically significant

Results and Discussion

Gene expression analysis

The relative expression of mRNA for AQP5 gene in Barbari and Sirohi breeds during different temperature and hyperosmotic treatments is presented in Table 2 and Fig 1. Relative mRNA expression of AQP5 gene in treatment 2 was taken as the calibrator. The relative expression of AQP5 mRNA was

observed to be lower during treatment 1 in both the Barbari and Sirohi breeds of goat (Fig 1). In both the breeds, the relative expression of AQP5 mRNA was found to be increased during treatment 3 (42°C) (Table 2). However, the mRNA expression value was significantly higher ($P < 0.05$) in Sirohi breed compared to the Barbari breed. During treatment 4 (hyperosmotic medium at 37°C) on the skin fibroblast cells of Barbari and Sirohi breeds, the relative expression of AQP5 mRNA was observed to be increased and mRNA expression value of Barbari breed was significantly higher ($P < 0.05$). The relative expression of AQP5 mRNA in skin fibroblast cells of Sirohi and Barbari breed was found to be decreased during treatment 5. The relative mRNA expression profile of AQP5 gene was observed to be similar in both the Barbari and Sirohi breed skin fibroblast cells (Figure 1).

Most of the studies on AQP 5 gene expression have been carried out in humans, rats, mice, etc. No study has been conducted to rule out the expression of AQP5 gene in skin fibroblast cells of goats. This is the first study that gives an insight into the expression of AQP5 gene in goat skin fibroblast cells and variations in the expression profile between Barbari and Sirohi breed. The study was performed on the cultured skin fibroblast cells of Barbari and Sirohi breeds of goat. The relative expression of AQP5 mRNA of treatment 2 having the optimum temperature and normal osmotic concentration was considered as standard. A variation was observed in the relative expression of AQP5 mRNA in all treatment conditions other than 2. This may be due to the stress observed on the skin fibroblast cells.

The relative mRNA expression of AQP5 gene was found to be increased at high temperature in both the Barbari and Sirohi breeds. The increase in the relative mRNA expression of AQP5 gene with increasing temperature may be an adaptive mechanism evolved by the animals to overcome the heat stress through evaporative water loss as AQP5 is a water specific channel protein

Table 1 Gene transcripts, primer sequence and their product size where F: Forward Primer. R: Reverse Primer. T_m : Annealing Temperature

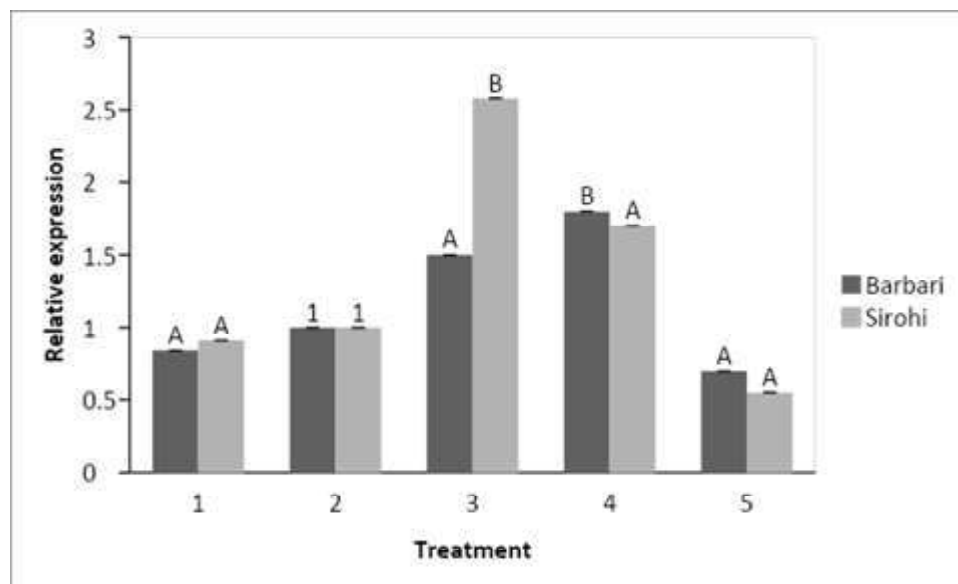
S. No	Primer name	F/R	Gene Sequence(5'-3')	T_m (°C)	Product size (bp).
1	AQP5	F	GATCACTGAGGGACCGAAAGATCGG	62	179
		R	ACTCCCGCCGCACCAGCCCTGT		
2	GAPDH	F	CCAACGTGTCTGTTGTGGATCTGA	62	218
		R	GAGCTTGACAAAGTGGTCGTTGAG		

Table 2 Relative expression values of AQP5 mRNA in skin fibroblast cells during different treatment conditions

Treatment	AQP5 gene				
	1	2	3	4	5
Barbari	0.84 ^A ±0.16	1.0±0	1.50 ^A ±0.26	1.80 ^B ±0.49	0.70 ^A ±0.19
Sirohi	0.91 ^A ±0.18	1.0±0	2.58 ^B ±0.27	1.70 ^A ±0.29	0.55 ^A ±0.11

The values are mean± SE, N= 3, ^{ABC}Bars with different superscripts are significantly different ($p < 0.05$) between treatments

Fig. 1 the relative mRNA expression analysis of AQP5 gene by quantitative polymerase chain reaction (qPCR) in skin fibroblast cells of Barbari and Sirohi breeds of goat during different treatment conditions (1,2,3,4 and 5) in *in-vitro* studies. Different superscripts denote statistically different values ($P<0.05$) in comparison to treatment 2.



of the membranes that is found to be associated with sweat physiology in the skin (Nejsum et al. 2002). But during the hyperosmotic conditions in the cell culture medium the relative expression of AQP5 mRNA was found to be decreasing with increase in the temperature. This reduction in the relative expression of the AQP5 mRNA during hyperosmotic condition helps to reduce the sweat production thus resulting in decreased water loss. Sirohi breeds had lower expression of AQP5 mRNA during hyperosmotic stress conditions suggesting that water loss through evaporation was more in Barbari breed. This reduced expression may be one of the reasons for this arid adapted goat breed for the better regulation of water balance. Increased expressions of AQP5 have been reported in *Xenopus tropicalis* when the animal is dehydrated (Shibata et al. 2014). The effects of osmotic stress on AQP5 expression have not been studied in detail (Hoffert et al. 2000).

Conclusions

The present study presents the expression of AQP5 mRNA in the Barbari and Sirohi breeds of goat and the expression profile of AQP5 demonstrates its breed specific character. The relative expression of AQP5 mRNA was found to be increased with increasing temperature in both the breeds. In hyperosmotic conditions, the decrease in the expression of AQP5 mRNA with an increase in the temperature helps the arid adapted goat breeds for their efficient water use. In comparison to the Barbari breed, Sirohi breed showed lower expression of AQP5 mRNA demonstrating its high endurance to thrive well in the water deficit areas. As these studies are conducted in *in-vitro* conditions, more *in-vivo* experiments are suggested for the better understanding of the role of the AQP5 gene in water regulation.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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RESEARCH ARTICLE

Supplementary effect of *K. alvarezii* based seaweed product on milk production, its composition and organoleptic appraisal in crossbred cows

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Abstract: Eighteen crossbred cows were divided into 3 groups of 6 animals each based on milk yield, body weight, parity and days in milk to study the effect of supplementation of *K. alvarezii* based seaweed product (SWP) on feed consumption, milk production and composition for a period of 150 days. The cows in group T₁ were fed rations as per their nutrient requirements (ICAR, 2013). The cows in groups T₂ and T₃ were fed the similar rations as in control group (T₁), however, the diets were supplemented with 1.5 and 3% of *K. alvarezii* based SWP (*K. alvarezii* powder: *Gracilaria salicornia* powder: *K. alvarezii* sap powder in 1: 1: 1 ratio) on DM basis through concentrate mixture in groups T₂ and T₃, respectively. Daily feed consumption, milk composition and organoleptic appraisal of milk were not influenced by SWP supplementation, however, persistency of lactation seemed to be better in group 3 supplemented with 3% SWP in the ration of crossbred cows particularly post 5th fortnight after start of the experiment.

Keywords: Crossbred cows, *K. Alvarezii*, Milk production and composition, Organoleptic evaluation, Seaweed

Introduction

Brown algae (Phaeophyceae), red algae (Rhodophyceae) and green algae (Chlorophyceae) are the common types of seaweeds (Chapman and Chapman, 1980; El Gamal, 2012). There are 434 species of red seaweeds (Rhodophyta), 194 species of brown seaweeds (Phaeophyta) and 216 species of green seaweeds in India (Modayil, 2004). Two red seaweed species i.e. *Kappaphycus alvarezii* and *Gracilaria salicornia* are important ones used for carrageenan production, which is mostly utilized as thickening agent and stabilizer in food industry (Sade et al. 2006; Pang et al. 2010, Mondal et al. 2015). Seaweeds and their by-products after extraction of carrageenan can be used as animal feed. The total production of seaweeds in world and India in 2018 was 32,386.2 thousand tonnes and 5.3 thousand tonnes, respectively, on fresh weight basis which for India comes out to be 0.02% of world's total seaweed production. China has the largest share with 57.36% of world seaweed production followed by Indonesia with 28.78% share (FAO, 2020).

The study of Franklin et al. (1999) showed that DM intake was significantly reduced due to supplementation of seaweeds in rations of milch cows. The cell walls of seaweeds contain alginic acid which has characteristics of forming viscous gel resulting in reduction in feed intakes (Beresford et al. 2000). Addition of brown seaweed by-products @ 2 or 4% in diet did not affect feed intake (Hong et al. 2015). Bendary et al. (2013) reported increased milk yield when dairy cows were supplemented with the mixture of seaweed and premix. Seaweed supplementation increased milk yield in high yielding dairy cows (Hostens et al. 2011). Contents of milk protein, milk fat, lactose, SNF were shown to be similar in Holstein cows given 0, 2 or 4% brown seaweed by product in diets of Holstein cows (Hong et al. 2015). Some researchers (Arieli et al. 1993; Ventura and Castanon 1998; Hasen et al. 2003; Wang et al. 2008; Bozic et al. 2009; Machado et al. 2014; El-Waziry et al. 2015) evaluated individual seaweeds, their combinations or their supplementary effect particularly while using brown and green algae and there is very scanty information available on red seaweeds (Sharma et al. 2019). Thus, the present study was undertaken to investigate the effect of inclusion of newly developed *Kappaphycus alvarezii* based seaweed product

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(SWP) in the diets of crossbred lactating cows on feed intake, milk production, its composition and organoleptic appraisal.

Materials and Methods

Experimental site and approval of animals for ethical considerations

All the animals were housed in the experimental shed of Animal Nutrition Division, Livestock Research Centre of ICAR-National Dairy Research Institute, Karnal situated at an altitude of 250 meter above mean sea level, latitude and longitude position being 29°42' N and 79°54' E, respectively. The maximum ambient temperature in summer goes up to 45°C and minimum temperature in winter goes down to about 4°C with a diurnal variation to the order of 15-20°C. Approval for conducting the study was sought from Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Govt. of India (386/01/ab/CPCSEA).

Animals and their feeding

Eighteen crossbred cows were procured and divided into 3 groups of 6 animals each based on milk yield, body weight, parity and days in milk (Table 1). Proper cleanliness and healthy surroundings were ensured throughout the experimental period. Deworming of animals was done before the feeding trial. All the animals were fed rations consisting of sugargraze (sorghum × sorgho × sudan grass hybrid) and concentrate mixture (% parts: maize 35, groundnut cake 15, soybean meal 15, wheat bran 32, mineral mixture 2, and common salt 1) as per their requirements (ICAR, 2013) in control group (T₁). However, the animals in groups T₂ and T₃ were also supplemented with 1.5 and 3% of *K. alvarezii* based seaweed powder (*K. alvarezii* powder: *Gracilaria salicornia* powder: *K. alvarezii* sap powder in 1: 1: 1 ratio), respectively on dietary DM basis through concentrate mixture. The SWP was obtained from AQUAAGRI Processing Pvt. Ltd., Manamadurai, Tamilnadu, India.

The animals were weighed before feeding and watering in the morning for two consecutive days at the start of experimental feeding and thereafter at fortnightly intervals during the experimental period of 150 days. DM intake was recorded daily by subtracting the residual DM from the quantity of DM offered.

Chemical analysis of feed samples

The samples of feeds offered and residues were collected daily and estimated for DM content by keeping in hot air oven at 65°C until constant weight, ground to pass through 1 mm sieve and analysed for proximate principles viz., DM, OM, CP, EE and total ash (AOAC, 2005) and cell wall constituents viz., NDF and ADF (Van Soest et al. 1991).

Milk composition and sensory evaluation

Milking was done in the morning at 5:00 h and evening at 17:00 h and milk was collected in milking vessels after screening through muslin cloth. Milk yield was recorded with help of digital platform weighing balance with accuracy of ±0.05 kg. Milk samples were analysed for fat, protein, lactose and solid-not-fat (SNF) contents at monthly intervals using pre-calibrated milk analyser (Lactostar, FUNKE GERBER, Article No 3510, Berlin).

For organoleptic test/sensory evaluation, individual milk sample from each animal was pasteurised in the laboratory and evaluated for quality by sensory evaluation by a panel of experts (n=3) from Dairy Technology Division, ICAR-NDRI, Karnal. Milk samples (~20 mL, presented in glass containers) were scored for quality attributes like flavour, consistency, colour and appearance as well as overall acceptability using 9-point hedonic scale (Peryam and Pilgrim, 1957).

Statistical analysis

The data were analyzed by two-way ANOVA as per Snedecor and Cochran (1994) using software package SPSS version 20.0 (2012).

Results and Discussion

Chemical composition of feeds offered to the animals

The chemical composition of different feed ingredients has been given in Table 2. The SWP contained 27.45, 5.58, 1.98, 72.55, 15.03 and 9.92% of OM, CP, EE, total ash, NDF and ADF, respectively. *K. alvarezii* collected from Semporna, (Malaysia) contained 66.66, 23.25, 5.35, 4.50, 0.23% of CHO, total ash, protein, fibre and lipid, respectively (Ahmad et al. 2012) indicating that type of seaweed or formulated products affect the chemical composition. The ingredient composition SWP used in this study has been explained in the previous section.

Fortnightly feed consumption

The body weights at the beginning of experiment were 415.86, 403.38 and 406.63 kg in treatments T₁, T₂ and T₃, respectively with corresponding values of 426.88, 417.19 and 418.40 kg at the end of 150 days of experiment. The DM intake varied from 11.10 to 13.19, 11.06-12.86 and 11.07-13.30 kg/d in treatments T₁, T₂ and T₃, respectively with corresponding values of 2.63-3.13, 2.56-3.12 and 2.65-3.21 kg/100 kg BW across 10 fortnights. The overall average DM intake in groups T₁, T₂ and T₃ was 12.22, 11.66 and 12.00 kg/d, respectively while the corresponding values of DM intake were found to be 2.91, 2.84 and 2.92 expressed on kg/100 kg BW basis. The feed intake (kg/100 kg BW) was found to be similar in all 3 groups irrespective of level of seaweed product supplementation (Fig. 1). Singh et al. (2016) also reported no

Fig. 1 Effect of supplementation of *K. alvarezii* based SWP on DM intake (kg/100 kg BW)

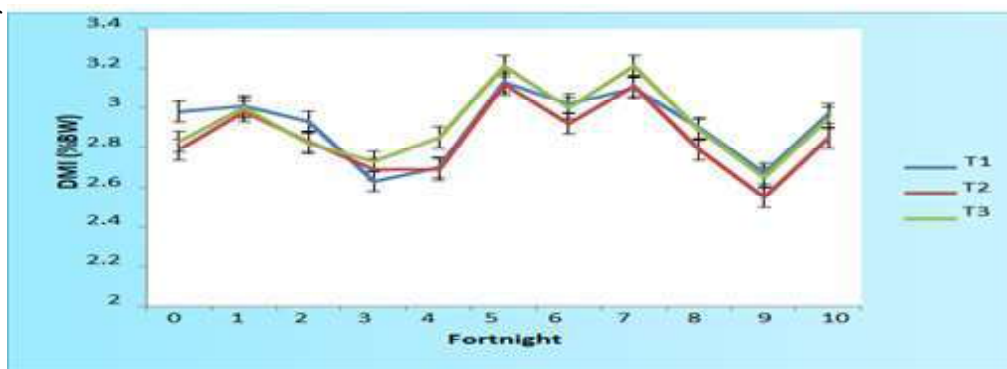


Fig. 2 Effect of supplementation of *K. alvarezii* based SWP on fortnightly milk yield (kg/d)

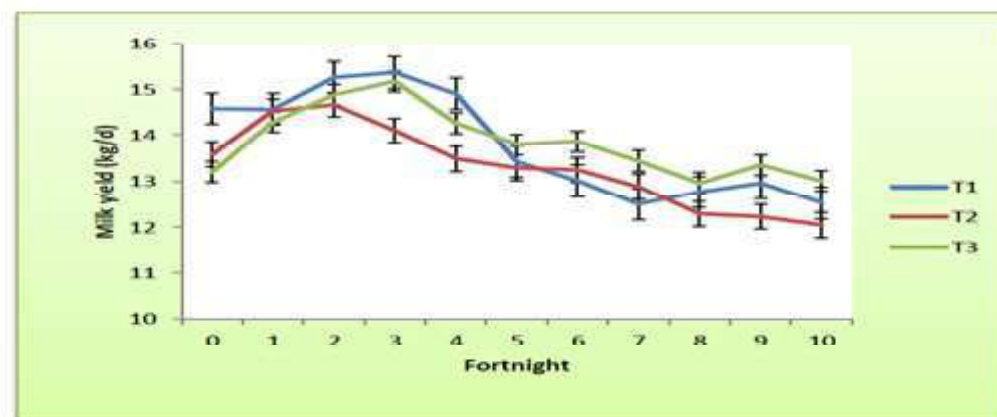


Table 1 Description of experimental animals

Group	Initial BW (kg)	Average milk yield (kg/d)	Days in milk	Parity
T ₁	415.86±5.35	14.58±0.61	52.66±5.99	2.16±0.30
T ₂	403.38±5.76	13.58±0.74	52.16±3.33	2.00±0.25
T ₃	406.63±4.15	13.20±0.30	51.83±0.60	1.83±0.30

Table 2 Chemical composition of feed ingredients

Parameter	Concentrate mixture	Sugargraze	Seaweed product
Proximate composition (% DM basis)			
DM	89.05	30.99	94.60
OM	93.12	92.83	27.45
CP	19.16	7.31	5.58
TA	6.88	7.17	72.55
EE	4.76	2.44	1.98
Cell wall constituents (% DM basis)			
NDF	27.37	56.72	15.03
ADF	13.33	32.69	9.92

significant effect on DM intake in lactating Sahiwal cows given *Sargassum wightii* seaweed powder in concentrate mixture at 20% level. On the other hand, Al-Shorpey et al. (2001) reported that supplementation of seaweed meal @ 1% in lambs diet increased DM intake, however, FCR was better in group given no seaweed meal.

