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Effect of incorporation of guava leaf powder on storage stability of curd balls under aerobic packaging condition at refrigeration temperature

Varsha Vihan, VP Singh, Akhilesh K Verma (✉), Pramila Umaraw, Chirag Singh and Shardanand Verma

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Abstract: The present study has undertaken for the evaluation of the impact of incorporating guava leaf powder (GLP) on the storage stability of developed curd balls. Four groups of curd balls were formulated with the addition of different levels of guava leaf powder: C (control without GLP), T1 (with 1.5% GLP), T2 (with 3.0% GLP), and T3 (with 4.5% GLP). The results revealed that pH and titratable acidity were significantly ($P \leq 0.05$) lower in the treated groups than in control. Peroxide value, thio-barbituric acid reactive substances, and free fatty acid content were significantly ($P \leq 0.05$) lower in GLP incorporated curd balls than in control. Guava leaf powder added curd balls has significantly ($P \leq 0.05$) higher DPPH, ABTS, and total phenolic content than the control. Among all samples, T3 (4.5%) recorded significantly ($P \leq 0.05$) lower microbial growth than the others groups of curd balls. However, sensory panelists rated significantly ($P \leq 0.05$) higher scores for T2 than T3. The sequent of the study concluded that the curd balls prepared with the inclusion of 3.0 % guava leaf powder prevent Physico-chemical quality deterioration, improve antioxidant capacity, reduced lipid oxidation and microbial growth with acceptable sensory attributes.

Keywords: Antioxidant activity, lipid oxidation, antimicrobial activity, sensory quality, guava leaf powder

Introduction

Curd is considered as a well-known traditional fermented milk product of Indian origin and as the most important dairy product in human diet. It is a semi-solid product obtained from boiled or pasteurized milk by souring, natural or using harmless lactic acid or other bacterial cultures. Besides direct consumption, curd also utilised in preparation of “chakka” by straining whey from curd. “Chakka” is an indigenous fermented dairy product and refers to a white to pale yellow semi-solid product of good texture and uniform consistency obtained after draining off the whey from the curd.

Natural products are of great interest for the integration of health-promoting substances in the diet as natural food additives, for the prevention of diseases and also for the improvement of general well-being. Consumers have additional considerations and recommendations to use natural antioxidants from food sources instead of artificial antioxidants that are restricted due to their toxic and carcinogenic effects (Abdel-Hameed et al. 2014). Dairy products are one among the foremost fascinating and promising foods with reference to their potential inhibitor activity, because of their wide diversity of antioxidant molecules like milk caseins and whey proteins. Medicinal plants abundant in natural antioxidants and phenolics compounds are gradually applied in the manufacturing of dairy foods to enhance their nutritional and therapeutic properties.

Guava is known as *Psidium guajava* from the Myrtaceae family, is a globally well-liked tropical fruit contains high amount of vitamins and phytochemicals. Guava is a natural product that possesses dietary fibre as well as antioxidant compounds. The extracts and metabolites of this plant, especially from the leaves, possess beneficial activities such as antioxidant properties, antimicrobial properties compared to other herbs. Studies have shown that guava leaves are advantageous as antibacterial agents Biswas et al. (2013), antioxidants (Chen and Yen, 20017). The strong antioxidant mechanisms acquired by guava leaf could be attributed to their free radical- scavenging ability. Additionally, phenolic compounds appear to be liable for the antioxidant activity of guava leaf.

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Considering the above facts, the present study is conducted to improve the shelf life of curd balls by incorporating different levels of guava leaf powder with the following objectives: To optimize the levels of guava leaf in prepared curd balls, to assess the physico-chemical and sensory parameters of the prepared product and to study the storage stability at refrigeration temperature ($4 \pm 1^\circ\text{C}$) under aerobic packaging condition.

Materials and methods

Chemicals and media

All chemicals and media utilised during the study were of analytical class and procured from standard firms like Hi-media, SRL, CDH and Merck, etc. Raw buffalo's milk was purchased from nearby village dairy plant of Modipuram, Meerut. Freeze-dried Lactic Culture was purchased from CHR Hensen, Denmark. Low density polyethylene films (200 μm gauge) were procured from local market and were sterilised by exposing to U.V. light for 30 minutes before use.

Preparation of guava leaf powder

Guava leaves were collected from Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut campus. Guava leaves were first cleaned with tap water and damaged, unwanted leaves were discarded. To remove the wash-water from the surface the leaves were then air dried for 1 hour and then leaves were oven (Meta-Lab Scientific Industries) dried for 48 hours at $50 \pm 1^\circ\text{C}$. Dried guava leaves were then grounded in a food mixer and strained through a stainless-steel sieve. It was packed in air tight container and stored in cool and dry place until used.

Preparation of chakka and curd balls

In summary, fresh buffalo milk was heated to $85^\circ\text{C} \pm 5$ for 20 minutes, followed by cooling to a temperature range of $43 \pm 2^\circ\text{C}$. Milk was then inoculated with 1.5% starter lactic culture to commence the fermentation process and incubation was allowed to be carried out at $37 \pm 2^\circ\text{C}$ for 5-6 hours. After that curd was strained with cheese cloth for 4 hours. The bulk was subsequently stored at 4°C overnight.