Milk production

The milk yield at the beginning of experiment was 14.58, 13.58 and 13.20 kg/d in treatments T₁, T₂ and T₃, respectively. The milk yield started decreasing after 4th fortnight of beginning of experiment till the end of experiment. The overall average milk yield across 10 fortnights in treatments T₁, T₂ and T₃ was 13.86, 13.31 and 13.84 kg/d, respectively. The milk yield was found to be similar in all the treatments irrespective of level of seaweed product addition in the diet. However, the persistency of lactation was

Table 3 Effect of supplementation of *K. alvarezii* based feed additive on milk yield (kg/d)

Fortnight	Group		
	T ₁	T ₂	T ₃
0	14.58±0.61	13.58±0.74	13.20±0.30
1	14.57±0.56	14.53±0.63	14.28±0.42
2	15.26±0.62	14.67±0.59	14.89±0.49
3	15.37±0.68	14.10±0.54	15.18±0.51
4	14.91±0.66	13.49±0.51	14.26±0.31
5	13.42±0.63	13.29±0.37	13.79±0.43
Mean (0-5 fortnight)	14.69±0.29	13.94±0.24	14.27±0.29
6	13.01±0.76	13.25±0.31	13.86±0.40
7	12.50±0.83	12.88±0.26	13.44±0.40
8	12.77±0.92	12.29±0.29	12.96±0.46
9	12.96±0.98	12.23±0.55	13.35±0.48
10	12.53±0.89	12.05±0.47	13.01±0.44
Mean*(6-10 fortnight)	12.75 ^a ±0.23	12.54 ^a ±0.11	13.32 ^a ±0.16

^{a,b}Values bearing different superscripts in a row differ significantly (P<0.05)

Table 4 Sensory evaluation of milk in different groups

Month	Group	Sensory attribute			
		Flavour	Consistency and texture	Colour and appearance	Overall acceptability
0	T ₁	8.5	8.5	8.5	8.5
	T ₂	8.5	8.5	8.5	8.5
	T ₃	8.5	8.5	8.5	8.5
1	T ₁	8	8	8	8
	T ₂	8	8	8	8
	T ₃	8.5	8.5	8.5	8.5
2	T ₁	8	8	8	8
	T ₂	8	8	8	8
	T ₃	8	8	8	8
3	T ₁	8.5	8.5	8.5	8.5
	T ₂	8.5	8.5	8.5	8.5
	T ₃	8.5	8.5	8.5	8.5
4	T ₁	8	8	8	8
	T ₂	8	8	8	8
	T ₃	8	8	8	8
5	T ₁	8.5	8.5	8.5	8.5
	T ₂	8	8	8	8
	T ₃	8	8	8	8

better (P<0.05) in group T₃ particularly after 5th fortnight (Table 3, Fig. 2) compared to other two groups. The SWP supplementation had no significant effect on milk yield expressed in different ways. However, Singh et al. (2015) reported increase in milk yield and 4% FCM yield in lactating Sahiwal cows given *Sargassum wightii* seaweed powder in concentrate mixture at 20% level. *Sargassum* sp. of seaweed could be fed up to the extent of 30% in the concentrate mixture without any significant changes in milk yield and FCM production in Kankrej cows (Desai and Shukla, 1975). Supplementation of *A. nodosum* @ 80 g/cow/day (Karatzia et al.

2012) or hydrolyzed *A. nodosum* (Cermak et al. 2011) did not affect milk production in cows. Increase in milk production was also observed due to brown seaweed waste supplementation (4% of diet) in Holstein dairy cows fed for 90 days (Lee et al. 2005). Likewise, milk production improved by supplementation of *A. nodosum* meal (Cvetkovic et al. 2004) or inclusion of 2% seaweed (*Macrocystis pyrifera*) meals in the concentrate ration in dairy cows (Succi and Zurla, 1967). The differences among studies could be due to source and nature of seaweed product presented to the lactating cows.

Milk composition and organoleptic evaluation

The average fat content across 5 months study was found to be 4.52, 4.49 and 4.33% in groups T₁, T₂ and T₃, respectively. In general, the milk fat content showed an increasing trend with progress in lactation. The average SNF level in the milk was 9.37, 9.45 and 9.41% in groups T₁, T₂ and T₃, respectively with corresponding values of 3.54, 3.53 and 3.52% for protein. The overall lactose content averaged 4.95, 5.03 and 4.97% in groups T₁, T₂ and T₃, respectively. The milk composition was not affected by supplementation of SWP @ 1.5 and 3.0% of ration in crossbred cows. Supplementation of SWP @ 1.5 or 3.0% of ration had no adverse effect on organoleptic/sensory appraisal of milk (Table 4). Singh et al. (2015) also reported that milk composition was not affected in lactating Sahiwal cows given *Sargassum wightii* seaweed powder in concentrate mixture at 20% level. Supplementation of *A. nodosum* @ of 80 g/cow/day did not affect milk protein and fat production in lactating Holstein cows (Karatzia et al. 2012). Daily milk yield and composition (fat, protein, SNF) were not affected by fermented brown seaweed waste (180 or 360 g; 1-2% of basal diet) supplementation in Holstein dairy cows for 60 days (Hong et al. 2010), inclusion of hydrolyzed *A. nodosum* in dairy cows (Cermak et al. 2011) or *A. nodosum* on heat stressed dairy cows (Pompeu et al. 2011). Seaweed (*Sargassum* sp.) could be fed to lactating Kankrej cows without any significant changes in milk composition up to the level of 30% in concentrate mixture (Desai and Shukla, 1975). However, seaweed feeding increased milk protein and fat content in cows (Succi and Zurla, 1967). *A. nodosum* meal supplementation increased milk production and milk protein content in dairy cows (Hong et al. 2010). The differences in studies could be due to source and level of seaweed product in the rations of dairy cows.

Conclusions

Supplementation of *K. alvarezii* based seaweed product at 1.5 or 3.0% level of diet did not influence feed intake, milk composition and organoleptic properties but persistency of lactation was better ($P < 0.05$) in group given seaweed product @ 3% of dietary DM particularly post 5th fortnight after initiation of study in crossbred cows.

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RESEARCH ARTICLE

Delayed apoptosis of buffalo neutrophil during mastitis and metritis

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Abstract: Mastitis and metritis are the common inflammatory diseases of buffalo and result huge economic losses to the small-scale farmers. Neutrophils are recruited during inflammation and cleared from site of inflammation by apoptosis to prevent further tissue damage. However, data are lacking on this aspect in buffalo. Hence, the study was aimed to determine the viability and apoptosis of neutrophils of buffalo during mastitis and metritis. Peripheral blood samples were collected from healthy, mastitis and metritis group. The viability of neutrophils at different time intervals (6, 12, 24, 36 and 48hr) was determined using trypan blue dye exclusion method. DNA laddering, light microscopy, caspase-3 and caspase-9 colorimetric assays were used to observe the apoptosis. Freshly isolated neutrophils showed 97% purity and 92% viability. Healthy group showed decrease ($p < 0.05$) in viable neutrophils at 12hr and onwards post-incubation. The number of apoptotic neutrophils was lower ($p < 0.05$) at 36hr and onwards post-incubation in mastitis group and at 48hr post-incubation in metritis group as compared to control group. DNA fragmentation was observed after 24hr post-incubation in healthy group and was absent in mastitis and metritis groups suggesting the delayed apoptosis during mastitis and metritis. Caspase-3 and caspase-9 activity did not show any change in healthy group, however, there was a significant increase at 24hr and 36hr post-incubation in mastitis and metritis group. Taken together, data suggest delayed apoptosis in mastitis and metritis.

Keywords: Apoptosis, Buffalo, Caspase-3 and Caspase-9, Neutrophils, Viability

Introduction

Milk producing animals such as buffalo and cattle significantly contribute to India's economy. However, high incidence of mortality and morbidity due to various diseases of livestock has drastically increased the therapeutic use of antibiotics that may create hazards of contaminated milk, bacterial resistance and development of super-resistant bacteria. Despite significant use of chemotherapeutics, we could not completely eliminate the incidence of bacterial diseases such as mastitis and metritis suggesting lack of complete understanding of the pathogenesis of these diseases that may be due to some inherent differences in the complement of various immune cells including neutrophils.

Polymorphonuclear leukocytes (PMNs or neutrophils) are the primary cells contributing to the host innate immune response. Neutrophils are recruited to the site of inflammation to kill and phagocytose invading pathogens thus plays an important role in the host innate immune system (Appelberg, 2007). The non-activated neutrophils in circulation have very short half-life of 6-8 hours and undergo the process of spontaneous apoptosis or programmed cell death before phagocytosed by tissue macrophages in liver, spleen and bone marrow (Martin et al. 2003). Apoptosis is critical for the resolution of inflammation during infection, as it would reduce local tissue destruction, diminish further inflammation and recruitment of immune cells, and facilitate the termination of the innate immune response by limiting pro-inflammatory capacity. Likewise, the neutrophil apoptosis safely clears the activated neutrophils from the site of infection and prevents the surrounding tissues from the pro-inflammatory activity of neutrophils (Savill and Fadok, 2000). In contrast with necrosis, apoptosis maintains plasma membrane integrity, so that release of harmful neutrophil contents is limited (Taylor et al. 2008).

Both mastitis and metritis are characterized by increased neutrophilic count. However, there is no systematic study on the dynamics of neutrophils during mastitis and metritis. Hence, we

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tested the hypothesis that neutrophil apoptosis is delayed during inflammatory conditions such as mastitis and metritis in buffalo.

Materials and Methods

Ethical approval

The experiment protocols were approved by Institutional Animal Ethical Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana.

Sampling

Blood samples were collected from healthy adult buffalo maintained at the dairy farm of Department of Animal Genetics and Breeding, GADVASU, Ludhiana and buffaloes infected with mastitis and metritis presented at the Teaching Veterinary hospital, GADVASU, Ludhiana (N=5, each group). Blood was collected aseptically from jugular vein in vacutainer tubes containing EDTA as anticoagulant and were immediately transported in ice packs and brought to the laboratory for further processing. The blood samples were kept at 4°C and were used within 1 hr of collection. The experiment was repeated five times on separate blood collections for each healthy, mastitis and metritis group.

Isolation of neutrophils

Histopaque density gradient centrifugation method was used for the isolation of neutrophils. Freshly collected blood (about 40 to 45 ml) was diluted in ratio (1:1) with sterile PBS and was then carefully layered over the HiSepLSM 1077 (2.5 ml) solution in a conical centrifuge tube. The tube was centrifuged to obtain the cell pellet. Erythrocytes lysis buffer was used to obtain the pure neutrophils that were maintained in RPMI-1640 media (Sigma Aldrich) supplemented with 5% fetal calf serum (Gibco) and antibiotic mixture 100X (Himedia).

Determination of cell purity

Slide smears of isolated cell suspension were prepared, air dried and stained with Hematoxylin and Eosin (H&E) staining. Stained slides were observed under light microscope and 100 cells were counted randomly on the H&E slides. The proportions of neutrophils (multilobed nucleus with intact cell membrane) were expressed in percentage.

Quantification of viable neutrophils

The pellet of neutrophils was re-suspended into 2 ml of RPMI-1640. Around 10 µL of the cell suspension was diluted with equal volume of trypan blue and 10 µl of this mixture was loaded on hemocytometer and the neutrophils were counted in all 16 squares located at the 4 corners of hemocytometer using hand tally counter. The addition of the count of 4 corner squares was divided by 2 and multiplied by 10^4 to obtain the required number (2×10^6

cells/ml) of cells per ml. The numbers of viable cells were counted after 6hr, 12hr, 24hr, 36hr and 48hr post-incubation in healthy, mastitis and metritis group.

Quantification of the apoptotic neutrophils

Neutrophil smears were prepared after 0hr, 6hr, 12hr, 24hr, 36hr and 48 hr post incubation from all the groups. Slides were air dried for 15-20 minutes and then stained with May-Grunwald Giemsa Stain. The stained slides were examined under light microscope under 40X and 100X and attention was given to normal and apoptotic cells as described earlier (Singh et al. 2015). Each slide from selected time interval was observed and randomly a total of 100 normal or apoptotic cells were counted by using hand tally counter and finally the percentage of apoptotic cells was calculated by following formula:

$$\text{Apoptotic \%} = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells (normal + apoptotic)}} \times 100$$

DNA laddering

The ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit was used to isolate the DNA from the neutrophils and to determine the level of DNA fragmentation. During apoptosis, the nucleases gets activated which degrade the higher order chromatin structure of DNA into fragments of 50 to 300 kb and subsequently into small DNA pieces of about 200 base pairs in length. These DNA fragments were extracted from the pellets of neutrophils from freshly isolated cells and after 6hr, 12hr, 24hr, 36hr and 48hr post-incubation from all the groups. DNA fragments were visualized by horizontal gel electrophoresis.

Caspase-3 colorimetric assay

Caspase-3 colorimetric assay was used to quantify the level of apoptosis by using ApoTarget™ Caspase-3 kit (cat no. KHZ0022). The amount of activated caspase-3 was quantified in freshly isolated and 12hr, 24 hr, 36 hr and 48 hr post-incubation cells in all groups as per manufacture's guidelines. Active caspase-3 hydrolyse the acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), resulting in the release of the p-nitroaniline (pNA) moiety which was detected at the wavelength of 405nm. Fold-change in Caspase-3 activity was determined by direct comparison to the level of the uninduced control in various groups at different time intervals as per manufacturer's instructions.

Caspase-9 colorimetric assay

Caspase-9 colorimetric assay was used to quantify the level of apoptosis by using ApoTarget™ Caspase-9 kit (cat no. KHZ0101). The amount of activated caspase-9 was quantified in freshly isolated and 12hr, 24 hr, 36 hr and 48 hr post-incubation cells in all groups as per manufacture's guidelines. Active caspase-9 hydrolyse Leu-Glu-His-Asp-pNA (LEHD) resulting in

the release of the p-nitroaniline (pNA) moiety which was detected at the wavelength of 405nm. Fold-change in Caspase-9 activity was determined by direct comparison to the level of the uninduced control in various groups at different time intervals as per manufacturer's instructions.

Statistical analysis

The data obtained from viability and apoptotic cell count from control and other groups were subjected to statistical analysis by using Fisher's Least Significance Difference test (Post Hoc test ANOVA) for multiple pair wise comparison among various groups for viable and apoptotic neutrophils. Results were considered statistically significant at $p < 0.05$.

Results and Discussion

The freshly isolated neutrophils showed viability (negative by trypan blue dye exclusion test) of more than 92.0% in all the groups. The purity of neutrophils was found to be 97%. Singh et al. (2015) reported a significant decrease in the viability following 24 hr of incubation. Taking lead from these observations, the viability in a narrow time window of 6hr and onwards post-incubation was examined. The neutrophils showed significant decrease ($p < 0.05$) in viability following 6hr and onward incubation compared to freshly isolated neutrophils in healthy group (Table 1). Half-life of the non-activated neutrophils is usually short due to very low levels of antiapoptotic proteins (Riedemann et al. 2004). Further, the number of viable neutrophils in mastitis as metritis group was significantly ($p < 0.05$) higher than the healthy group at 36hr and 48hr of incubation (Table 1). Sladek et al. (2006) reported the significance increase in the number of viable neutrophils in mastitis affected dairy cows as compared to the control neutrophils even after 24hr.

The healthy neutrophils were normal spherical cells with multilobed nucleus (Fig 1a) and apoptotic neutrophils showed characteristic morphology like intact cell membrane, chromatin condensation, blebbing, zeiosis, formation of apoptotic bodies, and karyopyknosis (Fig 1b). Light microscopy is equally effective as scanning electron microscopy to identify the various stages of apoptosis (Sladek and Rysanek, 2001). Few freshly isolated

neutrophils (10.65 ± 0.023) from healthy buffalo also showed apoptosis and there was a significant increase ($p < 0.05$) in the number of apoptotic neutrophils after 6hr and onwards incubation as compared to freshly isolated neutrophils in this group (Table 1). Neutrophils undergo spontaneous apoptosis due to co-expression of Fas and Fas ligand is responsible for the spontaneous apoptosis in the mature neutrophils (Liles et al. 1996; Kirschnek et al. 2011). In the present study, there was a significant decrease ($p < 0.05$) in the percentage of apoptotic neutrophils after 36hr and 48hr post-incubation (18.42 ± 0.017) in mastitis affected buffalo as compared to healthy group at this stage. Delayed apoptosis of polymorphonuclear leukocytes (PMN) during the neutrophil influx plays important role in the resolution of mastitis and the highest apoptotic number of neutrophils was attained at 72hr in LPS treated groups compared to 48hr in control/PBS treated group (Sladek and Rysanek, 2000, 2001). Similarly, 12hr and onwards incubation resulted significant increase ($p < 0.05$) in the percentage of apoptotic neutrophils in metritis affected buffaloes (Table 1). Neutrophils are activated by endotoxins/LPS and various microbial products that prolong their life span by delaying the spontaneous apoptosis (Lee et al. 1993).

In the present investigations, mastitis and metritis-affected groups did not reveal any DNA fragmentation (Fig 2) up to 48hr post-incubation indicating delayed apoptosis as reported earlier in mastitis and healthy buffaloes (Anwer et al. 2016). The delay in the natural apoptosis of neutrophils was found in patients with inflammatory diseases, including acute respiratory distress syndrome, sepsis and acute coronary artery disease (Garlich et al. 2004). Caspase 3 activity showed a significant ($p < 0.05$) increase after 36hr and 48hr post-incubation in healthy group (Fig 1c). Caspases are activated in a time dependent manner in neutrophils undergoing spontaneous apoptosis (Fadeel et al. 1998). Stimulus like toxins, radiation and free radicals changed the permeability of mitochondrial membrane leading to the release of proapoptotic proteins from the mitochondrial intermembrane space (Vermeulen et al. 2005). Mitochondria release cytochrome c in the cytosol that activates the apoptotic protease activating factor-1 (Apaf-1). Apaf-1 binds with the procaspase-9 (Zymogen form of caspase-9) by using ATP to form apoptosome and activates

Table 1: Percentage of viable neutrophils in various groups at different time intervals

Time	Percentage of viable neutrophils (Mean $\pm$ S.E.)			
	Healthy	Mastitis	Metritis	Positive control
0 hr	92.97 ^a $\pm$ 1.03	92.51 ^a $\pm$ 1.04	93.05 ^a $\pm$ 0.005	93.34 ^a $\pm$ 0.98
6 hr	90.73 ^b $\pm$ 1.00	91.23 ^a $\pm$ 1.01	90.22 ^b $\pm$ 0.005	92.21 ^b $\pm$ 0.25
12 hr	87.07 ^c $\pm$ 0.99	88.79 ^{ab} $\pm$ 1.95	85.74 ^c $\pm$ 0.005	88.25 ^c $\pm$ 0.01
24 hr	81.69 ^d $\pm$ 0.55	85.37 ^{bc} $\pm$ 1.40	79.25 ^{de} $\pm$ 0.003	80.58 ^d $\pm$ 1.03
36 hr	70.57 ^e $\pm$ 1.06	82.35 ^{cd} $\pm$ 1.10	71.52 ^{e*} $\pm$ 0.005	67.24 ^d $\pm$ 0.98
48 hr	51.77 ^f $\pm$ 1.07	72.77 ^{ds} $\pm$ 0.52	63.28 ^{fs} $\pm$ 0.004	48.79 ^f $\pm$ 0.07

a,b,c,d, e,f and #, *, \$no common subscript between two levels indicate the significant difference ($p \leq 0.05$) in a group and between group, respectively. Data represent the average from the five separate neutrophil preparations.

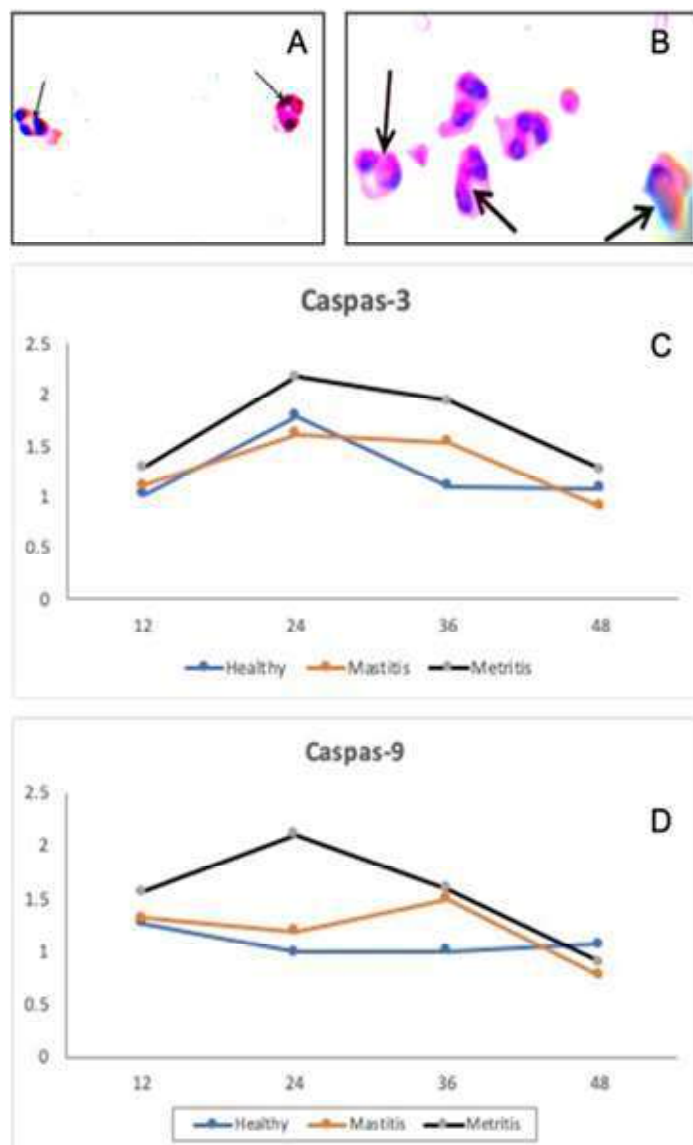


Fig 1. Multilobed neutrophils (thin arrow) with normal histo-architecture (a), chromatin condensation (thick arrow) in the neutrophils undergoing apoptosis (b); May Grunwald X 100, Fold-change in Caspase-3 (c) and Caspase-9 (d) activity determined by direct comparison to the level of the uninduced control in various groups at different time intervals

caspase-9. Caspase-9 then activates caspase-3 and caspase-7 leading to the onset of apoptosis (Fadeel and Orrenius, 2005). Further, there was a significant decrease in the caspase-3 and caspase -9 activity after 48hr post-incubation in mastitis and metritis group as compared to control group (Fig 1c,d). Swain et al. (2014) and Boutet et al. (2004) reported a significant delay in apoptosis of neutrophils isolated from the mastitis infected cows which was decreased to 44.8% after 24 hr. Delay in apoptosis of neutrophils may be due to GM-CSF which activates STAT5 with up-regulation of Bcl-xL mRNA (Boutet et al. 2004) or higher

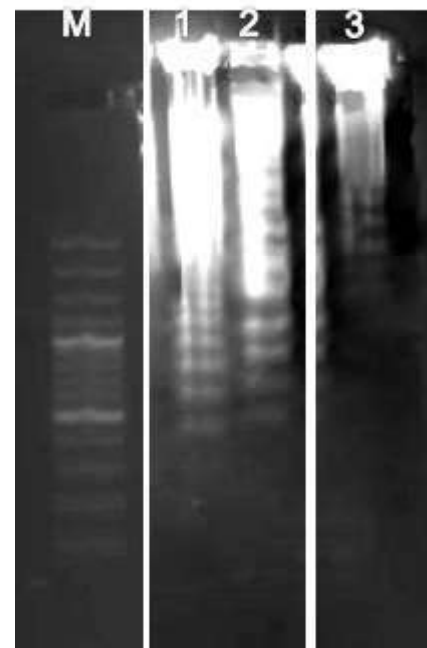


Fig. 2. Agarose gel electrophoresis showing DNA fragmentation. Lane M: DNA marker, Lane 1: 48hr normal group, Lane 2: 48hr mastitis group, Lane 3: 48hr metritis group

expression of TLR-2 and TLR-4 (Swain et al. 2014) in bovine neutrophils. The caspase 3 and 7 activity decreases during clinical mastitis in cow resulting delay in apoptosis (Swain et al. 2014). The data taken together with DNA fragmentation indicate prolonged neutrophil survival due to delayed apoptosis at 48hr post-incubation in mastitis and metritis affected buffalo.

Conclusion

The study reveals that the viability of the neutrophils showed prolonged maintenance during mastitis and metritis. Further, the delay in the apoptosis of neutrophils was found to be associated with mastitis and metritis suggesting the activation of neutrophils by these pro-inflammatory conditions. These findings are important as the delayed apoptosis of neutrophils can lead to the further tissue destruction and have the potential to transform the disease into severe chronic stage.

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RESEARCH ARTICLE

Impact of heat stress on reproductive performance of Sahiwal cows

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Abstract: The objective of the study was to investigate the effect of temperature humidity index (THI) and indicator of heat stress on reproductive performance i.e. calving interval, conception rate and pregnancy rate of Sahiwal cows maintained at Livestock Research Center, ICAR-National Dairy Research Center, Karnal. A total calving record pertaining to 3000 lactations of 847 Sahiwal cows, spread over 26 years from 1990 to 2015 were considered for the present study. The daily ambient temperature and relative humidity records were collected from ICAR-Central Soil Salinity and Research Institute, Karnal and NICRA center (Climate Resilient Livestock Research Center), NDRI, Karnal. Univariate logistic regression model was fitted to assess the effect of independent variable THI on the dependent variables conception and pregnancy status. A distinct relationship between THI and calving interval was observed. The cows had a significant longer calving interval at high THI as compared with low THI. It was observed that the average calving interval was increased by 2 days with per unit increase in THI value. Pregnancy rate and Conception rate of Sahiwal animals was significantly reduced from 35% at low THI to 18% at high THI and from 55% at low THI to 26% at high THI respectively with decreasing odd ratio. The high environmental temperature reduces the reproductive performance of animals.

Keywords: Calving interval, Conception rate, Fertility Sahiwal, Temperature–humidity index

Introduction

The efficient production system of dairy industry highly depends on reproductive efficiency of the animals. In the last 18 years conception rate of dairy cows has decreased from 42.6% to 27.7% worldwide (Roth, 2020). Kim and Jeong (2019) in Korean dairy cows reported that lower body condition score, heat stress due to hot weather and post partum disorders are risk factors limiting conception resulting in economic loss of 622.40 dollars per cow. Thus, heat stress is one important factor for reducing reproductive efficiency of animals. Increasing environmental temperature has a major effect on fertility and conception rate in farm animals in hot months and in seasons with higher temperature humidity index (Tarabany and Tarabany 2015) Climate change is also a challenge to the livestock and dairy sector in India. The rise in temperature over the entire country with increased humidity resulting from climate change is likely to affect the heat stress in dairy animals affecting the milk yield, growth and reproduction performance of animals and even the native breeds also suffers a major loss in their production and fertility. Therefore, the objective of the present study was to examine the effects of heat stress on reproductive performance of Sahiwal animals.

Materials and Methods

The present investigation is carried out in Sahiwal animals maintained at ICAR-National Dairy Research Institute, Karnal.

Collection of data and management of herd

The information related to the present study i.e. cow number, date of calving, date of service, bull number, date of successful insemination, pregnancy diagnosis, was collected from records maintained at Animal Genetics and Breeding Division, ICAR-National Dairy Research Institute (NDRI), Karnal from 1990 to 2015 pertaining to 3000 lactations of 847 Sahiwal cows. The reproductive performances calving interval, conception rate and pregnancy rates was estimated for all the Sahiwal animals under study. The conception and pregnancy of the animals was taken

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as a dichotomous variable means either animal conceived or not and become pregnant or not. The conception and pregnancy was confirmed at 30 days and 60 days after insemination respectively. The THI group was assigned on the basis of date of insemination i.e. THI value on the date of insemination. In case of calving interval THI was assigned on the basis of date of calving i.e. THI value on the date of calving.

The climatic parameters like dry bulb temperature (°C), wet bulb temperature (°C), and relative humidity (%) were collected for duration of twenty six years (1990-2015) and data were collected from Central Soil Salinity and Research Institute, Karnal for the years 1990-1999 and from ICAR-NICRA center (Climate Resilient Livestock Research Center), NDRI, Karnal for the years 2000-2015 of the study. The dry bulb and wet bulb temperature were measured twice a day (morning & afternoon). The daily average dry bulb and wet bulb temperature were calculated by combining morning & evening values. From the daily dry bulb and wet bulb temperature monthly average dry bulb and wet bulb temperature were estimated. Temperature Humidity Index (THI) was estimated on daily basis by the use of daily average dry bulb and wet bulb temperature.

From the daily dry bulb and wet bulb temperature monthly average dry bulb and wet bulb temperature were estimated. Temperature Humidity Index was estimated on daily basis was estimated by the use of daily average dry bulb and wet bulb temperature.

The Temperature Humidity Index (THI) was estimated by using the formula:

$$\text{THI} = 0.72 (\text{DBT} + \text{WBT}) + 40.6 \text{ (Johnson et.al, 1973)}$$

Where, DBT = Dry bulb temperature and WBT = Wet bulb temperature

The Temperature Humidity Index calculated for the Karnal region ranged from 45.12 in winter season to 85.27 in summer season. The THI data set was classified into 2 sets as follows:

1. Data set 1- The THI data was classified into 7 different THI subclasses (≥ 45 to ≤ 50 , ≥ 51 to ≤ 56 , ≥ 57 to ≤ 62 , ≥ 63 to ≤ 68 , ≥ 69 to ≤ 73 , ≥ 74 to ≤ 79 and ≥ 80 to ≤ 85 to analyze the effect of THI on calving interval.
2. Data set 2- The THI data was also categorized in to three levels as low less than 70; moderate more than 70 but less than 80; and high more than 80 to assess the effect of THI on dichotomous variables conception and pregnancy status.

The THI groups were statistically classified using Struge's Formula (1926)

The climate of the farm is subtropical in nature. The minimum temperature falls to 2°C in winter and maximum goes up to 47°C in

summer. Selective breeding is being followed for Sahiwal cows. Artificial insemination is practiced for breeding. The nutritional requirements are met through a standardized balanced ration of green and dry fodders along with concentrates. All Sahiwal cows are exclusively stall fed in open paddock at the farm.

Statistical Analysis

All statistical procedures were performed using SAS Statistical Package (SAS Institute Inc. version 9.2).

The Univariate fixed effect model was fitted through the maximum likelihood procedure to access the effect of THI on calving interval (Harvey 1990).

The univariate logistic regression model was fitted to assess the effect of independent variable THI on the dependent variables conception and pregnancy status. The results are expressed as percentages along with crude odds ratios and their 95% confidence interval. Probability values of less than 0.05 considered to have a statistically significant association.

Results and Discussion

The value of THI is greatly influenced by the temperature and humidity parameters. These parameters vary widely over the months. The production and reproduction parameters of animals are also influenced by temperature and humidity to which they are expressed. The THI in different months of the year around Karnal is presented in Fig 1. The results revealed that THI values varied from 57.13 in the month of January to 81.84 in the month of July. The maximum THI value was observed in the month of July as 81.84 and minimum in the month of January.

Effect of THI on Calving Interval

The analysis of variance reveals that the effect of THI groups were found to be highly significant ($p < 0.01$) on calving interval of the Sahiwal animals over the years. The overall least square mean of calving interval average of Sahiwal cows along with the standard error is presented in Table 1. A distinct relationship between THI and calving interval was observed in the present

Table 1: Least-squares means and Standard Error of Calving Interval of Sahiwal cows

Effect	Mean & S.E.
Overall	444.80±5.41
THI group	
45.00 – 50.00	424.81 ^a ±11.21(512)
51.00 – 56.00	433.02 ^b ±8.14(486)
57.00 – 62.00	436.37 ^b ±6.12(415)
63.00 – 68.00	458.7 ^c ±5.45(374)
69.00 – 73.00	465.74 ^d ±4.89(682)
74.00 – 79.00	486.53 ^e ±9.6(432)
80.00 – 85.00	492.66 ^e ±4.32(215)

Fig. 1 Monthly Average THI values over years in farm area of NDRI, Karnal

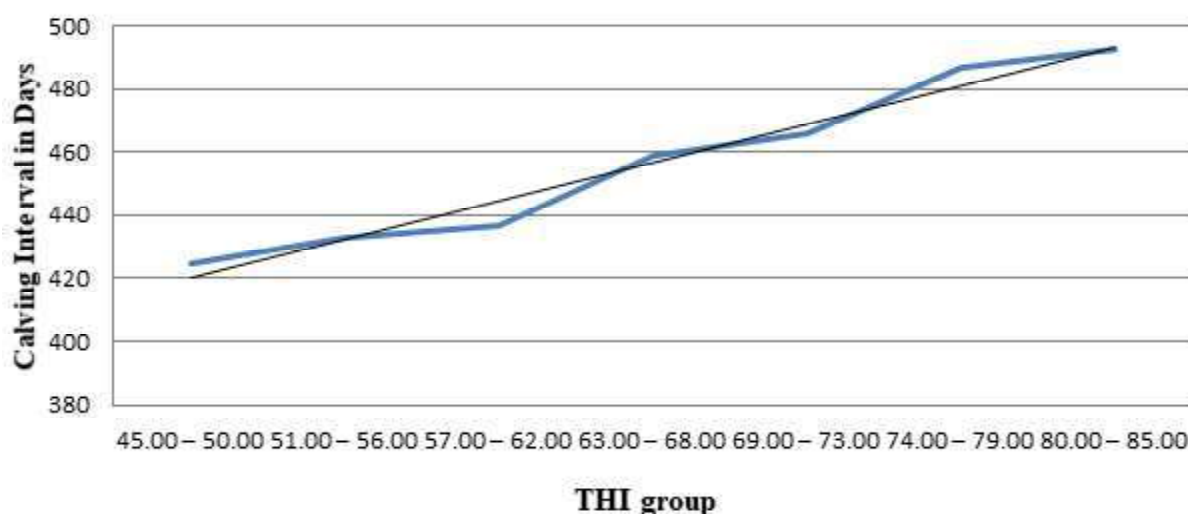
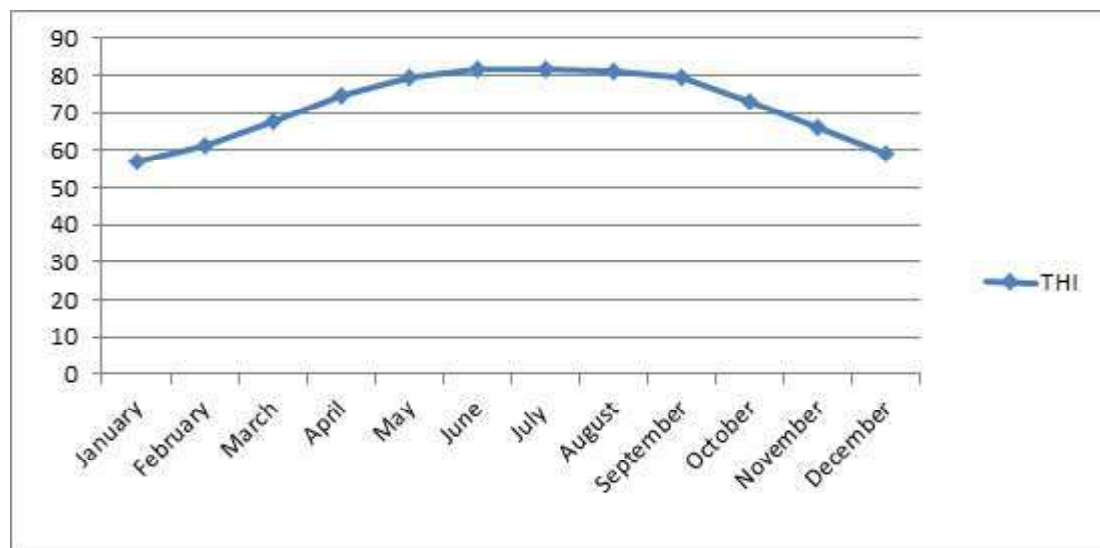


Fig. 2 Effect of THI group on Calving Interval of Sahiwal Cows

study (Fig 2). The perusal of Table 1 and Fig 2 reveals that the cows had a significant longer calving interval at high THI as compared with low THI.

The effect of heat stress (measured as THI) on calving interval was also estimated on the basis of month of calving for each month during twenty six years period. The month wise average calving interval during twenty six years 1990 to 2015 along with monthly average THI values are presented in Table 2. The average calving interval of Sahiwal cows was found to be the highest as 492.66 days in the month of August. The average THI value in the month of August was estimated as 81.11. The lowest calving interval of Sahiwal cows was 422.4 days in the month of March. The average THI value in the month of March was 67.68. The present showed that the calving interval was higher in the animals that are calved in the months with high THI values whereas the

value calving interval was continued to be higher in the months with higher average THI values.