Curd balls were prepared by incorporation of three different levels of guava leaf powder viz., (T1) 1.5%, (T2) 3% and (T3) 4.5%, the levels of guava leaf powder and control without guava leaf powder (C). All ingredients were weighed and thoroughly mixed till uniform batter formation and then shaped into balls (Table 1). These curd balls were then cooked by convection cooking in a preheated oven (Meta-Lab Scientific Industries) at 75°C for 30 minutes and then turned and again cooked for 15 minutes. The cooked curd balls were then cooled to room temperature and then each group was separately packed under aerobic packaging in low density polyethylene bags (LDPE) and stored under refrigeration temperature at $4 \pm 2^\circ\text{C}$ for further study.

Physico-chemical analysis

pH and titratable acidity value

The pH of the sample was determined by dipping the combined glass electrode of digital pH meter (ESICO, Model-1012). The titratable acidity in terms of percent lactic acid was determined by method as described by Shelef and Jay (1970).

Antioxidant activity

Total phenolic content

The total phenolic content of control products and treated groups was analyzed by Folin-Ciocalteu's method as prescribed by Zhang et al. (2006) with slight modification and gallic acid was used as standard.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity of curd balls were analyzed using stable radical (2, 2-diphenyl-1-picrylhydrazyl) as determined by Brand-Williams et al. (1995).

$$\text{DPPH Scavenging activity (\% inhibition)} = 100 - \frac{[At_{20}]}{At_0} \times 100$$

2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS⁺) radical scavenging activity

ABTS⁺ radical scavenging activity was determined as per the procedure outlined by Umaraw et al. (2023) using spectrophotometry. The ABTS⁺ activity was expressed as % inhibition using formula –

$$\text{ABTS}^+ \text{ activity (\% inhibition)} = \frac{0.7 - At_{20}}{0.7} \times 100$$

Lipid oxidation

Peroxide value, Thiobarbituric acid reacting substances (TBARS) value and Free fatty acids (FFAs)

Peroxide value was analyzed as per the procedure of Koniecko (1979). The peroxide value expressed as meq/kg of sample was calculated using the formula:

$$\text{PV (meq/kg sample)} = \frac{0.1 \times \text{mL } 0.1\text{N sodium thiosulphate}}{\text{Sample weight (g)}} \times 100$$

Thiobarbituric acid reacting substances value was analyzed as

per the procedure of Witte et al. (1970).

TBARS value (mg malonaldehyde/ kg of sample) = O.D. of the sample $\times$ 5.2

Free fatty acids value of the sample was determined by modified Koniecko (1979). The amount of potassium hydroxide consumed for titration was noted and then the free fatty acids content of the sample was calculated as follows:

$$\text{Free fatty acid (\% oleic acid)} = \frac{0.1 \times \text{mL } 0.1 \text{ N alcoholic KOH} \times 0.282}{\text{Sample weight (g)}} \times 100$$

Microbiological analysis

Standard plate count, psychrophilic count, coliforms count and yeast and moulds count of the samples were conducted as per the method prescribed by American Public Health Association (1992).

Sensory evaluation

The sensory quality of the samples was evaluated by using 9-point hedonic scale as presented in sensory evaluation scoring sheet. A nine-point hedonic scale, varying from extremely undesirable (score 1) to extremely desirable (score 9) was used. Sensory parameters such as colour, taste, aroma, texture and overall acceptability were used to assess the curd balls. Precooked curd balls from each batch were heated in microwave oven for 1-2 minutes and then presented to sensory panelist with 2-digit random code for evaluation. A sensory panel (semi-trained) was drawn from post-graduates' students and staff of college. After briefing properly about the product, the panelists were requested to evaluate the product to determine their organoleptic characteristics in terms of their colour, taste, aroma, texture and overall acceptability.

Statistical analysis

Table: 1 Formulation for the preparation of curd balls incorporated with guava leaf powder

Ingredients	Control	T1	T2	T3
Chakka	77.0	75.5	74.0	72.5
Refined oil	3.0	3.0	3.0	3.0
Flour	3.0	3.0	3.0	3.0
Salt	1.5	1.5	1.5	1.5
Spices	1.0	1.0	1.0	1.0
Condiments	2.0	2.0	2.0	2.0
Carrot	12.5	12.5	12.5	12.5
Guava leaf	0.0	1.5	3	4.5

C: Control curd balls without guava leaf powder; T1: curd balls with 1.5 % guava leaf powder; T2: curd balls with 3.0 % guava leaf powder; T3: curd balls with 4.5 % guava leaf powder.

Experiment was carried out three times and data were collected two times for every attributes. Two-way ANOVA was used for the analysis of recorded data using SPSS 22 statistical software (SPSS Inc., Chicago, IL, USA). Means of attributes were correlated using Duncan's multiple range test (DMRT), at the (Pdd0.05) level of significance.