To analyze the association of monthly averaged THI values and monthly average calving interval (Table 3) and to quantify the change in calving interval with unit change in the THI value regression analysis was done taking calving interval of the animals as dependent variable and THI as an independent co-variable. The analysis revealed that the regression of calving interval on THI was highly significant ($P < 0.01$). It was observed that the average calving interval was increased by 2 days with per unit increase in THI value.

Extremely high environmental temperature reduces the reproductive performance of animals and also influences the body temperature balance resulting in impaired reproductive cycle, delayed follicular development, repeat breeding, lack of heat and

Table 2 Month wise average THI values and average first calving interval during twenty six year period (1990-2015)

MON	THI	CI (days)
January	57.13	424.81
February	61.2	433.02
March	67.68	422.4
April	74.7	422.45
May	79.74	436.37
June	81.61	465.74
July	81.84	486.53
August	81.11	492.66
September	79.28	458.7
October	73.36	436.26
November	66.28	443.25
December	59.37	438.8

longer calving interval and days open. Fertility traits have low heritability value therefore greatly influenced by environmental factors and heat stress is one of the important factors that affects the fertility and reproductive ability of the animals. Zadeh et al. (2013). Tarabany and Tarabany (2015) reported that cows had a significant longer calving interval at high THI (449 and 173 days, respectively), compared with low THI (421 and 146 days, respectively) in Holstein cows. Zewdu et al. 2014 reported a increase in calving interval by 23%-25% in Deoni crossbred cows; and Habeeb et al. (2018) in local cattle of Egypt; Rhoads (2020) reported a increase in calving interval due to heat stress in Holstein cows. Purohit et al. (2020) reported that an increase in THI simultaneously increases calving interval in dairy animals and decreases conception rate, pregnancy rate, lactation length in females.

Effect of THI on Conception and Pregnancy

The effect of THI on conception and pregnancy is given in Table 4. Pregnancy rate of Sahiwal animals was significantly reduced from 35% at low THI to 18% at high THI with decreasing odd ratio indicating the more effect of THI value on pregnancy rate of

Table 3 Regression of calving interval on THI

SN.	Source of Variance	MSS			
1	Regression (1)	2785.30**			
2	Error (10)	3556.62			
Regression	Intercept	b	S.E	R ²	
THI	323.07	1.71	0.44	0.43	

Figures in paratheses indicates respective degree of freedom
*P<0.05, **P<0.01

Sahiwal animals. The effect of THI on conception and pregnancy is depicted in Fig 4. In the present study it was found that conception rate is strongly dependent on mean daily THI at the day of insemination. Conception rate decreased continuously with increasing THI value indicating a negative association with THI. The odd ratios and probability values of different THI groups is given in Table 4. Conception rate of Sahiwal animals was significantly reduced from 55% at low THI to 26% at high THI with decreasing odd ratio indicating the more effect of THI value on conception rate of Sahiwal animals. Both the conception rate and pregnancy rate are reduced by 50% in response to increasing THI value.

High body temperature results in increased uterine temperature, due to the fact that blood supply is directed to the body periphery to promote body heat loss, so the blood flow in the uterus is reduced. Consequently, there is a decrease in gestation rate, embryonic loss, and reduced availability of nutrients and hormones due to decreased blood supply to uterus. This makes the reproductive environment unsuitable for the sperm to fertilize the ova and the embryo development. The higher ambient temperatures have deleterious effect on ovarian and physiological cycles that are important for conception and maintenance of pregnancy. The heat stress also cause stress in animals and reduces dry matter intake and may increase negative energy balance which in turn may affect entire body metabolism of animals including oocyte development, uterus synchronization,

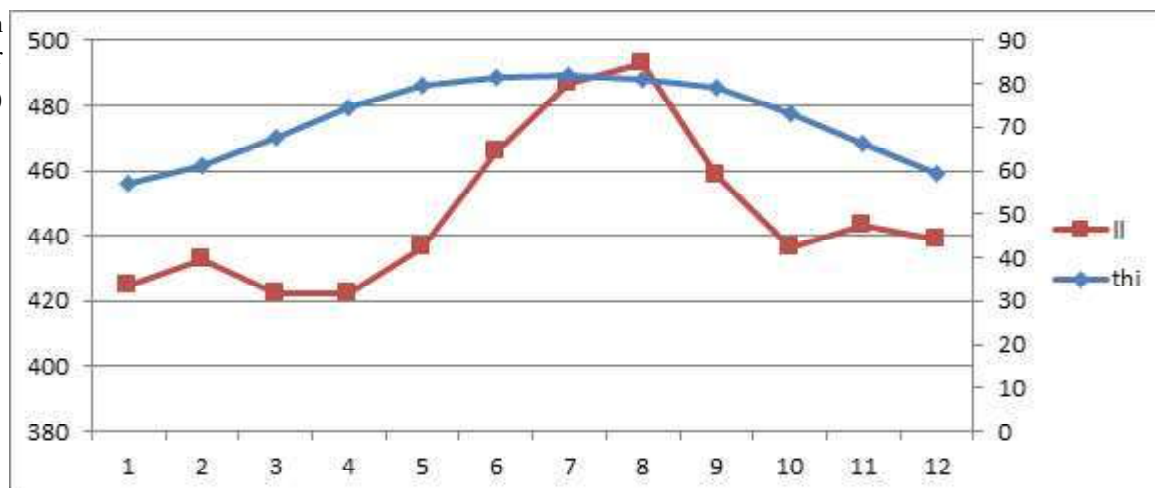
Fig. 3 Effect of THI on Calving Interval over the years (1990-2015)

Table 4 Odd ratios for the effect of temperature humidity index (THI) on Conception Rate and Pregnancy Rate in Sahiwal Animals over Years

	Conception Rate			Pregnancy Rate		
	Low	Moderate	High	Low	Moderate	High
Value	35.21 ^a	31.54 ^a	18.37 ^b	55.77 ^a	49.66 ^b	26.12 ^c
Odd Ratio	-	1.10	0.94	-	0.85	0.54
P-value	-	0.032	0.014	-	0.048	0.026
N	874	1245	881	858	1187	839

conception, fertilization, embryo development resulting in cases of abortion, miscarriage, anestrus and repeat breeding.

Many workers {Costa et al. (2015), Tarabany and Bayoumi (2015), Tarabany and Nasr (2015), Schuller et al. (2016), Hadi et al. (2016), Alves et al. (2017), Liu et al. (2018), Silva et al. (2018), Gernand et al. (2019), Mahyari et al. (2019), Rhoads (2020), Kim and Jeong (2019) and Purohit et al. (2020)} have reported a decrease in conception rate with increase in THI value. Costa et al. (2015) reported reduction in conception rate of Girolando Cows from 67.14% to 58.26% ;Tarabany and Bayoumi (2015) reported that conception and the pregnancy rate of the Holstein cows decreased from 35.8% and 29.4%, respectively, at low THI to 16.1% and 12.1%, respectively, at high THI; Tarabany and Nasr (2015) reported that at high THI conception rate decreased from 43.1% at low to 24.1%; Schuller et al. 2016 reported a decrease in conception rate from 31% to 12% with increasing THI; Alves et al. (2017) reported that the heat stress reduced pregnancy rate in Nellore cows from 70.37% to 51.85% respectively; Liu et al. 2018 reported that conception rate of Taiwan cows reduced from 57.14% to 47.26% with increasing THI; Rhoads (2020) reported decrease in conception rate of Holstein cows by 50.12% to 61% due to increasing THI values. Hence, heat stress significantly affects the reproductive efficiency of animals.

Conclusion

Extremely high environmental temperature reduces the reproductive performance of animals and resulting in impaired reproductive cycle, repeat breeding and longer calving interval . Hence, managemental strategies are needed to plan and implement to minimize heat stress and attain optimal animal productivity.

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Effect of feeding slow release non protein nitrogen sources on milk production and milk quality parameters in Cross bred dairy cows

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Abstract: A study of 90 days duration was conducted on 18 multiparous crossbred cows (363±14kg, body weight; in II or III lactation; 3,000–4,500kg milk yield per lactation) randomly allotted to 3 dietary treatments with 6 animals in each group in a completely randomized design to investigate the effect of two slow release non protein nitrogen (SRNPN) products on feed intake, milk yield, milk composition, blood biochemical parameters and cost economics. The concentrate mixture for control group was formulated with maize, gram husk, ground nut cake, wheat bran, deoiled rice bran, salt and mineral mixture. The other two experimental concentrate mixtures were prepared by totally replacing ground nut cake with calcium salts of *fatty acid coated urea* (CFCU) and *Hydroxypropyl methylcellulose coated urea* (HMCCU) at 2.42%. The cows were offered fixed quantity of Napier green fodder (10kg/cow/day) and *ad libitum* paddy straw. The daily allowance of respective concentrate mixture offered to each cow was calculated based on body weight, previous week milk yield and roughage intake to meet the nutrient requirements of as per ICAR (2013). Incorporation of either of the coated urea products in concentrate mixtures did not affect the total DM intake, milk yield (13.94 to 14.11kg/d), milk composition (Milk fat, Protein, SNF and Lactose) and blood biochemical (Blood urea

nitrogen, Glucose and total protein). Though non significant, the average daily cost of feeding (Rs/d) was lowered in CFCU (228.14) and HMCCU (215.98) diet fed cows compared to control group cows (237.08) by 3.77 and 8.89% respectively. Whereas, the cost of feeding (Rs) per kg milk production differed significantly ($P<0.01$) with highest in control diet fed group (16.80) while in CFCU (15.43) and HMCCU (15.49) fed group it was lower. CFCU and HMCCU can be included at 1% of dry matter or 2.42% in the concentrate replacing the conventional protein sources, without affecting the milk yield and its composition in cross bred cows and reducing the feed cost per kg milk production.

Keywords: Cost economics, Milk yield, Milk composition, Dairy cow, Slow release urea

Introduction

The rumen microbes can utilise the non-protein nitrogen (NPN) which is not presented as assembled amino acids along with carbohydrate forming high-quality protein. Among several compounds characterized as NPN, urea is highlighted by the low cost, availability and ease of use, being widely included in animal nutrition, especially in ruminants (Santos et al. 2001). Urea has a high density of protein equivalent (262 to 292%) however, urea is used inefficiently by ruminants when compared with other true protein sources (Broderick and Reynal, 2009), and this is due to the rate at which urea is degraded in the rumen. Urea rapidly solubilizes in the ruminal environment and the utilization of NH_3 may be incomplete by rumen bacteria, leading to accumulation and absorption of ammonia and subsequent excretion of nitrogen in the urine (Highstreet et al. 2010).

During the past 40 years, several technologies have been developed to synchronize the rate of NPN release with the degradation rate of carbohydrates in the rumen to maximize the efficiency of microbial protein synthesis. Compounds with slow release of NPN developed previously, such as biuret (Loest et al. 2001), Starea (Bartley and Deyoe, 1975), urea-formaldehyde (Prokop and Klopfenstein, 1977), and urea covered by linseed oil (Forero et al. 1980) did not present the same advantages as urea when used in ruminant nutrition. This fact was probably due to a

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large portion of NPN by these compounds bypassing the rumen without being converted into ammonia, reducing microbial protein synthesis.

Hence considering the above limitations and based on the hypothesis of synchronization in the ruminal degradation of nutrients, nitrogen sources that maintain ruminal ammonia levels constant throughout the days have been researched and a possible alternative to improve ruminal nutrient utilization is using calcium salts of fatty acid and polymer-coated slow-release urea were developed. Cherdthong et al. (2011) reported increased dry matter intake, digestibility, and 3.5% fat-corrected milk yield when dairy cattle were fed urea calcium slow-release urea when compared with feed-grade urea. In addition, Xin et al. (2010) found that slow-release urea increased the milk protein content and decreased milk urea N without changing milk yield, when compared with feed-grade urea. However, Galo et al. (2003) did not report any effect of a polymer-coated slow release urea on dry matter intake and digestibility. The comparison between experiments is complex because the result depends on forage source, concentrate level of diet, type of slow-release urea used, and level of inclusion. Several experiments reported decreased ruminal NH_3 concentrations when slow-release urea was fed (Cherdthong et al. 2011; Ribeiro et al. 2011; Taylor-Edwards et al. 2014) compared with feed-grade urea.

The objectives of the present study were to quantify the effects of two types of slow-release urea in diets for lactating cows primarily on feed intake, milk yield, milk composition, blood biochemical parameters and cost economics.

Materials and Methods

Source of Slow Release Urea Products and Other Feed Ingredients

Slow release urea products (SRUP) sources having 40% nitrogen under the investigation were *calcium salts of fatty acids coated urea* (CFCU) and *Hydroxypropyl methylcellulose coated urea* (HMCCU) (M/s Kemin Industries South Asia Pvt. Ltd, Tamil Nadu, India). The concentrate feed ingredients were procured from the local market. Paddy straw was purchased from local farmers and hybrid Napier was obtained on daily basis from College Farm.

Experimental Diets

The concentrate mixture for control group was formulated with maize, gram husk, ground nut cake, wheat bran, salt and mineral mixture (Table. 1). The other two experimental concentrate mixtures were prepared by totally replacing ground nut cake with CFCU and HMCCU at 2.42% and balance protein requirement was met by adding deoiled rice bran. All the concentrate mixtures

were iso-nitrogenous and iso-caloric and formulated to contain 2365 kcal ME/kg and 18.5% CP. The cows were fed to meet the nutrient requirement for maintenance and 10 kg/d of milk production as per ICAR (2013).

Experimental Animals and Management

Eighteen cross bred dairy cows (363 ± 14 kg, body weight; in II or III lactation; 3,000–4,500 kg milk yield per lactation) were randomly allotted to 3 dietary treatments with 6 animals in each group in a completely randomized design. The cows were offered fixed quantity of Napier green fodder (10 kg/cow/day) at 12.00 noon, paddy straw was offered in *ad libitum* at 8.00 AM, 4.00 PM and 8.00 PM. The daily allowance of respective concentrate mixture offered at 6 am and 6 pm... to each cow was calculated based on body weight, previous week milk yield and roughage intake.

Recording of Milk Production and Milk Sampling

The cows were hand milked twice daily at 6.00 AM and 6.00 PM. Daily milk yield was recorded using a digital weighing balance (M/s Essey Company Ltd). The 4% fat corrected milk (FCM) yield was calculated using the formula: $4\% \text{ FCM} = (0.4 \times \text{kg milk yield}) + (15 \times \text{kg of fat yield})$ according to NRC (2001). Milk samples were collected before start of the experiment and at the end of the 90 days experiment period for 3 consecutive days. The pooled samples of milk obtained from mixing of morning and evening in proportion to corresponding yield were used for further analysis. Milk samples were analysed for its composition (SNF, protein, fat and lactose) by using automatic electronic milk analyzer based on principle of ultrasonic sensors (M/S Vector automation system). Milk urea content was determined by a modified colorimetric DMAB (p Dimethyl aminobenzaldehyde) assay (Bector et al. 1998).

Blood Collection

Blood samples were collected aseptically from jugular vein of all cows in to clean sterilized glass tubes at start of the experiment and at end of 90 days feeding trial for analysis of blood urea nitrogen, glucose and total protein.

Statistical Analysis

The data was subjected to one-way analysis of variance under a completely randomized design. Milk composition and blood biochemical constituents collected at different intervals of time were analysed in two way analysis of variance for studying the interaction effects as per the procedure of Snedecor and Cochran (1980) by using SPSS 20. The difference between means was tested by significance using Duncan's multiple range test (Duncan, 1955).

Results and Discussion

Chemical Composition of Experimental Diets

The chemical composition of three experimental concentrate mixtures were similar (Table 2), as only the ground nut cake in the control diet was totally replaced with SRNPN sources and the rations were formulated to be iso-nitrogenous and iso-caloric with adjustments in the level of inclusion of deoiled rice bran and gram husk. The CP % in the concentrate mixture ranged between 17.81 to 18.12 on DM basis.

Dry Matter Intake

Incorporation of either of the coated urea products in concentrate mixtures at 2.42% did not affect ($P>0.05$) the DM intake of paddy straw, napier green grass and concentrate mixture in dairy cows throughout the six fortnights (Fig1). No effect on DM intake could be due to its lower level of inclusion *i.e.*, 2.42% of concentrate mixture which was amounting to on average 1.16 and 1.19% of total dietary DM intake in CFCU and HMCCU fed cows. Hence the average DM intake for the overall period was comparable among the three groups (12.15 - 12.78kg) and similar trend was observed when expressed in terms of percent body

Table 1: Ingredient composition (%) of concentrate mixture containing slow release non protein nitrogen products fed to lactating dairy cows

Ingredient	Control	CFCU ¹	HMCCU ²
Maize grain	24.91	24.91	24.91
Gram husk	25.33	30.11	30.11
Wheat bran	20.70	20.14	20.14
Deoiled rice bran	0	20.14	20.14
Ground nut cake	26.99	0	0
Mineral and vitamin mixture*	1.03	1.03	1.03
Salt	1.04	1.04	1.04
Non protein nitrogen source	0.00	2.42 ¹	2.42 ²
Sodium Sulphate	0.00	0.21	0.21
Total	100.00	100.00	100.00

*Mineral and vitamin mixture provided per kg of concentrate feed: Calcium 2.5g, Phosphorus 1.25g, Magnesium 0.62g, Iron 62.4mg, Zinc 22.88mg, Copper 41.6mg, Iodine 2.08mg, Cobalt 1.04mg, Vitamin B₁ 13.52mg, Vitamin B₆ 1.35mg, Vitamin B₁₂ 31.2mg, Vitamin A 11440IU, Vitamin D₃ 2080IU and Vitamin E 2.08IU.

¹Calcium salts of fatty acids coated urea

²Hydroxypropyl methylcellulose coated urea

Table 2: Chemical composition (% DM basis) of concentrate mixture containing slow release non protein nitrogen products and other roughages fed during lactation trial

Constituent	Concentrate mixture			Paddy straw	Green Hybrid Napier
	Control	CFCU ¹	HMCCU ²		
Dry matter	89.21	88.34	89.83	91.28	28.41
Organic matter	94.40	94.19	94.14	86.48	87.46
Crude protein	18.01	17.81	18.12	3.70	8.06
Ether extract	3.11	3.06	3.13	0.84	2.77
Crude fibre	4.30	4.54	4.61	32.08	27.69
Nitrogen free extract	68.99	68.78	68.26	49.86	48.94
Total ash	5.60	5.81	5.86	13.52	12.54
Neutral detergent fiber	37.62	36.51	35.89	71.49	58.33
Acid detergent fiber	6.33	7.04	6.82	42.14	32.60
Hemicellulose	31.29	29.47	29.07	29.35	25.73
Cellulose	5.09	5.31	5.11	35.68	28.84
Lignin	0.83	0.92	0.96	2.87	2.78

Each mean value is an average of 2 values.

¹ Calcium salts of fatty acids coated non protein nitrogen

² Hydroxypropyl methylcellulose coated non protein nitrogen

Fig. 1 Average dry matter intake (kg) of paddy straw, napier green, concentrate mixture and total ration by dairy cows fed with diets containing various slow release non protein nitrogen products

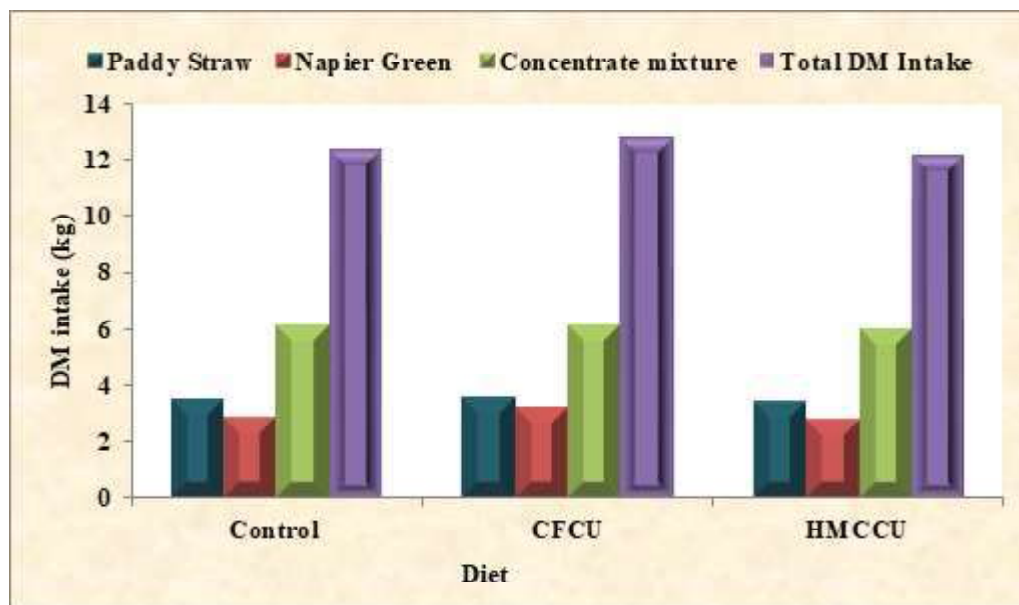


Table 3: Average fortnightly milk yield (kg) of cows fed diets containing various slow release non protein nitrogen products

Fortnight	Control	CFCU ¹	HMCCU ²	SEM	P value
Start	11.33±1.67	13.08±0.89	12.92±1.57	0.796	0.64
I	13.20±1.21	14.13±1.26	12.87±0.82	0.621	0.72
II	14.07±1.28	14.44±1.26	12.96±1.05	0.670	0.67
III	14.46±1.14	14.88±0.99	13.73±0.82	0.550	0.72
IV	14.22±1.24	14.40±0.96	13.65±1.21	0.626	0.89
V	14.39±1.33	15.36±0.86	14.58±1.28	0.646	0.83
VI	14.31±1.35	15.81±0.57	15.84±0.92	0.570	0.48
Mean±SEM	14.11±1.20	14.83±0.91	13.94±0.88	0.562	0.80

Each dietary treatment contains 6 replicates

¹ Calcium salts of fatty acids coated non protein nitrogen

² Hydroxypropyl methylcellulose coated non protein nitrogen

weight. Kertz et al. (2010) reported that, urea can be fed to lactating dairy cows up to a level of 1.0% of the total ration without any negative effects on DMI. While higher dietary inclusion of (>2.0%) of urea or slow-release urea decreased the DMI in beef steers (Taylor-Edwards et al. 2009). Gardinal et al. (2017) incorporated urea or SRU (with or without sulphur) in rations at 2% DM and observed decrease DM intake in steers. In agreement with present study, Santiago et al. (2015) reported no significant difference in the DM intake in cross bred cows fed with polymer coated slow-release urea replacing the soybean meal at 0, 34, 66 and 100% levels. Similarly, Calomeni et al. (2015) reported comparable DM intake in Holstein cows fed control (without urea addition) or diets containing 1% urea, 1% polymer-coated slow release urea or 1% polymer-coated slow release urea with sulphur. Replacing SBM with Optigen in diets of cows had no effect on DMI (Raouf et al. 2017). However in contrast to above findings, Cherdthong et al. (2013) reported increase in DM intakes with the increasing level of calcium sulphate mixture (U-cas) supplementation in the urea molasses feed block and the intakes were highest with 180

g/kg followed by 150, 120, and 0 g/kg DM inclusion in Thai male native beef cattle.

Milk Production

Fortnightly milk yield and average milk yield during the study period was not affected ($P>0.05$) by replacing the conventional protein with SRNPN sources (Table 3). This could be as a result of better synchrony in utilisation of ammonia released from SRNPN sources along with energy sources added in the feed, as better as any conventional protein sources. Abreu et al. (2014) also observed no difference in milk yield of crossbred cows when SBM was replaced with polymer coated urea up to 6%. Likewise, Santiago et al. (2015) observed no effect on milk yield in crossbred cow when SBM in the control diet was replaced with polymer coated urea at graded levels (0, 34, 66 and 100%). Further, in the present study, the cows were medium milk yielders (average milk yield 14.29kg) and thus, have lower requirement of nutrients by these animals makes it possible to infer that the SRNPN met the

rumen-digestible protein requirements and consequently allowed for an adequate microbial protein synthesis. Some authors (Souza et al. 2010; Santos et al. 2011; Sinclair et al. 2012) have worked with high-milk-yield cows (>30 kg/cow/day) with slow release urea products and observed decrease in milk yield in the animals. While, Inostroza et al. (2010) observed greater ($P < 0.01$) milk yield in lactating cows by 0.5 kg/d per cow, when animals were fed polymer coated urea (Optigen) compared to SBM based control group, in a feeding trial with inclusion of Optigen at rate of 114 g/d as a replacement to SBM.

Milk Composition

Supplementation of SRNPN sources did not affect the milk fat, SNF, protein, lactose and milk urea nitrogen (Table 4) significantly ($P > 0.05$) and was comparable with that of control. However the total yield of fat, protein and lactose increased on 90th day by 11.89, 8.15 and 14.32% respectively compared to 0 day of feeding trial in all the treatments. This asserts that, the amount of microbial protein produced in SRNPN supplemented group is better than the control diet in maintaining the milk composition. Xin et al.

Table 4: Milk composition and milk component yield in dairy cows fed with diets containing various slow release non protein nitrogen products

Attribute	Control	CFCU ¹	HMCCU ²	Mean±SEM
Fat (%)				
0 day	3.26±0.46	3.42±0.63	2.98±0.48	3.22±0.41
90 day	3.10±0.47	3.32±0.95	2.98±0.57	3.13±0.41
Mean±SEM	3.18±0.51	3.37±0.51	2.98±0.51	
Fat yield (g/d)				
0 day	351.53±41.41	433.34±77.12	364.97±60.81	383.28±50.71
90 day	419.55±44.10	464.20±85.04	402.90±67.21	428.88±50.71
Mean±SEM	385.54±62.10	448.77±62.10	383.94±62.10	
SNF (%)				
0 day	8.50±0.13	8.10±0.057	8.26±0.067	8.29±0.07
90 day	8.38±0.21	8.20±0.07	8.32±0.13	8.30±0.07
Mean±SEM	8.44±0.09	8.15±0.09	8.29±0.09	
SNF yield (g/d)				
0 day	958.32±133.70	1060.12±74.88	1072.54±135.31	1030.17±57.85
90 day	1180.64±97.90	1215.54±71.97	1154.42±60.56	1183.53±57.85
Mean±SEM	1069.49±70.85	1137.98±70.85	1113.09±70.85	
Protein (%)				
0 day	3.05±0.176	3.17±0.08	3.30±0.082	3.17±0.07
90 day	2.95±0.167	3.03±0.042	3.00±0.052	2.99±0.07
Mean±SEM	3.00±0.08	3.10±0.08	3.15±0.08	
Protein yield (g/d)				
0 day	339.82±49.57	411.31±21.17	428.13±55.04	393.06±20.80
90 day	410.68±30.23	448.33±22.02	416.29±21.36	425.10±20.80
Mean±SEM	375.26±25.48	429.79±25.48	422.19±25.48	
Lactose (%)				
0 day	4.65±0.09	4.63±0.067	4.40±0.12	4.56±0.06
90 day	4.52±0.16	4.57±0.06	4.50±0.08	4.55±0.06
Mean±SEM	4.58±0.07	4.60±0.07	4.48±0.07	
Lactose yield (g/d)				
0 day	526.22±77.63	603.51±34.17	569.65±72.14	566.46±40.54
90 day	633.98±50.09	675.16±34.05	633.62±32.37	647.58±40.54
Mean±SEM	580.01±49.65	639.33±49.65	601.63±49.65	
Milk urea nitrogen (mg/dl)				
0 day	12.42±1.06	12.56±0.81	13.07±0.91	12.68±0.55
90 day	12.20±1.00	13.12±1.03	13.78±0.88	13.03±0.55
Mean±SEM	12.31±0.67	12.84±0.67	13.43±0.67	

Each mean value is an average of 2 values.

¹ Calcium salts of fatty acids coated non protein nitrogen

² Hydroxypropyl methylcellulose coated non protein nitrogen

(2010) also reported higher fat and protein yield in polyurethane-coated urea (0.6% of diet) and isolated soy protein (2.78% of diet) fed Holstein cows compared to feed-grade urea fed cows (0.6%). While, Santiago et al. (2015) also reported, no effect ($P>0.05$) of replacement of SBM with SRU at varying levels in the diets (0, 34, 66 and 100%) on percentages of fat, protein, total solids and solids non fat in the milk of cross bred cows. Similarly, Raouf et al. (2017) too reported no effect of supplementation of

Optigen @ 125 and 62.5 g with replacement of 1 and 0.5 kg soybean meal in diets of lactating Holstein cows on milk fat, protein, lactose and SNF. In contrast to above observations, Calomeni et al. (2015) also observed lower milk protein and its yield but similar fat yield in treatment group of Holstein cows fed either polymer-coated slow release urea with sulphur or without sulphur (2.95%) compared to conventional diet fed control group of cows.

Table 5: Blood biochemical constituents in dairy cows fed diets containing various slow release non protein nitrogen products

Attribute	Control	CFCU ¹	HMCCU ²	Mean±SEM
Blood urea nitrogen (mg/dl)				
0 d	21.79±1.08	22.31±0.77	21.28±1.15	21.79±0.57
90 d	21.25±0.72	20.42±0.83	21.04±1.23	20.90±0.57
Mean±SEM	21.52±0.70	21.36±0.70	21.16±0.70	
Glucose (mg/dl)				
0 d	43.96±1.27	45.53±1.01	43.35±1.11	44.28±0.81
90 d	43.73±1.69	45.09±1.76	45.25±1.37	44.69±0.81
Mean±SEM	43.85±0.99	45.31±0.99	44.30±0.99	
Total protein g/dl				
0 d	7.55±0.12	7.06±0.25	7.92±0.22	7.51±0.13
90 d	7.79±0.22	7.27±0.26	7.58±0.27	7.55±0.13
Mean±SEM	7.67±0.16	7.16±0.16	7.75±0.16	

Each dietary treatment contains 6 replicates

¹Calcium salts of fatty acids coated non protein nitrogen

²Hydroxypropyl methylcellulose coated non protein nitrogen

Table 6: Cost economics of feeding and milk production in dairy cows fed diets containing various slow release non protein nitrogen products

Attribute	Control	CFCU ¹	HMCCU ²	SEM	P
Average feed intake (kg/d)					
Paddy straw	4.02±0.34	4.08±0.19	3.89±0.26	0.148	0.88
Green Napier fodder	14.08±1.18	15.73±2.00	13.62±0.92	0.812	0.57
Concentrate mixture	7.04±0.59	7.02±0.27	6.81±0.46	0.250	0.93
Total ration intake	21.25±3.15	26.82±2.36	24.32±1.65	1.445	0.31
Cost of concentrate mixture (Rs/kg)	24.25	22.29	22.29	-	-
*Average cost of feeding (Rs/d)					
Paddy straw	24.13±2.03	24.47±1.16	23.34±1.58	0.891	0.88
Napier green fodder	42.23±3.55	47.19±5.99	40.85±2.77	2.437	0.57
Concentrate mixture	170.72±14.36	156.48±5.72	151.79±9.93	6.285	0.27
Total ration	237.08±19.94	228.14±11.83	215.98±14.28	8.913	0.52
Cost of milk production per day (Rs/kg)					
Average milk production during trial (kg/d)	14.11±1.20	14.83±0.91	13.94±0.88	0.562	0.80
Average 4% FCM milk production during trial (kg/d)	12.37±0.96	13.43±1.89	11.81±1.85	0.872	0.34
Cost of feeding per kg milk yield (Rs/d)	16.80±0.07	15.43±0.18	15.49±0.14	0.175	0.001
Cost of feeding per kg 4%	19.16±1.54	16.98±2.32	18.29±2.66	1.281	0.67
FCM milk yield (Rs/d)					

Each mean value is an average of 6 values.

¹ Calcium salts of fatty acids coated non protein nitrogen

²Hydroxypropyl methylcellulose coated non protein nitrogen

* Cost of ration includes cost of paddy straw was 6Rs/kg and Napier grass was 3Rs/kg.

The milk urea nitrogen (mg/dl) was comparable among the different dietary groups and it ranged from 12.31 to 13.43. This may be due to nearly similar quantities of DM intakes and consequently similar CP intakes. Urea concentration in milk of cow is influenced by the amount of crude protein in the diet, as well as by degradable and undegradable protein intake (Ropstad et al. 1989). Oltner and Wiktorsson (1983) have postulated that a surplus of N intake increases blood urea nitrogen (BUN) which has a close relationship with milk urea nitrogen (Dhali, 2001), because urea freely diffuses from blood to milk. Golombeski et al. (2006) compared two diets containing nitrogen sources either as slow release urea diet @ 0.61% of total diet or no slow urea diet, which partially replaced soya bean meal SBM and reported no effect on milk urea nitrogen. Similarly, Calomeni et al. (2015) also observed no effect on MUN in Holstein cows fed either polymer-coated slow release urea with sulphur or without sulphur (2.95%) compared to conventional diet fed control group of cows. Raouf et al. (2017) too reported no effect of supplementation of Optigen @ 125 and 62.5 g with replacement of 1 and 0.5 kg soybean meal in diets of lactating Holstein cows on MUN.

Blood Biochemical Constituents

Replacing the conventional protein source with SRNPN sources did not affect the blood urea nitrogen, glucose and total protein in serum of dairy cows (Table 5). Incorporation of FCU and HMCCU at rate of 2.42% in concentrate mixture had no adverse effect on BUN in cows.

The BUN increased in buffalo heifers fed with diets containing 1% urea compared to control (Sorathiyai and Fulsoundar, 2011), while such increase in BUN was not observed in present study with inclusion of SRNPN at 1% of DM intake. Corroborating with the present study findings, replacement of regular urea with Optigen at 0,40,80 and 100% levels in beef steers (Gonçalves et al. 2015) and dietary inclusion of urea and Optigen at 1% in Nili-Ravi buffalo calves diets (Muhammad et al. 2015) showed no effect on blood glucose, total protein and blood urea nitrogen concentration. Similarly, dietary inclusion of feed grade urea, polymer-coated slow-release urea with or without sulphur @ 20 g/kg DM to Nellore steers also showed no ($P>0.05$) effect on blood glucose and blood urea concentration (Gardinal et al. 2017). While, Alves et al. (2014) observed an increase in BUN values with inclusion of Optigen at the rate of 0, 20, 40, 60 and 80% as a replacement to urea in the diet of sheep.

Cost Economics

The cost of concentrate mixture of control group (Rs. 24.25/kg) was higher compared to CFCU and HMCCU (Rs. 22.29/kg) based concentrate mixture due to higher inclusion level of ground nut cake (Table 6). Though non significant, the average daily cost of feeding (Rs/d) was lowered in CFCU (228.14) and HMCCU (215.98) diet fed cows compared to control group cows (237.08)

by 3.77 and 8.89% respectively, due to higher cost of concentrate mixture of control diet. Whereas, the cost of feeding (Rs) per kg milk production differed significantly ($P<0.01$) with highest in control diet fed group (16.80) while in CFCU (15.43) and HMCCU (15.49) fed group it was lower. No difference in feed cost of milk production was observed between the 2 SRUP groups. Higher cost of feeding per kg milk production in control group was mainly due to high cost of concentrate mixture consumed in comparison to two SRNPN groups. The cost of feeding per kg 4% fat corrected milk was though non-significant among the three dietary groups, it was lowered by 11.38 and 4.54% when fed CFCU and HMCCU diets, respectively compared to control. Abreu et al. (2014) also reported lower cost of production per kg milk production in cows with supplementation of polymer coated SRU was replacing SBM up to 6% of control diet. While, Raouf et al. (2017) observed that replacement of SBM with Optigen did not affect the cost of feeding of Holstein cows.

Conclusion

Based on the above findings it can be concluded that, SRNPN sources (Calcium salts of fatty acids coated urea and Hydroxypropyl methylcellulose coated urea) can be included at 1% of dry matter replacing the conventional protein sources, without affecting the milk yield and its composition in cross bred cows. Replacing the conventional protein sources with SRNPN sources reduced the feed cost per kg milk production by 8.87 and 8.46%, respectively.

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Economic analysis of different milk products manufacture at farms in Punjab State

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Abstract: Value addition has greater importance for dairy producers particularly in situations of poor market for raw liquid milk. Keeping in view the benefits of the value addition of milk, the present study was undertaken to assess the economic feasibility of value addition of milk at farm level which could be considered as the best alternative in order to sustain and increase the income level of the dairy farmers. Total numbers of 50 farm households were selected from 8 districts of Punjab state, of which constitute 19 small farmers, 16 medium farmers and 15 large farmers. Around 69.39 per cent of liquid milk was sold out as such while 18 per cent was used for manufacturing of milk products in the study area. The average quantity of milk used for manufacturing of milk products was 3.34, 8.30 and 14.12 litres per farm in the case of small, medium and large farms, respectively. In summer and winter season, average quantity of 5.57 and 10.8 litres of milk respectively was used for manufacturing of milk products. The value addition of milk could generate about 62 per cent of more income as against the sale of liquid milk by small farmers, while medium and large farmers earned 68.67 and 58.88 per cent more income, respectively. The value addition of milk was generating higher income than that of sale of liquid milk and therefore, the study strongly recommends that value addition of milk at farm level is beneficial for earning sustainable income from dairy farming in the Punjab state.