Results and Discussion

Change in pH and titratable acidity

The pH value varied significantly ($P \leq 0.05$) among the groups (Table 2). The decrease in pH value and a corresponding increase in titratable acidity were observed in all groups. However, the pH values decreased significantly ($P \leq 0.05$) during storage which might be due to proliferation of *Lactobacillus sp.* of the microbes. Titratable acidity value among the groups differed significantly from the 5th day of storage to end of the storage time (Table 2). Titratable acidity values increased significantly ($P \leq 0.05$) during the storage. The decrease in pH value of the curd ball samples might be due to an increase in production of the acidic compounds during the proliferation of microorganisms. However, the rate of decrement in pH value of treated samples was lower than the control which might due to slower rate of growth of spoilage microbes. Similar results were also reported by Najgebauer-Lejko et al. (2011) for yoghurt prepared with incorporation of tea polyphenols during storage. Our findings were in accordance with the results of Qureshi et al. (2019) who reported decreasing trend of pH value in paneer prepared with the extracts during storage. The increase in titratable acidity value might be due to growth of lactic acid producing microbes during the storage. Our finding is in harmony with the results reported by Ahuja and Goyal (2013). Kumar et al. (2019) also reported increasing trend for the titratable acidity during the storage study of milk products.

Change in antioxidant parameters

Total phenolic content

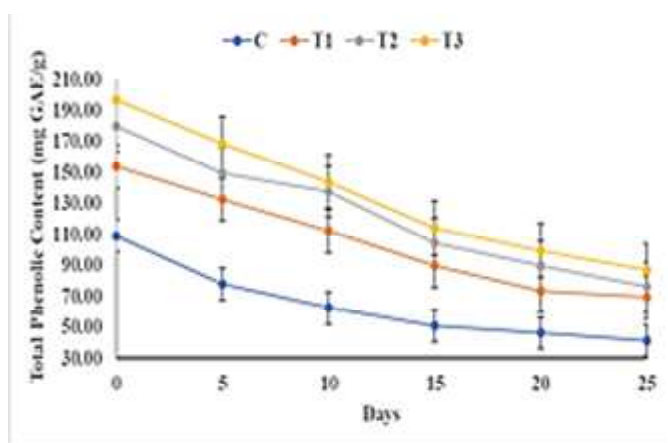


Fig. 1 Change in total phenolic content of curd balls prepared with guava leaf powder

C: Control curd balls without guava leaf powder; T1: Curd balls with 1.5 % guava leaf powder; T2: Curd balls with 3.0 % guava leaf powder; T3: Curd balls with 4.5 % guava leaf powder. n=6

On day 0, total phenolic content exhibited by treated curd balls followed the order T3>T2>T1>C as depicted in Fig. 1. The total phenolic content of guava leaf powder added curd balls was considerably ($P \leq 0.05$) higher than that of control curd balls, 109.17 (C), 154.00 (T1), 180.00 (T2), and 196.67 (T3) mg GAE/g, respectively. The total phenolic content of the groups varied significantly ($P \leq 0.05$) across the storage days. Refrigerated curd balls prepared with inclusion of guava leaf powder and control curd balls showed significantly ($P \leq 0.05$) declining trends for total phenolic content during the storage. The decreased total phenolic content during storage of curd balls might be due to

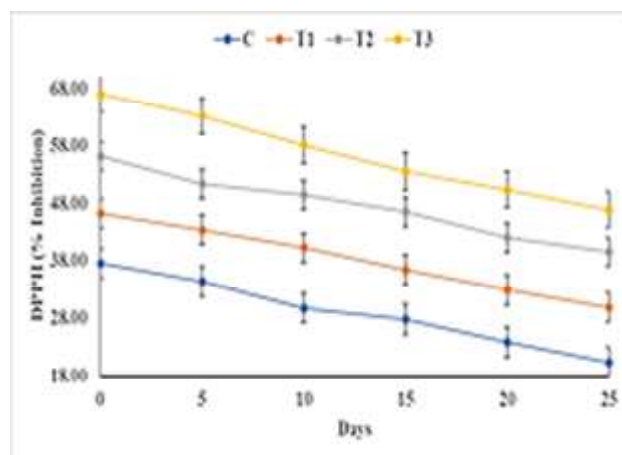


Fig. 2 Change in DPPH (% inhibition) of curd balls prepared with guava leaf powder

C: Control curd balls without guava leaf powder; T1: Curd balls with 1.5 % guava leaf powder; T2: Curd balls with 3.0 % guava leaf powder; T3: Curd balls with 4.5 % guava leaf powder. n=6

lipid oxidation and microbial degradation of phenolic compounds during storage. However, T3 showed highest total phenolic content amongst treatments at the end of storage. Phenolic content has linear association between scavenging activity. Lee et al. (2016) also reported decreased total phenolic content with increased storage time.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Da Porto et al. (2000) reported that 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical activity has been widely used to assess the free

Table: 2 Change in pH and titratable acidity of curd balls incorporated with guava leaf powder during refrigeration ($4 \pm 1^\circ\text{C}$) storage