Keywords: Cost-Benefit analysis, Economic feasibility, Milk products, Value addition

Introduction

Value addition is an innovative practice that enhances or improves quality of an existing product or introduces new products or creates new product uses. Small scale dairy market agents and chains supplying fresh milk and traditionally processed dairy products still play a significant role in most of the developing countries (Alvarez et al. 2018). They often provide the main market outlet for small scale dairy producers, and the main source of supplying fresh milk for consumers. In current scenario of rising input costs and lower profit margins, it becomes very important to study the dynamics of value addition of milk at farm level as it offers a strategy for transforming a sick enterprise into a profitable one. Therefore, lot of opportunity created for dairy farmers to adopt the value addition of milk by converting the raw liquid milk into milk products and can earn up to 25-50 per cent more profit as compared to selling the milk directly.

In India, numbers of animals in milk Exotic/Crossbred cows were 15.96 million numbers in 2016-17 which increased to 17.67 million numbers in 2018-19, whereas Non-descript/Indigenous cows were 33.16 million numbers in 2016-17 which increased to 35.16 million numbers in 2018-19. Also, the numbers of animals in milk of buffaloes in 2016-17 were 42.56 million which increased to 44.76 million numbers in 2018-19 (Anonymous, 2019a). Punjab livestock sector contribution to agriculture and allied GDP increased from ₹7698 crore (29 per cent) during 2000-01 to 33,422 crore (35.90 per cent) during 2015-16 (Anonymous, 2018). Punjab is producing 6.7 per cent of milk production of India with only 2.53 per cent dairy animals and stands first in terms of per capita milk availability (1181 gm/day vs. 394 gm/day) in the country (Anonymous, 2019b).

The Indian dairy industry is characterized by the existence of organized or formal sector and unorganized or informal sectors. Formal dairy sector is under developed basically in local remote areas where sale of milk occurred in distant markets with higher transaction cost compels small dairy farmers for informal sectors (BIRTHAL et al. 2005). Formal sector consists of dairy cooperatives

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and private processor which procures about 23 per cent of total milk production and remaining 77 per cent is being procured by informal dairy sector which consists of small dairy processing units, milk vendors and halwai shops (Sharma, 2015; BIRTHAL et al. 2017).

Till now, studies conducted so far have primarily focused on production side of dairy farming and value addition at plant level but there is no study that generated field level baseline data about value addition of milk at farm. Therefore, present study has been carried out to generate the data on the actual status of value addition of milk in the Punjab state. The cost-benefit analysis of processing of milk products at farm level would help dairy farmers in choosing right kind of product portfolio and this

would also generate a package of practices for farmers who are in business of liquid milk processing.

Materials and Methods

Selection of the study area

The present study was conducted in Punjab State. Total samples size of 50 farmers were selected by random sampling method as there was no such area where farmers were doing value addition of milk. The farmers were scattered all over the Punjab state therefore, from 8 districts and 35 villages, total 50 farmers were selected for the present study. All the dairy farmers were classified into three categories i.e. small, medium, and large by using

Table 1: Categorization of small, medium and large size farms in Punjab state

Category of farm	Small	Medium	Large
Number of dairy farmers	19	16	15
Herd Size (Avg. no. of animals)	09	15	19
Total milk production (litres/day/farm)	Up to 41.52	41.52 – 51.29	> 51.29
Average Total milk production (litres/day/farm)	24.05	46.45	70.76
Product manufactured	Milk Cake, Dahi Plain, Lassi Plain, Paneer, Ghee and Khoa	Milk Cake, Dahi Plain, Lassi Plain, Paneer, Ghee, Khoa, Pasteurized Flavoured Milk (PFM) and Sterilized Flavoured Milk (SFM)	Milk Cake, Dahi Plain, Lassi Plain, Paneer, Ghee and Khoa
Quantity of milk used for manufacturing of value added milk products (lit)	3.34	8.30	17.87
Level of value addition of milk (%)	13.89	17.87	19.95

cumulative cube root method (Singh, 1975). Total daily milk production was taken as the base criteria for the categorization of sample households. All sample households were sorted out in ascending order according to their quantity of milk production at farm and then by using cumulative cube root method, different categories of farms i.e. small, medium and large have been made.

Data Collection & Analytical Framework

The interview schedule was designed to collect the data from the selected dairy farmers. The field data on fixed investments, working capital, milk products, and price of inputs and outputs for the two season's viz. summer and winter of the year 2018 were collected from the respondent dairy farmers by personal interview method using a specially designed and pre-tested questionnaire. The required information regarding various costs of different milk products were collected from sample households. Averages and percentage analysis were used in the computation.

Value Addition

It reflected the difference between price for which a firm sold its products and the cost incurred on the purchased inputs by it. This difference represented the value addition by the productive activities of the firm (Kohls and Uhl, 1967).

Value addition = Selling price of the product – Cost of the total inputs

Table 1 showed that, total sample of 50 dairy farmers constituted 19 small farmers, 16 medium farmers and 15 large farmers. An average herd size constituted by small, medium and large dairy farms worked out to be 9, 15, and 19, respectively. The dairy farms having daily milk production up to 41.52 litres were classified as small farms; those having milk production in between 41.52 – 51.29 litres were categorized as medium farms while the dairy farms having milk production more than 51.29 litres of milk production were grouped under large dairy farms.

The products manufactured at small, medium, and large farms were Milk Cake, Dahi Plain, Lassi Plain, Paneer, Ghee, and Khoa. On medium farm only, Pasteurized Flavoured Milk (PFM) and Sterilized Flavoured Milk (SFM) was prepared. The level of value addition of milk was highest on large farm i.e. 19.95% followed by 17.87% at medium farm and 13.89% at small farm (Kaur, 2020; Kaur et al. 2021).

Table 2: Milk yield of different breeds on selected farms

Category	Milk yield per in-milk animal (litres/day)			Milk yield per milch animal (litres/day)		
	Indigenous	Crossbred	Buffalo	Indigenous	Crossbred	Buffalo
Small	4.83	11.17	8.87	4.63	7.44	6.04
Medium	5.00	11.00	8.88	3.82	7.87	6.57
Large	5.50	11.33	11.03	2.75	9.09	6.76
Average	5.11	11.17	9.59	3.73	8.13	6.45

Results and Discussion

Amongst different subsidiary occupation, dairy farming can easily be adopted in the Punjab state which could be helpful in generating employment and supplement income of the rural households at sustainable level. It generates cash income for the rural people to meet their daily needs. The present study highlighted the level of value addition of milk on different farms and cost-benefit analysis of value added milk products by the dairy farmers at different farms in the Punjab state.

Table 2 showed that, among the different farms, on large farm, crossbred cattle had highest milk yield per in-milk animal (11.33 litres/day) followed by in buffalo (11.03 litres/day) and in indigenous cattle (5.50 litres/day); on medium size farm, crossbred cattle have highest milk yield per in-milk animal (11.00 litres/day), then buffalo (8.88 litres/day) and indigenous cattle (5.00 litres/day) and on small size farm, crossbred cattle have highest milk yield per in-milk animal (11.17 litres/day), then buffalo (8.87 litres/day) and indigenous cattle (4.83 litres/day). The average milk yield per in-milk animal was highest in crossbred cattle (11.17 litres/day), followed by in buffalo (9.59 litres/day) and in indigenous cattle (5.11 litres/day). The average milk yield per milch animal was also highest in crossbred cattle (8.13 litres/day), followed by buffalo (6.45 litres/day) and indigenous cattle (3.73 litres/day).

Table 3 showed that, total milk production produced per day per farm was 24.05, 46.45 and 70.76 litres of milk on small, medium, and large farms, respectively. The total milk production was highest on large farm from crossbred cattle (70.76 litres/day/farm), out of which the percentage share of crossbred cattle was highest i.e. 56.09 per cent. On medium size farm, out of total milk production (46.45 litres/day/farm), the percentage share was maximum by buffalo i.e. 51.64 per cent, and on small size farm, out of total milk produced (24.05 litres/day/farm), the contribution was maximum by buffalo i.e. 76.85 per cent.

Level of value addition

Quantity of milk used for converting into value added milk and milk products represent level of value addition (Kaur et al. 2020).

The value addition would benefit who can convert liquid raw milk into value added milk products which could generate more

Fig. 1. Level of value addition of milk at sample farms**Table 3:** Species-wise milk production on selected farms

Category	Total milk Production (litres/day/farm)	Indigenous cattle (%)	Crossbred cattle(%)	Buffaloes (%)
Small	24.05	4.13	19.02	76.85
Medium	46.45	3.69	44.67	51.64
Large	70.76	0.37	56.09	43.54

profits to dairy farmers. The value addition at farm level is being promoted due to different reasons but the most important reason is that it will increase the per liter returns of the farm and increases the shelf life of milk and ultimately increase the socio-economic status of dairy farmers by generating additional source of income. Level of value addition of milk calculated from the below formula.

$$\text{Level of value addition (\%)} = \frac{\text{Quantity of value added milk and milk products produced} \times 100}{\text{Quantity of milk used for production}}$$

Fig. 1 showed that, highest percentage i.e. 19.94% of value addition of milk was done at large farms, followed by 17.87% at medium farm and 13.9% at small farms. The dairy farmers were engaged in preparation of different kind of value added milk and milk products such as Milk Cake, Dahi Plain, Lassi Plain, Paneer, Ghee and Khoa. Medium size farm households in addition to above mentioned products, also prepared Pasteurized flavoured milk (PFM) and Sterilized flavoured milk (SFM). Small and medium dairy farms were engaged in preparation of mainly dahi and lassi as their major milk products while large farms were involved in preparation of paneer, ghee and khoa as their main products.

Overall, majority of the farmers i.e. 15 (30 per cent) were involved in preparation of dahi followed by 13 (26 per cent) prepared lassi and ghee and 11 (22 per cent) prepared paneer and khoa at their farms. Small-scale dairy enterprises contributed between 35 and 40 per cent of the milk production (Val-Arreola et al. 2006). The study on value addition and performance of informal dairy enterprises in Kenya concluded that product diversification in the form of value added milk products yielded better performance compared to the performance of raw fresh milk in the informal sector (Kariuki et al. 2015). The study found that significantly superior performance of value added milk products as compared to raw fresh milk in terms of profit per litre gain. It was also found

that, raw fresh milk contributed largely to the total sales of the business compared to value added milk products.

Table 4 revealed that an average daily milk production was 45.24 litres per day per farm. Out of total milk production, 39.55 litres per day per farm (87.42 per cent) was surplus quantity (either for sale as liquid milk or used for milk products) after retaining 5.53 litres (12.22 per cent) for self-use and 0.16 litre (0.35 per cent) for calves suckling. The results showed that the maximum quantity i.e. 31.39 litres per day per farm (69.39 per cent) was sold as liquid milk; however, about 8.16 litres (18.04 per cent) of the milk was used for manufacturing of milk products. Category wise, per day production of milk was 24.05, 46.45 and 70.76 litres per day on small, medium and large farms respectively. The quantity of milk sold as liquid milk was 16.29, 31.73 and 50.14 litres per farm in the case of small, medium and large farms, which constituted 67.73, 68.31 and 70.86 per cent share to the total milk production. An average quantity of milk used for preparation of milk products was 3.34, 8.30 and 14.12 litres per farm per day in the case of small, medium and large farms, which constituted 13.89, 17.87 19.95 per cent share to the total milk production, respectively. Season-wise, an average quantity of milk production worked out to be 48.83 litres per day in winter season and 41.70 litres per day in summer season.

Out of the total milk production, the per cent share of surplus milk was observed to vary with farm size. It was the highest on large farms 71.25 litres (90.80 per cent), followed by medium 42.86 litres (86.19 per cent) and least on small farms i.e. 31.61 litres (81.61 per cent) respectively in winter season. Similar pattern can be seen in the case of quantity of milk sold as liquid milk and quantity of milk used for milk products. Out of total milk production in summer season, 87.29 per cent of the milk was left as surplus quantity after retaining 12.35 per cent for self-use and 0.36 per cent for calves suckling. Maximum quantity i.e. 73.96 per cent was reported to be sold out as liquid milk; however, about

The per unit cost for manufacturing of one kg of milk cake was worked out to be Rs 277.70, and medium farmers earned Rs 22.30 margin per litre of milk as net income after selling one kg of milk

cake on medium farm. The per unit cost for producing one kg of dahi plain was worked out to be Rs 32.58, of which medium farmers received Rs 17.42 margin per litre of milk as net income after

Table 5: Cost-Benefit analysis of different milk products manufactured on small size farms of Punjab state

Particulars	Milk Cake	Dahi Plain	Lassi Plain	Paneer	Ghee	Khoa
Total annual quantity produced (in kg)	465	5840	4980	364	546	213
(A) Fixed cost (in Rs.)						
Interest on Capital Investment	360	190	785	800	1275	1800
Depreciation on building & equipment	120	110	615	200	225	600
Total fixed cost (A) (in Rs.)	480	300	1400	1000	1500	2400
(B) Variable cost (in Rs.)						
Cost of raw milk	110550	172525	144000	93800	192960	37989
Citric acid	4600	-	-	300	-	-
Cost of extracting cream	-	-	-	-	48240	-
Boiling of cream	-	-	-	-	900	-
Sugar price/salt	5610	-	6120	-	-	-
Fuel cost	165	6145	-	600	-	1565
Labour cost	3485	12305	5472	1828	11400	2794
Packaging cost	660	6116	3870	253	396	649
Total variable cost (B) (in ¹)	125070	197091	159462	96781	253896	42997
Total cost (A+B) (in Rs.)	125550	197391	160862	97781	255396	45397
Unit cost ((Rs./kg or litres)	270.0	33.80	32.30	268.63	467.76	213.13
Price of Product(Rs./kg or litres)	290.0	45.0	48.0	280.0	480.0	230.0
Margin per litre of milk (in Rs.)	20.0	11.20	15.70	11.37	12.24	16.87

Table 6: Cost-Benefit analysis of different milk products manufactured on medium size farms of Punjab State

Particulars	Milk Cake	Dahi Plain	Lassi Plain	Paneer	Ghee	Khoa	PFM	SFM
Total annual quantity produced (in kg)	688	6638	6047	688	1725	242	2274	1037
(A) Fixed cost (in Rs.)								
Interest on Machines and equipments	390	252	975	900	1440	2400	10930	5387
Depreciation on equipment & Machines	130	108	525	300	360	800	2732	951
Total fixed cost (A) (in Rs.)	520	360	1500	1200	1800	3200	13662	6338
(B) Variable cost (in Rs.)								
Cost of milk	168300	189750	172000	180180	625152	44352	64020	29700
Citric acid	8200	-	-	500	-	-	-	-
Cost of extracting cream	-	-	-	-	156288	-	-	-
Boiling of cream	-	-	-	-	2700	-	-	-
Sugar price/salt	8670	-	7310	-	-	-	-	-
Fuel cost	255	6395	-	1100	-	1670	3500	6300
Labour cost	4193	13597	6192	3595	33750	3525	1290	774
Flavour used	-	-	-	-	-	-	776	360
Packaging cost	918	6163	4364	455	1184	660	9700	4500
Total variable cost (B) (in Rs.)	190536	215905	189866	185830	819074	50207	79286	41634
Total cost (A+B) (in Rs.)	191056	216265	191366	187030	820874	53407	92948	47972
Unit cost (Rs. /kg or litres)	277.70	32.58	31.65	271.85	475.87	220.69	40.87	46.26
Price of Product(Rs. /litres)	300	50	50	285	490	240	80	90
Margin per litre of milk (in Rs.)	22.30	17.42	18.35	13.15	14.13	19.31	39.13	43.74

*SFM (Sterilized Flavoured Milk); PFM (Pasteurized Flavoured Milk)

selling one kg of dahi plain on medium farm. The per unit cost for producing one kg of lassi plain was worked out to be Rs 31.65, and medium farmers received Rs 18.35 margin per litre of milk as net income after selling one litre of lassi plain on medium farm. The per unit cost of manufacturing one kg of paneer was worked out to be Rs. 271.85, and medium farmers earned Rs. 13.15 margin per litre of milk as net income from selling one kg of paneer on medium farm. The per unit cost of manufacturing one kg of ghee worked out to be Rs. 475.87, and medium farmers earned Rs. 14.13 margin per litre of milk as net income after selling one kg of ghee on medium farm. The per unit cost of producing one kg of khoa was worked out to be Rs. 220.69, and medium farmers received Rs. 19.31 margin per litre of milk as net income after selling one kg of khoa on medium farm. The per unit cost of producing one litre of pasteurized flavoured milk was worked out to be Rs. 40.87, and medium farmers received Rs.39.13 margin per litre of milk as net income after selling one litre of pasteurized

flavoured milk on medium farm. The per unit cost of producing one litre of sterilized flavoured milk was worked out to be Rs. 46.26, and medium farmers received Rs 43.74 margin per litre of milk as net income after selling one litre of sterilized flavoured milk on medium farm.

The product-wise results with respect to cost-benefit analysis of different products produced on large farms such as milk cake, dahi, lassi, paneer, ghee, and khoa is given Table 7.

The per unit cost for manufacturing of one kg of milk cake was worked out to be Rs. 289.45, and large farmers earned ¹ 25.63 margin per litre of milk as net income by producing one kg of milk cake on large farm. The per unit cost for producing one kg of dahi plain was worked out to be Rs. 32.07, and large farmers earned Rs. 22.93 margin per litre of milk as net income by producing one kg of dahi plain on large farm. The per unit cost of manufacturing

Table 7: Cost-Benefit analysis of different milk products manufactured on large size farms of Punjab State

Particulars	Milk Cake	Dahi Plain	Lassi Plain	Paneer	Ghee	Khoa
Total annual quantity produced (in kg)	370	6380	7344	3260	3936	1140
(A) Fixed cost (in Rs.)						
Interest on Machines and Equipments	245	252	1200	1092	1640	2470
Depreciation on equipment & Machines (A)	105	108	300	308	360	830
Total fixed cost (A) (in Rs.)	350	360	1500	1400	2000	3300
(B) Variable cost (in Rs.)						
Cost of milk	61050	191400	235000	646800	1039104	158004
Citric acid	2700	-	-	1200	-	-
Cost of extracting cream from milk	-	-	-	-	259776	-
Boiling of cream	-	-	-	-	4200	-
Sugar price	3145	-	9996	-	-	-
Fuel cost	95	6415	-	3800	-	3375
Labour cost	1796	13769	8460	13304	51800	15575
Packaging cost	333	6190	5963	1630	1968	1570
Total variable cost (B) (in Rs.)	69119	217774	259419	666734	1356848	178524
Total cost (A+B) (in Rs.)	69469	218134	260919	668134	1358848	181824
Unit cost ((Rs. /kg or litres)	289.45	32.07	31.13	275.75	484.44	227.85
Price of Product (Rs. /kg or litres)	315	55	52	290	500	250
Margin per litre of milk (in Rs.)	25.55	22.93	20.87	14.25	15.56	22.15

Table 8: Income from value addition of milk against income from the sale of liquid milk in Punjab State

Category	₹ /farm			₹ /kg of milk		
	Income from value addition of milk	Income from sale of liquid milk*	% increase over liquid milk sale	Income from value addition of milk	Income from sale of liquid	% increase over liquid milk sale
Small	63096	38925	62.10	51.71	31.90	62.10
Medium	163979	97221	68.67	54.13	32.09	68.67
Large	269849	169846	58.88	52.38	32.97	58.88

* Income estimates based on the milk quantity used for conversion of milk products only

of one kg of lassi plain was worked out to be Rs. 31.13, and large farmers earned Rs. 20.87 margin per litre of milk as net income by producing one litre of lassi on large farm. The per unit cost for producing one kg of paneer was worked out to be Rs. 275.75, and large farmers earned Rs. 14.25 margin per litre of milk as net income by producing one kg of paneer on large farm. The per unit cost of manufacturing one kg of ghee worked out to be Rs. 484.44, and large farmers earned Rs. 15.56 margin per litre of milk as net income for producing one kg of ghee on large farm. The per unit cost of producing one kg of khoa was worked out to be Rs. 227.85, and large farmers earned Rs. 22.23 margin per litre of milk as net income for producing one kg of khoa on large farm.

Comparing the results of the present study with respect to previous studies carried, it was seen that processing of raw milk into value added milk and milk products was seen mainly in organized sector of co-operative and private dairy plants. Narnaware and Sonwane (2021) worked out the cost of production of cow ghee was Rs. 126.53 but, Chauhan et al. (2006) calculated unit cost of manufacturing of ghee was Rs. 120.97 and stated that profit margin of 1.65 per kg of ghee production while Babu and Verma (2010) calculated the total cost of per unit production of ghee (Rs./kg) in co-operative and private dairy plant was Rs. 175.91 and Rs. 176.12, respectively and earned Rs. 34.09 and Rs. 28.28 margin respectively. Rangasamy (2006) worked out cost of per kg of ghee in co-operative and private dairy plant as Rs. 129.49 and Rs. 129.89, respectively. Doni and Chauhan (2018) calculated the unit cost of ghee production in co-operative milk plant was Rs. 320 per kg, Rs. 205.09 per kg of panner and Rs. 10.35 per litre of dahi produced. Babu and Verma (2010) calculated the unit cost of khoa produced in co-operative dairy plant was Rs. 169.41 and stated margin of Rs. 10.59. Rangasamy (2006) calculated per unit cost of favoured milk in co-operative and private dairy plant as Rs. 50.05 and Rs. 29.14, respectively. Thakur et al. (2020) worked out the cost of different dairy products such as dahi, paneer and ghee and stated that per unit cost of dahi was Rs. 39.30, paneer was Rs. 217.78 and ghee was Rs. 425.24 and generated profit per kg of Rs. 20.70 from dahi, Rs. 27.22 from paneer and Rs. 54.24 from ghee, which is higher as compared to the present study.

Category-wise product-wise income from sale of milk products

The extent of income generated from sale of milk products turned out to be Rs. 66171, Rs. 167251 and Rs. 262989 on small, medium and large farms. Income from all milk products as Milk Cake, Dahi Plain, Lassi Plain, Paneer, Ghee, Khoa, Pasteurized flavoured milk (PFM) and Sterilized flavoured milk (SFM) was calculated that comes out to be increased with increase in farm size as it is more on large farms than medium and small farms.

Table 8 showed that, value addition of milk has been generating more income as against the sale of liquid milk. The extent of income generated per annum from the value addition of milk turned

out to be Rs. 63096, Rs. 163979 and Rs. 269849 per farm on small, medium and large farms. However, the quantity of milk used for the conversion of milk products if sold in liquid form it is assumed to be generated Rs. 38925, Rs. 97221 and Rs. 169846 per farm on small, medium and large farms respectively.

Conclusions

Around 69.39 per cent of liquid milk was sold out as such while 18 per cent was used for manufacturing of milk products in the study area. The value addition of milk could generate about 62 per cent more income as against the sale of liquid milk on small farms, while medium and large farmers earned 68.67 and 58.88 per cent more income from value addition of milk. There were total 73 labourers working per month which include 7 permanent labourers, 62 contract labourers and 4 daily paid labourers. Each permanent labour get Rs. 6000/month salary, contract labour get Rs. 5322/month salary and daily paid labour get Rs. 3750/month salary. Therefore during the value addition of milk, the labourers were utilized which generates employment on the farm. The value addition of milk has been generating higher income than that of sale of liquid milk and therefore, the study strongly recommends the value addition of milk at farm level for earning sustainable source of income at farms in the Punjab state.

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SHORT COMMUNICATION

Labour absorption in livestock activities in arid Western and Northern region of Rajasthan

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Abstract: In the arid western and northern regions of Rajasthan livestock activity is a complementary activity to farming and it provides much needed risk cover in case of crop failure. The present investigation was undertaken to study the labour absorption in different livestock activities in Arid western and northern regions of Rajasthan. The study was conducted based on primary data. The primary data were collected from the 200 households of 10 villages during year of the 2018-2019. The average utilization of human labour in livestock activities was found 54.48 man-days/animal/year in arid western and northern region. The participation of woman labour in all livestock activities was found more than man labour in all herd size group in arid western and northern region. The annual average human labour absorption was maximum in site preparation activity i.e., 28.76 hours/animal/year for male and 101.63 hours/animal/year for female, then other activities in all size of farms.

Keywords: Labour absorption, Livestock activity, Health care, Handling, Site preparation

Livestock population status of major milch animal of Rajasthan (In million) was presented in Figure 1. Category wise Labour absorption in livestock activities in arid western and northern region are presented in Table 1. On all the size categories of farmer's total human labour used in livestock activities was family labour. The annual average human labour absorption in livestock activities in arid western and northern region was 54.48 man-days/animal/year, out of which man labour contributes 12.60 man-days/animal/year while woman labour contributes 41.88 man-days/animal/year. The annual average absorption of man labour was the highest in herding (33.52 hours/animal/year) followed by site preparation (28.76 hours/animal/year) and feeding (12.40 hours/animal/year) while the lowest in health care (3.19 hours/animal/year) among all livestock activities. In case of woman labour it was the highest in site preparation (101.63 hours/animal/year) followed by production (61.41 hours/animal/year), watering (57.44 hours/animal/year) and feeding (54.39 hours/animal/year) and the lowest in health care (6.08 hours/animal/year). The annual average utilization of woman labour was higher than man labour among in the all livestock activities in arid western and northern region. It was observed that the women are involved in livestock production activities since a long time. There are some factors i.e. social, cultural and economic that are responsible for women participation in all livestock activities. Because most of the animal farming activities such as fodder collection, feeding, watering, health care, management, milking and household-level processing, value addition and marketing are performed by women.

In arid western and northern region, the utilization of human labour for small, medium and large herd category was 71.28 man-days/animal/year, 45.95 man-days/animal/year and 23.81 man-days/animal/year, respectively. It was noticed that the absorption of total human labour in livestock activities (family man and woman) was maximum on small herd and minimum on large farm. There is considerable variation in human labour absorption among the small, medium and large herd category. It was due to some livestock activities i.e. herding and site preparation in which time taken was same regardless number of animals.

In case of small farms, average number of animals were 1 to 4 while the time taken for feeding, watering, production, herding and site preparation are almost same whether number of animal is

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Table 5: Category wise Labour Absorption in Livestock Activities in Arid Western and Northern Region for the year 2018-2019.

Hard size	Animals category	Livestock activities (hours/animal/year)												Total (Man-days/animal/year)						
		Feeding			Watering			Production			Handling			Health care			Herding		Site preparation	
		M	W		M	W		M	W		M	W		M	W		M	W		
Small (=4 animals)	Young	10.58	47.17	9.67	48.01	2.53	14.60	0.00	0.00	0.00	2.99	6.74	40.69	53.08	33.00	112.55	12.43	35.27	47.70	
	Mature	18.75	79.96	14.15	87.10	17.27	118.22	7.12	18.01	8.48	4.69	8.48	59.75	77.67	45.50	159.63	20.90	68.63	89.53	
	Average	16.03	69.03	12.65	74.07	12.35	83.68	4.75	12.01	4.12	7.90	47.04	61.27	37.17	128.24	16.76	54.52	71.28		
Medium (5-6 animals)	Young	4.56	21.75	4.47	22.07	1.86	14.41	0.00	0.00	0.00	1.48	3.28	33.79	44.08	22.28	73.45	8.56	22.38	30.94	
	Mature	14.95	68.44	11.78	71.28	15.16	98.68	6.93	18.01	3.89	7.12	35.33	47.60	27.39	93.14	14.43	50.53	64.96		
	Average	9.14	42.35	7.70	43.78	7.73	51.59	3.06	7.94	2.54	4.97	34.47	45.63	24.53	82.14	11.15	34.80	45.95		
Large (=7 animals)	Young	3.58	17.08	3.58	17.08	0.72	2.76	0.00	0.00	0.00	1.03	1.83	0.00	0.00	13.12	59.46	2.75	12.28	15.03	
	Mature	9.89	43.44	8.76	43.61	2.05	30.00	5.48	14.52	2.12	3.74	0.00	0.00	13.54	60.09	5.23	24.43	29.65		
	Average	7.37	32.90	6.69	33.00	1.52	19.10	3.29	8.71	1.68	2.98	0.00	0.00	13.37	59.84	4.24	19.57	23.81		
Annual Average		12.40	54.39	10.10	57.44	8.81	61.41	4.00	10.26	3.19	6.08	33.52	43.85	28.76	101.63	12.60	41.88	54.48		

Animals = *Cattle (Cow & Buffalo); M=Man; W=Woman; Young= (< 3 year); Mature= (e"3 year); M* = It includes child labour by converting into standard unit

one or more. Due to less number of animals the absorption of human labour in livestock activities was more on small farms as compared to medium and large farms.

The annual average absorption of man and woman labour in all livestock activities was 16.76 man-days/animal/year and 54.52 man-days/animal/year, respectively on small farms (Table 5). The annual average man labour was used maximum in the herding (47.04 hours/animal/year) followed by site preparation (37.17 hours/animal/year) and minimum in health care (4.12 hours/animal/year). While in case of woman labour it was maximum in site preparation (128.24 hours/animal/year) and minimum in health care (7.90 hours/animal/year). The absorption of total man and woman labour in all activities in young stock was 12.43 man-days/animal/year and 35.27 man-days/animal/year, respectively while in mature stock it was 20.90 man-days/animal/year and 68.63 man-days/animal/year, respectively on small farms. The utilization of total human labour was more in mature stock (89.53 man-days/animal/year) than young stock (47.70 man-days/animal/year). It was due to some animal specific activities which was performed only in mature stock like oil cakes & concentrates to milch cattle, milking and handling.

The annual average absorption of man and woman labour in all livestock activities was 11.15 man-days/animal/year and 34.80 man-days/animal/year, respectively on medium farms. The annual average absorption of man labour was the highest in the herding 34.47 hours/animal/year and minimum in health care 2.54 hours/animal/year. While in case of woman labour it was maximum in site preparation (82.14 hours/animal/year) and minimum in health care (4.97 hours/animal/year). The absorption of total man and woman labour in all activities in young stock was 8.56 man-days/animal/year and 22.38 man-days/animal/year, respectively while in mature stock it was 14.43 man-days/animal/year and 50.53 man-days/animal/year, respectively on medium farms. The utilization of total human labour was more in mature stock (64.96 man-days/animal/year) than young stock (30.94 man-days/animal/year) on medium farms. There was same reason for higher utilization of human labour in mature stock than young stock, as found on small farms.

The annual average absorption of man and woman labour in all livestock activities was 4.24 man-days/animal/year and 19.57 man-days/animal/year, respectively on large farms. The annual average absorption of man labour was the highest in the site preparation 13.37 hours/animal/year and minimum in production 1.52 hours/animal/year. While in case of woman labour it was maximum in site preparation (59.84 hours/animal/year) and minimum in health care (2.98 hours/animal/year). While there was no use of human labour in herding activity on large farms. The absorption of total man and woman labour in all activities in young stock was 2.75 man-days/animal/year and 12.28 man-days/animal/year, respectively while in mature stock it was 5.23 man-days/animal/year and 24.43 man-days/animal/year, respectively on large farms.

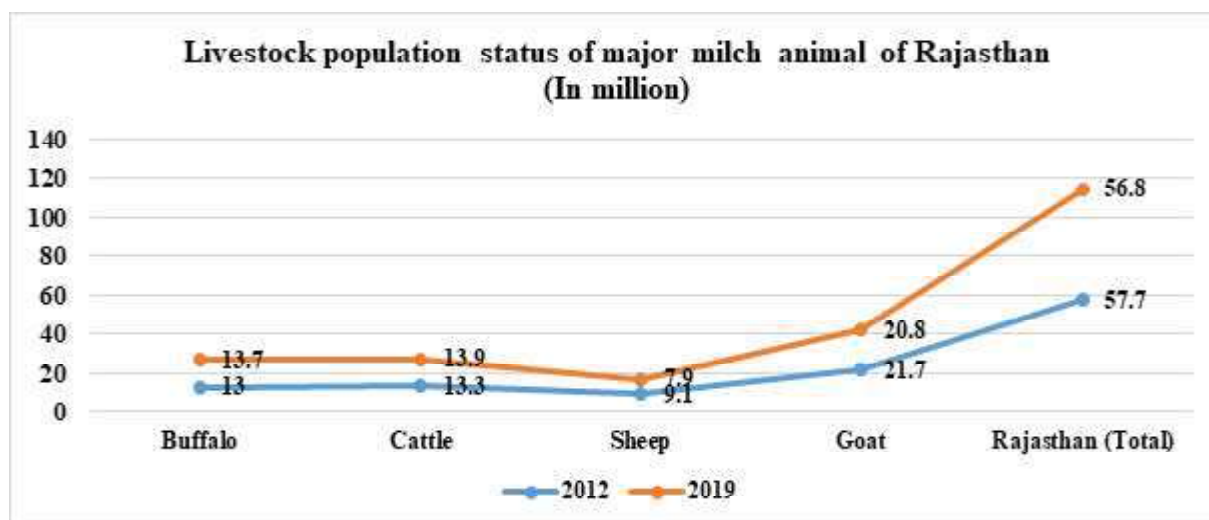


Fig. 1 Livestock population status of major milch animal of Rajasthan (In million)

The utilization of total human labour was more in mature stock (29.65 man-days/animal/year) than young stock (15.03 man-days/animal/year) on large farms. There was the same reason for higher utilization of human labour in mature stock than young stock, as found on small and medium farms on arid western and northern region.

Thus, it can be concluded that the participation of women labour in all livestock activities was found maximum than man labour in all herd size group in arid western and northern region. Total man-days contributed by women labour per animal per year were three times that of man labour in livestock activities. These results were in conformity with Upadhyay and Desai (2011), Mulugeta and Amsalu (2014) and Patel et al. (2016). The annual average human labour absorption was maximum in site preparation activity than other activities in all herd size group. It was observed that the herding activity was absent on large herd size group. As the number of animal increased from small to large herd size category the utilization of average human labour was decreased in arid western and northern region.

Conclusions

The absorption of family labour was found more for mature animal than the young animal on all herd size category arid western and northern regions in the state of Rajasthan. This was due to some specific activities such as milking, feed & fodder, handling which was more frequently carried out for mature animal than the young animal. The average utilization of family labour was found maximum on small herd size category while minimum was found on large herd size category. As the number of animal increased from small to large herd size category the utilization of average human labour was decreased in arid western and northern regions. The utilization of average human labour (includes only family labour) in livestock activities was found 54.48 man-days per animal per

year in arid western and northern region (Region-I). Out of this, the average absorption of male and female labour was found 12.60 man-days per animal per year and 41.88 man-days per animal per year, respectively. The participation of woman labour in all livestock activities was found more than man labour in all herd size group in arid western and northern region. The annual average human labour absorption was maximum in site preparation activity i.e., 28.76 hours per animal per year for man and 101.63 hours per animal per year for woman, then other activities in all herd size.

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SHORT COMMUNICATION

Role and motivational factors of vendors in milk marketing system

Awadhesh Dixit and Kuppusamy Ponnusamy

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Abstract: Vendors are important players in milk procurement and distribution among the consumers especially in urban and semi-urban areas. In order to understand their role and motivational factors in milk marketing, a study was conducted in four regions of Uttar Pradesh during 2020-2021. A sample of 120 was considered for the study by randomly selecting 30 respondents each from western, eastern, central and Bundelkhand regions of Uttar Pradesh. The primary data were collected and analysed using frequency and percentage method. The findings revealed that all the vendors had performed the 'milk procurement and milk distribution activities', 'serving as major source of information' as major roles and 'provision of farm inputs and advisory services' as minor role across the four regions. Adulteration detection was reported as major role by 16.67 per cent of respondents and minor role by 80.83 per of them and 2.5 per cent were not considering it as role, link to government officials as major role by only 2.5 per cent of the vendors and 97.50 per cent of them reported it as a minor role. Majority of the vendors in Central region (76.67%), Eastern region (86.67%), Bundelkhand region (73.33%) and Western region (66.67%) were motivated by seeing their previous generations who were running the vendorship successfully with good income and therefore

they adopted it as family tradition. Good income in vendorship motivated 56.57 per cent of them in Central region, 50 per cent in Eastern region, 40 per cent in Bundelkhand region and 46.67 per cent in Western region. The study warrants promotion of milk vendors in the formal dairy value chain by providing training and other support services for development of sustainable milk marketing.

Keywords: Milk distribution, Milk marketing, Unorganized dairy sector, Vendors

Dairy development entails involvement of several stakeholders who contribute directly and indirectly in various ways. India maintains first rank in the world milk production since 1998, with the production of 198.4 million tonnes in 2019-20 (GOI, 2020). While world's milk production is increasing at the rate 2.3 per cent per annum, the rate of increase is 6.62 per cent per annum in India (Kale et al. 2016). This magnificent performance is contributed by several stakeholders in a process of dairy development including milk vendors. About 80 per cent of the milk produced in the country is marketed through the unorganized sector and remaining 20 per cent is shared by co-operative and private sectors for processing. The organised sectors comprising dairy co-operatives and private dairies handle 10 - 12 per cent of the total milk production or 15 per cent of the marketed surplus (Ponnusamy et al, 2020). The rest is handled by large, complex, highly differentiated traditional private traders of milk and dairy products. These private traders are mainly the milk vendors operating in rural and urban areas.