Groups	0 Day	5 Days	10 Days	15 Days	20 Days	25 Days
pH						
C	5.50 ^{Yf} ±0.05	5.29 ^{Yc} ±0.03	5.13 ^{Yd} ±0.03	4.89 ^{Xc} ±0.02	4.72 ^{XYb} ±0.03	4.53 ^{Wa} ±0.05
T1	5.27 ^{Xc} ±0.03	5.11 ^{Xd} ±0.02	4.92 ^{Xc} ±0.03	4.85 ^{Xbc} ±0.03	4.80 ^{Yb} ±0.03	4.58 ^{WXa} ±0.02
T2	5.18 ^{Xc} ±0.02	5.01 ^{Wd} ±0.03	4.86 ^{WXc} ±0.02	4.67 ^{Wb} ±0.02	4.68 ^{WXb} ±0.04	4.59 ^{WXa} ±0.03
T3	5.06 ^{Wc} ±0.03	4.95 ^{Wd} ±0.03	4.81 ^{Wc} ±0.02	4.72 ^{Wb} ±0.02	4.62 ^{Wab} ±0.03	4.66 ^{Xa} ±0.02
Titratable acidity (% lactic acid)						
C	0.74 ^a ±0.003	0.77 ^{Ya} ±0.004	0.87 ^{Zb} ±0.003	0.94 ^{Zc} ±0.003	1.05 ^{Zd} ±0.004	1.24 ^{Ye} ±0.048
T1	0.74 ^a ±0.002	0.76 ^{Xa} ±0.003	0.85 ^{Yb} ±0.003	0.92 ^{Yc} ±0.003	1.03 ^{Yd} ±0.002	1.11 ^{Xe} ±0.031
T2	0.74 ^a ±0.002	0.75 ^{Xa} ±0.003	0.84 ^{Xb} ±0.002	0.90 ^{Xc} ±0.003	0.97 ^{Xd} ±0.003	1.06 ^{WXe} ±0.031
T3	0.74 ^a ±0.003	0.73 ^{Wa} ±0.004	0.82 ^{Wb} ±0.002	0.88 ^{Wc} ±0.004	0.93 ^{Wd} ±0.002	0.96 ^{Wd} ±0.029

Means values bearing small letters (a, b, c, d.....) days wise and capital letters (W, X, Y and Z) groups wise indicate differ significantly ($P \leq 0.05$) n=6; C: Control curd balls without guava leaf powder; T1: curd balls with 1.5 % guava leaf powder; T2: curd balls with 3.0 % guava leaf powder; T3: curd balls with 4.5 % guava leaf powder.

radical scavenging capacity of several compounds and has been recognized as a method for free radicals originating in lipids during oxidation. 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity of curd balls prepared with inclusion of guava leaf powder are presented in Fig. 2. Among all group's DPPH value differed significantly ($P \leq 0.05$) across the all-storage days. However, the DPPH value decreased significantly ($P \leq 0.05$) for all groups during the entire storage. Guava leaf powder exhibited concentration-based DPPH radicals scavenging activity. The DPPH free radical scavenging activity of guava leaf powder might be due to their hydrogen donating capacity. Presence of higher quantity of hydroxyl groups, resulted greater ability of free radical scavenging capacity. Guava leaf is rich source of phenolic compounds, like gallic acid, ellagic acid, ferulic acid, pyrocatechol and taxifolin Chen and Yen (2007) and presence of these phyto-active compounds is primarily responsible for the antioxidant activity (Farag et al. 2020).

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

Perusal of Fig. 3 results revealed that guava leaf powder added groups had shown significantly ($P \leq 0.05$) higher ABTS scavenging activity as compared to control. Comparatively higher ABTS scavenging activity of treated groups might be due to presence of higher phenolics contents. All guava powder added curd balls groups showed significantly ($P \leq 0.05$) higher ABTS scavenging

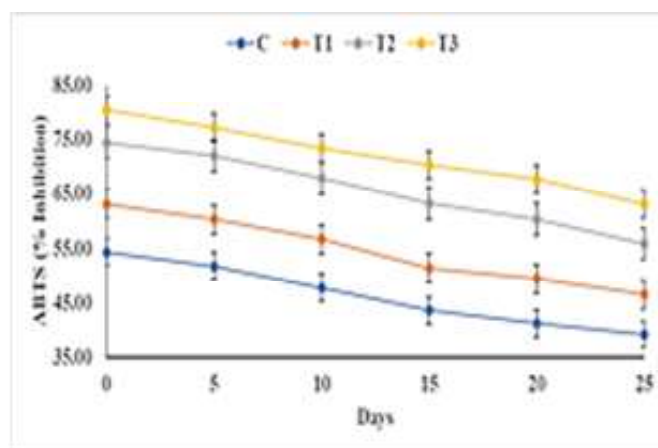


Fig. 3 Change in ABTS (% inhibition) of curd balls prepared with guava leaf powder

C: Control curd balls without guava leaf powder; T1: Curd balls with 1.5 % guava leaf powder; T2: Curd balls with 3.0 % guava leaf powder; T3: Curd balls with 4.5 % guava leaf powder. n=6

activity than control. However, ABTS values followed a decreasing pattern in all the samples during the storage study. Olatunde et al. (2021) reported that the antioxidant activity of guava leaf is due to presence of various phyto-active compounds

Table: 3 Effect of guava leaf powder incorporation on lipid oxidation of curd balls during refrigeration ($4 \pm 1^\circ\text{C}$) storage

Groups	0 Day	5 Days	10 Days	15 Days	20 Days	25 Days
Peroxide value (meq/kg)						
C	2.06 ^{Xa} ±0.05	2.66 ^{Yb} ±0.06	3.60 ^{Yc} ±0.08	7.45 ^{Yd} ±0.05	9.27 ^{Ze} ±0.05	13.56 ^{Zf} ±0.05
T1	1.95 ^{Wxa} ±0.04	2.47 ^{Yb} ±0.05	3.19 ^{Xc} ±0.08	6.88 ^{Xd} ±0.08	7.68 ^{Ye} ±0.06	10.87 ^{Yf} ±0.06
T2	1.79 ^{Wa} ±0.04	2.24 ^{Xb} ±0.09	2.84 ^{Wc} ±0.06	6.44 ^{Wd} ±0.07	7.05 ^{Xe} ±0.07	9.79 ^{Xf} ±0.14
T3	1.86 ^{Wa} ±0.08	1.91 ^{Wa} ±0.07	2.65 ^{Wb} ±0.05	6.27 ^{Wc} ±0.04	6.52 ^{Wd} ±0.06	8.90 ^{We} ±0.06
TBARS (mg malonaldehyde/ kg)						
C	0.17 ^a ±0.01	0.40 ^{Zb} ±0.01	0.50 ^{Yc} ±0.01	0.63 ^{Zd} ±0.01	0.83 ^{Zc} ±0.02	1.14 ^{Yf} ±0.08
T1	0.16 ^a ±0.01	0.30 ^{Yb} ±0.01	0.37 ^{Xc} ±0.01	0.54 ^{Yd} ±0.01	0.76 ^{Ye} ±0.01	0.90 ^{Xf} ±0.02
T2	0.16 ^a ±0.01	0.25 ^{Xb} ±0.01	0.33 ^{Wc} ±0.01	0.48 ^{Xd} ±0.01	0.55 ^{Xe} ±0.01	0.75 ^{Wf} ±0.01
T3	0.15 ^a ±0.01	0.21 ^{Wb} ±0.01	0.34 ^{WXc} ±0.02	0.36 ^{Wc} ±0.01	0.49 ^{Wd} ±0.01	0.66 ^{We} ±0.01
FFA (% oleic acid)						
C	0.08 ^{Xa} ±0.00	0.16 ^{Yb} ±0.01	0.24 ^{Zc} ±0.01	0.36 ^{Yd} ±0.01	0.48 ^{Ze} ±0.01	0.74 ^{Zf} ±0.01
T1	0.08 ^{Wxa} ±0.00	0.13 ^{Xb} ±0.01	0.20 ^{Yc} ±0.01	0.30 ^{Xd} ±0.01	0.37 ^{Ye} ±0.01	0.58 ^{Yf} ±0.01
T2	0.07 ^{Wa} ±0.00	0.10 ^{Wb} ±0.01	0.16 ^{Xc} ±0.01	0.27 ^{Xd} ±0.02	0.32 ^{Xe} ±0.01	0.51 ^{Xf} ±0.01
T3	0.07 ^{Wa} ±0.01	0.09 ^{Wa} ±0.00	0.13 ^{Wb} ±0.01	0.18 ^{Wc} ±0.01	0.26 ^{Wd} ±0.02	0.43 ^{We} ±0.02

Means values bearing small letters (a, b, c, d,) days wise and capital letters (W, X, Y and Z) groups wise indicate differ significantly ($P \leq 0.05$) n=6; C: Control curd balls without guava leaf powder; T1: curd balls with 1.5 % guava leaf powder; T2: curd balls with 3.0 % guava leaf powder; T3: curd balls with 4.5 % guava leaf powder.

like piceatannol 40-galloylglucoside, quercetin 3-(23 -galloyl- α -L-arabinopyranoside), epicatechin, 8-hydroxyluteolin 8-sulphate, and aclurin 3-C-(63 -p-hydroxybenzoyl-glucoside). In addition to these compounds Taha et al. (2019) also identified various flavonoid compounds in guava leaf such as quercetin, kaempferol, hesperetin, catchin, quercitrin, rutin and apigenin and postulated that these compounds are accountable for the antioxidant activity. The decreased ABTS values during the storage might be due to the decrease in the concentration of the phyto-active compounds during the neutralizing of the free radicals which was formed during oxidation.

Change in lipid oxidation (PV, TBARS and FFA) values

The peroxide value differed significantly ($P \leq 0.05$) among the groups and comparatively higher peroxide value was estimated in control than the treatment (Table 3). All the groups showed significantly ($P \leq 0.05$) increasing trends for peroxide value with progression of the storage period. The increased peroxide value during storage might be attributed primary oxidation of fat molecules and formation of hydroxy-peroxide molecules during storage. The comparatively lower peroxide value in guava leaf powder added sample was due to the presence of phyto-active compounds in guava leaf powder such as poly-phenolic compounds, triterpenoids, flavonoids, alkaloids, saponins and sesquiterpenes (Kumar et al. 2021). These phyto-active compounds have capacity to inhibit the generation of hydroperoxide reducing the formation of free radicals and/or

terminating the free radicals, therefore, lower peroxide value recorded in treated groups.