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Quality and traceability solutions can make India an export hub in dairy and data driven decisions in dairy farming could lead to building smart dairy value chain (Ponnusamy, 2019). There are two types of milk marketing system in India namely organized and unorganized. Unorganised system comprised of milk vendors who mostly hail from rural areas, gather the milk directly from farmers and then directly sell to the consumers and left over milk to sweet meat makers (*Halwais*) in the urban areas. Despite its spectacular achievement in milk production, farmers are invariably distressed about realization of non-remunerative prices for liquid milk. The major chunk of profit is said to be cornered by middlemen who collect the milk in rural areas and sell it in urban and peri-

urban areas, at a rate which is 30-40 per cent more than what was paid to the producers. These middlemen are called as milk vendors or locally as 'Doodhia' who are mostly hailing from the same locality of farmers. Milk vendors occupy a central stage in connecting dairy farmers and consumers. The milk vendor constitutes an important element in the unorganized dairy production system. However, these vendors are always left out in this process of knowledge mainstreaming wherein they could have played a vital role in diffusion of scientific dairy management practices. Vendors are critical link between producers and consumers and are considered as missing links in the dairy development process. A major shift in thought process is required not to consider them as nemesis in the milk distribution system. Any research and development work towards making vendors as essential service providers would result in supply of quality dairy products to the consumers and improve the dairy value chain. In this context, a study was undertaken to ascertain the role and motivational factors of vendors in the milk marketing system.

The study was conducted during 2020-2021 in four different regions of Uttar Pradesh state. This state is largely known as the land of Dhudiyas (Milk vendors) and catchment for few private dairy players. The total annual milk production in the state was 30.52 million metric tonnes in 2019 which ranked 1st in India (GoI 2020). It is home of the highest buffalo population and second highest cattle population in India. It contributed about 18 per cent of total milk production in India with per capita availability of milk 371 gms/day which is relatively low as compared to national average. Therefore, four different economic regions were purposively selected for assessing the role and motivation of vendors in milk marketing system.

Selection of districts: The State of Uttar Pradesh (U.P) consists of 75 Districts which are grouped in four economic regions namely Western U. P., Central U. P., Eastern U. P. and Bundelkhand region. The Western, Central, Eastern and Bundelkhand regions comprises 30, 10, 28 and 7 Districts, respectively. One District from each region viz. Ayodhya, Lucknow, Meerut and Banda Districts representing Eastern U. P., Central U. P., Western U. P. and Bundelkhand region of U.P were randomly selected, respectively.

Selection of block and a peri-urban area: One block from each District viz. Rudauli in Ayodhya District, Malihabad in Lucknow District, Daurala in Meerut District and Atarra in Banda District were randomly selected. Within the block, a town with peri-urban characteristic was selected. Almost all the selected blocks represented such characteristics.

Selection of respondents: From each town, 30 Milk vendors were drawn randomly who were undertaking milk vendorship for continuously at least five years. Therefore, the total sample size was 120 respondents covering all four regions of the state.

Research design and statistical analysis: A survey research design was used to carry out the present study. Relevant variables were selected after thorough review of literature and consultation with the experts. The percentage and frequency were followed for data analysis.

Measurement of roles of vendors in milk marketing: It refers to the different activities played by the vendor from the point of milk collection to milk distribution to the doorsteps of the customers such as milk procurement, milk distribution, provision of inputs, advisory services, adulteration detection, link to government officials, and source of information. The respondents were provided with a list of roles and were asked to indicate their weightage for each item of roles. It was measured on three-point continuum i.e., major, minor, and not a role with score of 2, 1 and 0 respectively. Then, the frequencies and percentage were calculated for each item of roles.

Assessment of motivational factors for the involvement in milk vendorship

It was operationalised as different factors which attract the vendors' involvement in milk marketing system as well as running the vendorship. The reasons which were considered for assessing the motivation of vendors include good income, family tradition, easy going or hassle free type of work, social prestige, no alternate livelihood opportunity, maintenance of public relation as well as comparison of their social and economic status with other white collar jobs. To measure this variable, the responses were categorised in frequency and percentage.

The data on the roles and motivational factor of vendors in the milk marketing system are discussed as under:

Role of vendors in milk marketing system

While practising the milk vendorship, there is a need to take care of several activities in order to attain the greater effectiveness and attract the potential new customers and retain the existing customer base. So vendors are carrying out various recurring activities in their venture. The findings from Fig 1 and Fig 2 showed that all the vendors had performed the milk procurement and milk distribution activities across the four regions. Vendors acting as 'source of information' were reported as major role by 81.67 per cent of respondents while 18.33 per cent of them reported it as minor role. 'Provision of inputs to the milk producer clients' was reported as major role by 40.83 per cent vendors while 59.17 per cent of vendors reported as minor role. 'Performing advisory role to the milk producers' was reported as major role by 29.17 per cent of the vendors while 70.83 per cent of them considered it as minor role. 'Adulteration detection' was reported as major role by 16.67 per cent of respondents and minor role by 80.83 per cent of them and 2.5 per cent were not considering it as role, 'link to government officials' as major role by only 2.5 per cent of the vendors and 97.50 per cent of the reported it as minor role. These

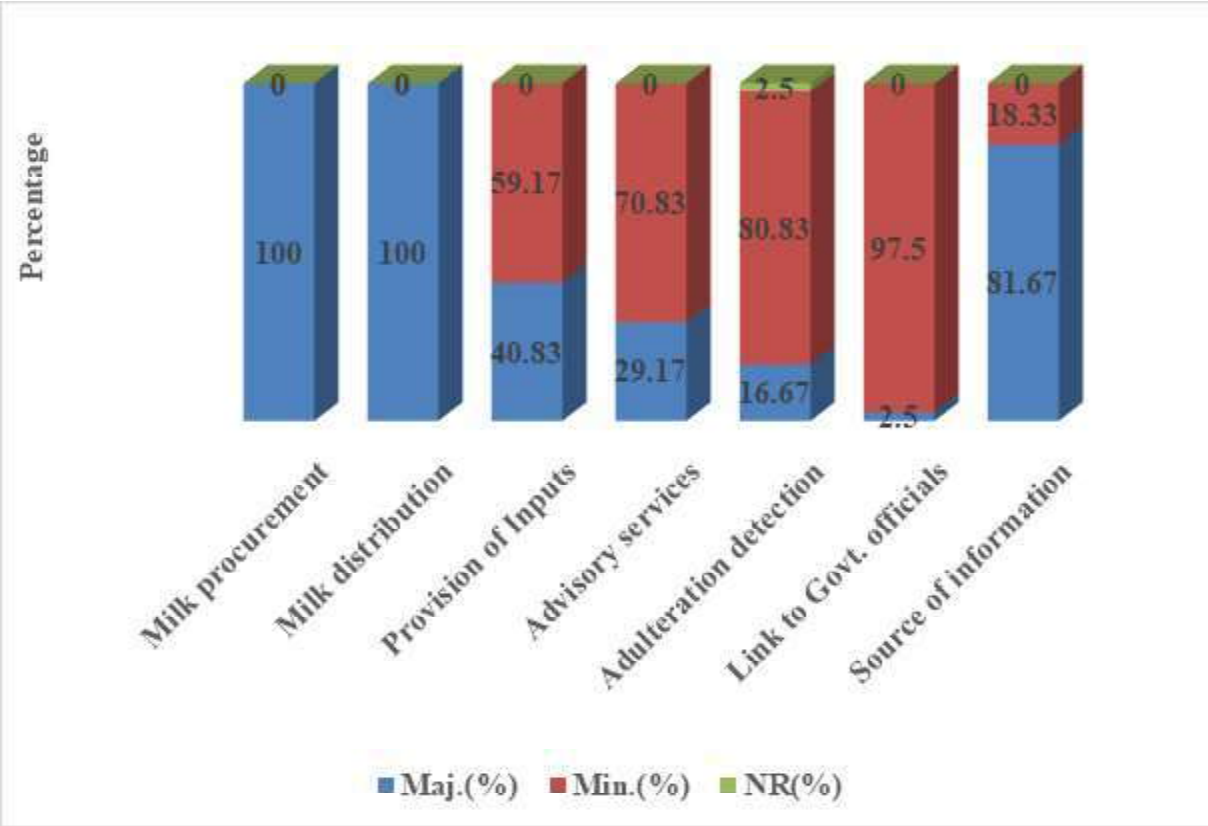
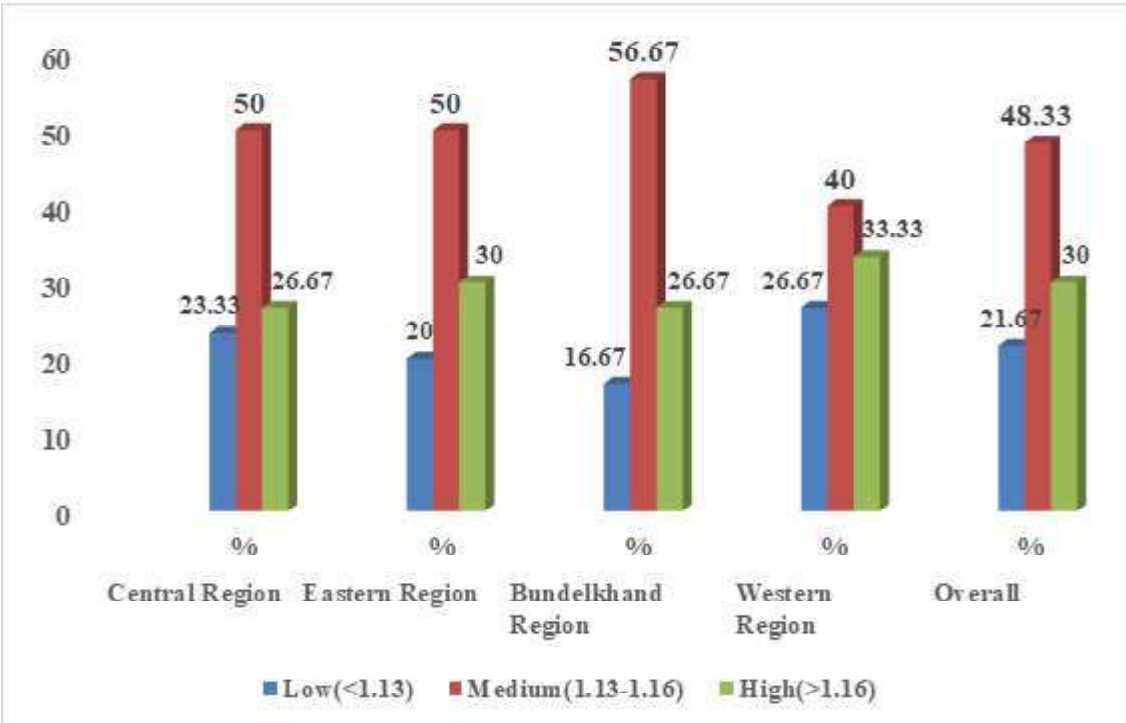


Fig 1. Representation of various roles of vendor in milk marketing system

Fig 2. Categorization of vendors as per their level of marketing efficiency



roles become essential for vendors in order to earn and maintain trust among clients and customers. It is to be noted that as most

of the milk producers were hailing from relatively distant locations where there was poor infrastructure facilities, majority of milk

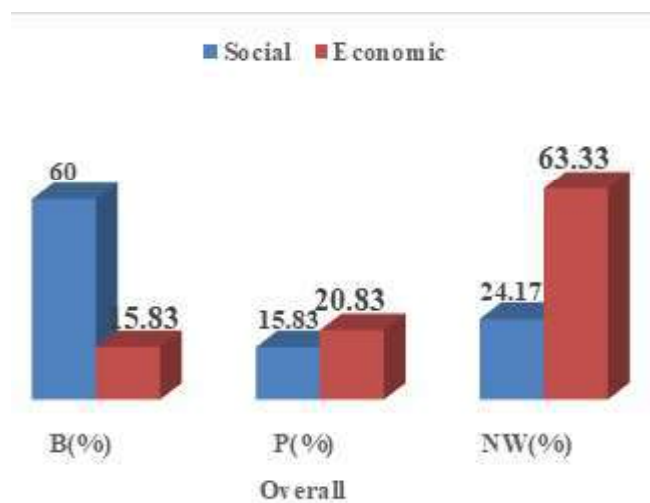


Fig 3. Expression of social and economic status of vendors (%)

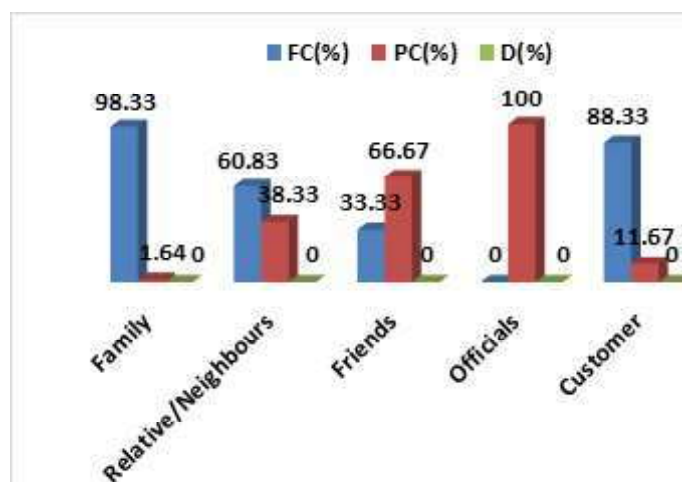


Fig 4. Representation of level of support for running the milk vendorship (%)

Table 1. Motivational factors of vendors in milk marketing system

S.No.	Motivational factors*	Central Region (n=30)		Eastern Region (n=30)		Bundelkhand Region (n=30)		Western Region (n=30)		Overall (n=120)	
		Freq	%	Freq	%	Freq	%	Freq	%	Freq	%
1.	Good income	17	56.67	15	50	12	40	14	46.67	58	48.33
2.	Family tradition	23	76.67	26	86.67	22	73.33	20	66.67	91	75.83
3.	Easy going or Hassle free	16	53.33	19	63.33	21	70.00	19	63.33	75	62.50
4.	Social prestige	11	36.67	18	60.00	9	30.00	12	40.00	50	41.67
5.	No other opportunities	1	3.33	2	6.67	3	10.00	1	3.33	7	5.83
6.	Public relation	16	53.33	15	50.00	19	63.33	21	70.00	71	59.17

* Multiple responses

producers depends on vendors because they were more frequently visiting them during milk procurement and distribution. In this process, milk producers obtain certain critical inputs from vendors and also act as source of information and advisory services due to better linkage of few vendors with government officials. Therefore, the role performed by every vendor from collection and distribution of milk in addition to supply of critical inputs immensely benefited both the customers and producer clients while continuing running the vendorship. About 55 per cent of the vendors provided monetary assistance sometimes to their dairy farmers (from whom vendors were collecting the milk). It is followed by 45 per cent of vendors who gave monetary assistance always. Monthly payment was commonly followed.

Motivational factors of vendors in milk marketing system

Motivation refers to the process that stimulates, guides and sustains goal oriented behaviour within individual. While undertaking any task, people need encouragement and same is

relevant in the case of starting dairy based vendorship. Study of motivational factors would throw light on support system which helped to promote the vendorship in the study area.

Various sources of motivational factors

For a credible success in any self-employed venture, motivational sources play key role in initiation and sustenance of the venture. Such sources of motivation would provide inspiration to further proceed with succeeding tasks. Table 1 revealed that majority of the vendors in Central region (76.67%), Eastern region (86.67%), Bundelkhand region (73.33%) and Western region (66.67%) were motivated by seeing their previous generations who were running the vendorship successfully with good income and therefore they adopted it as family tradition. Good income in vendorship motivated 56.57 per cent of them in Central region, 50 per cent in Eastern region, 40 per cent in Bundelkhand region and 46.67 per cent in Western region. About 53.33 per cent of them were motivated by easy going or hassle free nature of milk vendorship

in Central region followed by 63.33 per cent in Eastern region, 70 per cent in Bundelkhand region and 63.33 per cent in Western region. Maintenance of public relation motivated 53.33 per cent of vendors in Central region, 50 per cent in Eastern region, 63.33 per cent in Bundelkhand region and 70 per cent in Western region as they move to different places for distribution of milk. Therefore, they maintain good relations with key stakeholders of the society by which they could get certain help for other personal activities too. Although various sources created interest in starting the vendorship, actual stimulation had come from the self-motivation. In-depth observation coupled with curious mind can trigger new sources of motivation that eventually leading to starting and continuing milk vendorship.

Comparison of social and economic status of milk vendorship with white collar jobs

Social and economic status often play crucial role in starting any venture. In the case of milk vendorship also, these factors determine the active involvement in the venture. Majority (63.33 %) of the respondents had better perception of social status while 73.33 per cent of them perceived no worry at all about economic status in central region and 60 per cent of respondents expressed better social status while 63.33 per cent of them felt no worry at all about their economic status in Eastern region (Fig 3). In Bundelkhand region, 56.67 per cent respondents opined better social status while 53.33 per cent of them considered no worry at all about economic status. In Western region, 63.33 per cent of the respondents expressed better social status and no worry at all about economic status. The data indicate that earning a better and decent livelihood is the primary concern of milk vendors by living in local area or own village rather earning a higher income through private jobs where there are chances of job insecurity and living away from own villages.

Level of support for running the milk vendorship

While initiating and running vendorship, every vendor need a minimum level of support from different stakeholders to handle the difficult activities on daily basis. It is known from the Fig 4 that 98.33 per cent of the vendors got full cooperation from their family members while 88.33 per cent vendors were obtaining full cooperation from their customers and 60.83 per cent vendors took the same from their relative / neighbours, 33.33 per cent of them were obtaining the same from their friends whereas all vendors invariably obtained only partial cooperation from government officials in their vendorship which might be due to poor linkages or communication gap between vendors and officials. However, the extension functionaries should maintain better linkage with vendors in order to understand the dynamics of milk production, processing and marketing in both rural and urban areas. Further, they can implement certain schemes with the active support of vendors. It is worthwhile to note that family members are extending whole hearted support for milk vendorship

as it gives them regular flow of income which often helps them to continue the regular activities. Similar findings were reported by Chahal and Ponnusamy (2014).

Conclusions

Most of the vendors in all the four regions of Uttar Pradesh prompted the vendors to adopt milk vendorship as a family tradition by seeing their success of previous generations with good income. They perceived better social and economic status. Under the level of support from different stakeholders for running the vendorship, all the respondents (100%) were getting full cooperation from their family members in all regions except Eastern region (93.33%). Vendors performed their duty as source of information while carrying out milk procurement and milk distribution.

Limited contact of milk vendors with government officials' especially animal husbandry functionaries led to poor knowledge in scientific dairying and limited utilization of available technologies which calls for alternative extension approaches like linking vendors with implementing programs related to dairy farming. Milk adulteration being a big menace across the study area, capacity building of vendors on diversification in their produce with brand promotion would be major boon for them as they started selling only raw milk under their vendorship. Credit and infrastructure facilities would further motivate the vendors to improve their effectiveness level and effectively serve their clients. Marketing skills need further strengthening for vendors especially introducing innovativeness in vendorship.

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