Thiobarbituric acid reactive substances value varied significantly ($P \leq 0.05$) among groups throughout storage and highest value was observed for control at last day of storage (Table 3). The increased TBARS value was observed on increase in storage time for all the curd ball samples. The rate of upsurge in TBARS formation was normally lower for guava leaf treated groups in a dose-dependent manner than control. Guava plant enriched with various natural antioxidant substances such as phenolic compounds, alkaloids, chlorophyll derivatives, carotenoids, and ascorbic acid. Paganga et al. (1999) reported that antioxidant action of phenolic compounds was due to their redox activity and play significant role in sequestering and deactivating free radicals or disintegrating peroxide substances. Lower TBARS values in treated groups might be due to the presence of high antioxidant compounds of guava leaf powder.

Free fatty acid content of all samples increased during refrigerated storage (Table 3). However, the incorporation of guava leaf powder at different levels in the curd balls had a significant effect on the FFA formation with concentration dependent manner. Chen and Yen (2007) reported that the guava leaf is rich source of antioxidants and have capacity to reduce the lipid oxidation in food products. Various phyto-active compounds were also isolated by Nantitanon and Okonogi (2012) like morin, quercetin and quercetin-3-O-glucopyranoside from leaf of guava and

Table: 4 Microbiological changes in curd balls incorporated with guava leaf powder during refrigeration (4 ± 1 °C) storage

Groups	0 Day	5 Days	10 Days	15 Days	20 Days	25 Days
SPC count (cfu/g)						
C	2.12 ^a ±0.42	3.02 ^{Yb} ±0.11	4.11 ^{Xc} ±0.09	5.00 ^{Yd} ±0.06	5.34 ^{Yd} ±0.04	6.27 ^{Yc} ±0.07
T1	2.08 ^a ±0.42	2.89 ^{XYb} ±2.80	3.81 ^{Wc} ±3.59	4.80 ^{XYd} ±0.09	4.96 ^{Xd} ±0.07	5.90 ^{Yc} ±0.02
T2	1.70 ^a ±0.54	2.76 ^{WXb} ±0.06	3.67 ^{Wc} ±0.08	4.73 ^{Xd} ±0.07	4.83 ^{Xd} ±0.08	5.71 ^{Xc} ±0.07
T3	1.67 ^a ±0.52	2.64 ^{Wb} ±0.06	3.71 ^{Wc} ±0.07	4.08 ^{Wc} ±0.05	4.34 ^{Wc} ±0.03	5.40 ^{Wd} ±0.03
Psychrophilic count (cfu/g)						
C	ND	ND	1.25 ^a ±0.56	2.12 ^b ±0.42	2.78 ^{Xbc} ±0.09	3.28 ^{Xc} ±0.08
T1	ND	ND	ND	1.67 ^a ±0.53	2.25 ^{WXab} ±0.46	2.74 ^{WXb} ±0.06
T2	ND	ND	ND	1.30±0.58	1.70 ^{WX} ±0.54	2.22 ^{WX} ±0.45
T3	ND	ND	ND	0.83 ^{ab} ±0.53	1.28 ^{Wb} ±0.57	1.77 ^{Wb} ±0.56
Coliform count (cfu/g)						
C	ND	ND	ND	ND	0.88 ^{ab} ±0.55	1.70 ^b ±0.54
T1	ND	ND	ND	ND	0.83 ^{ab} ±0.53	1.26 ^b ±0.57
T2	ND	ND	ND	ND	ND	0.94±0.59
T3	ND	ND	ND	ND	ND	0.87±0.55
Yeast and moulds count (cfu/g)						
C	ND	ND	ND	1.26 ^a ±0.56	2.10 ^b ±0.42	2.56 ^b ±0.03
T1	ND	ND	ND	0.90 ^{ab} ±0.57	1.68 ^{bc} ±0.53	2.08 ^c ±0.42
T2	ND	ND	ND	0.84 ^{ab} ±0.53	1.27 ^b ±0.57	1.72 ^b ±0.54
T3	ND	ND	ND	0.87 ^{ab} ±0.55	1.26 ^{ab} ±0.56	1.38 ^b ±0.62

Means values bearing small letters (a, b, c, d,.....) days wise and capital letters (W, X, Y and Z) groups wise indicate differ significantly ($P \leq 0.05$) $n=6$; C: Control curd balls without guava leaf powder; T1: curd balls with 1.5 % guava leaf powder; T2: curd balls with 3.0 % guava leaf powder; T3: curd balls with 4.5 % guava leaf powder.

confirmed that quercetin is the most effective antioxidant substance among all phyto-active substances. Similar finding was also reported by Tachakittirungrod et al. (2007) that the presence of flavonoids, morin and quercetin-3-oglucopyranoside in guava leaf possessed scavenging activity.

Change in microbial quality parameters

Microbial quality (standard plate count, psychophilic count, coliform count and yeast and mould count) of curd balls was evaluated and the data is depicted in Table 4. Among groups, during initial day of storage, data for SPC did not differ significantly ($P \geq 0.05$) and further it was found that from 5th day of storage onward the SPC count increased significantly ($P \leq 0.05$). The SPC value increased significantly ($P \leq 0.05$) throughout storage in all groups. The SPC counts was observed significantly ($P \leq 0.05$) lower in T3 than T2, T1 and control group. The antimicrobial activity of guava leaf might be due to presence of phenolic compounds in the leaves, primarily gallic acid. Similarly, Rattanachaikunsopon and Phumkhachorn (2007) reported the antibacterial activity of guava leaf. Ozcelik et al. (2008) postulated that phenolic compounds act as inhibition of nucleotides and

depolarization of microbial membrane followed by inhibition of macromolecular synthesis. Ahuja et al. (2012) also found increased total plate count during the storage of paneer tikka.

The psychophilic count was not detected up to 5th day of storage in control sample and up to 10th day of storage in treated groups (Table 4). Among the groups psychophilic count differed significantly ($P \leq 0.05$) at 20th and 25th day of storage. Psychophilic count increased significantly ($P \leq 0.05$) during the storage of curd balls in each group. However, comparatively lower psychophilic count was observed in guava leaf powder added curd balls than control. Olatunde et al. (2018) reported that polyphenolic compounds, especially quercetin 32 -xyloside present in guava leaf extract was attributed the antimicrobial activity. Some other antimicrobial phyto-active compounds also present in guava leaf such as quercetin and its glycosides having an antimicrobial activity (Gorniak et al. 2019).

The coliform count was not detected up to 15th day of storage in control and T1 sample and it was not detected in T2 and T3 up to 20th days of storage (Table 4). Among the groups coliform count did not differ significantly ($P \geq 0.05$) during storage. Coliform count

Table: 5 Sensory attributes of curd balls incorporated with guava leaf during refrigeration ($4 \pm 1^\circ\text{C}$) storage

Groups	0 Day	5 Days	10 Days	15 Days	20 Days	25 Days
Colour and appearance						
C	8.18 ^e ±0.19	7.96 ^{Wc} ±0.09	7.25 ^{Wd} ±0.09	6.93 ^{Wc} ±0.09	6.57 ^{Wb} ±0.12	5.96 ^{Wa} ±0.11
T1	8.36 ^d ±0.14	8.04 ^{Wcd} ±0.14	7.68 ^{Wxc} ±0.19	7.25 ^{Wxb} ±0.11	6.64 ^{Wa} ±0.14	6.25 ^{Wxa} ±0.12
T2	8.46 ^e ±0.14	8.39 ^{Xdc} ±0.11	8.14 ^{Ycd} ±0.07	7.93 ^{Yc} ±0.09	7.36 ^{Xb} ±0.11	6.93 ^{Ya} ±0.07
T3	8.39 ^e ±0.13	8.11 ^{WXdc} ±0.07	7.86 ^{Xd} ±0.09	7.36 ^{Xc} ±0.18	6.86 ^{Wb} ±0.07	6.46 ^{Xa} ±0.16
Taste						
C	8.11 ^e ±0.07	7.96 ^{Wc} ±0.09	7.21 ^{Wd} ±0.09	6.89 ^{Wc} ±0.12	6.57 ^{Wb} ±0.12	5.93 ^{Wa} ±0.13
T1	8.36 ^e ±0.14	7.89 ^{Wd} ±0.13	7.64 ^{WXd} ±0.18	7.21 ^{Wxc} ±0.10	6.64 ^{Wb} ±0.14	6.14 ^{Wxa} ±0.09
T2	8.46 ^d ±0.14	8.39 ^{Xd} ±0.11	8.11 ^{Yc} ±0.07	8.04 ^{Yc} ±0.07	7.43 ^{Xb} ±0.09	6.90 ^{Ya} ±0.07
T3	8.39 ^d ±0.13	8.00 ^{Wcd} ±0.12	7.86 ^{Xc} ±0.09	7.32 ^{Xb} ±0.18	6.79 ^{Wa} ±0.14	6.46 ^{Xa} ±0.16
Aroma						
C	8.18 ^d ±0.09	7.96 ^{Wd} ±0.09	7.14 ^{Wc} ±0.07	6.86 ^{Wc} ±0.11	6.54 ^{Wb} ±0.11	5.86 ^{Wa} ±0.14
T1	8.39 ^e ±0.13	8.00 ^{Wde} ±0.15	7.68 ^{Xd} ±0.19	7.25 ^{Xc} ±0.11	6.71 ^{Wb} ±0.10	6.18 ^{Wxa} ±0.12
T2	8.46 ^d ±0.14	8.39 ^{Xd} ±0.11	8.11 ^{Yc} ±0.07	8.04 ^{Yc} ±0.06	7.43 ^{Xb} ±0.09	6.86 ^{Ya} ±0.09
T3	8.36 ^d ±0.14	8.04 ^{WXcd} ±0.14	7.75 ^{Xc} ±0.16	7.25 ^{Xb} ±0.14	6.75 ^{Wa} ±0.14	6.43 ^{Xa} ±0.18
Texture						
C	8.29 ^f ±0.09	7.86 ^{Wc} ±0.13	7.21 ^{Wd} ±0.10	6.86 ^{Wc} ±0.07	6.39 ^{Wb} ±0.11	6.00 ^{Wa} ±0.09
T1	8.11 ^d ±0.13	7.89 ^{Wcd} ±0.13	7.57 ^{Xc} ±0.16	7.14 ^{Xb} ±0.09	6.71 ^{Wa} ±0.18	6.32 ^{Wxa} ±0.12
T2	8.39 ^e ±0.11	8.54 ^{Xc} ±0.04	8.14 ^{Yd} ±0.07	7.79 ^{Yc} ±0.11	7.29 ^{Xb} ±0.09	6.82 ^{Ya} ±0.07
T3	8.25 ^d ±0.09	7.89 ^{Wcd} ±0.16	7.71 ^{Xc} ±0.16	7.18 ^{Xb} ±0.09	6.71 ^{Wa} ±0.15	6.39 ^{Xa} ±0.15
Overall acceptability						
C	8.11 ^d ±0.07	7.82 ^{Wd} ±0.12	7.11 ^{Wc} ±0.07	6.75 ^{Wb} ±0.13	6.50 ^{Wb} ±0.11	5.82 ^{Wa} ±0.09
T1	8.14 ^e ±0.13	7.93 ^{Wxc} ±0.13	7.54 ^{Xd} ±0.14	7.11 ^{Xc} ±0.07	6.71 ^{Wb} ±0.15	6.21 ^{Xa} ±0.10
T2	8.43 ^e ±0.12	8.29 ^{Xdc} ±0.10	8.07 ^{Yd} ±0.05	7.64 ^{Yc} ±0.13	7.25 ^{Xb} ±0.09	6.75 ^{Ya} ±0.12
T3	8.25 ^e ±0.08	8.00 ^{WXdc} ±0.14	7.79 ^{Xd} ±0.09	7.25 ^{Xc} ±0.12	6.82 ^{Wb} ±0.09	6.43 ^{XYa} ±0.13

Means values bearing small letters (a, b, c, d,.....) days wise and capital letters (W, X, Y and Z) groups wise indicate differ significantly ($P \leq 0.05$) $n=21$; C: Control curd balls without guava leaf powder; T1: curd balls with 1.5 % guava leaf powder; T2: curd balls with 3.0 % guava leaf powder; T3: curd balls with 4.5 % guava leaf powder.

increased during storage of curd balls from 20th to 25th days in control and T1 but the value did not differ significantly ($P \geq 0.05$). Phyto-active compounds present in the guava leaf powder was attributed to lower count of coliform in treated curd balls than control. Chanda and Kaneria (2011) reported that extract of guava leaf exhibited antimicrobial activity against *E. coli* and other microbes.

The yeast and mould count were absent up to 10th days of storage among all the groups (Table 4). Among the groups yeast and mould count did not differ significantly ($P \geq 0.05$) throughout storage. Yeast and mould count were increased in curd ball groups from 15th day of storage onwards though the rate of their increase did not differ significantly ($P \geq 0.05$). Treated curd balls showed lower yeast and mould count than control which might be due to antifungal activity of guava leaf powder. Morais-Braga et al. (2017) reported that guava leaf extracts exhibited antifungal activity against *C. albicans* and *C. tropicalis* by significant reduction in percentage of viability of yeast and mould during their study. Beatriz et al. (2012) stated in their study that guava leaf showed antifungal activity against various fungi.

Change in sensory attributes

All sensory attributes among the groups did not differ significantly ($P \geq 0.05$) at 0 day of storage and varied significantly ($P \leq 0.05$) from 5th day to 25th day of storage (Table 5). Among all samples, T2 sample was rated highest score than control, T3 and T1. The sensory score was lower for T3 group than T2 which might be due to higher level of guava leaf powder and formation of oxidative compounds during storage which could be responsible for bitter taste. Sensory scores for colour and appearance, taste, aroma, texture and overall acceptability exhibited decreasing trend during the storage. The decreased taste, aroma and overall acceptability scores of sensory attributes might be due to oxidation of lipid and formation of volatile free fatty acid content in the aforementioned curd ball samples on day 25th than initial day of storage. Kumar et al. (2019) also reported declined trend of sensory parameters during the storage of milk smoothies prepared with the addition of tulsi, lemon grass and aloe vera. The production of lactic acid due to growth of lactic acid producing as well as non-starter lactic acid bacteria during the storage of curd balls producing repulsive odour in curd balls resulted decrease in overall acceptability score by the evaluators. Oxidation of lipid and fat molecule of curd balls further attributed to decreased textural quality of the products. Ahuja et al. (2012) also reported decreased sensory score during the storage of paneer tikka.

Conclusions

The results revealed that curd ball prepared with addition of guava leaf powder T3 (4.5%) were recorded significantly lower lipid oxidation, microbial growth however, sensory panelist rated

comparatively lower sensory score for T3 than control (0 %), T1 (1.5 %) and T2 (3.0 %) during refrigerated storage. Also, treated curd balls with GLP were significantly higher in antioxidant profiles (Total phenolics content, DPPH % inhibition and ABTS % inhibition) than control, therefore guava leaf powder could be utilized as potential natural antioxidant. Results concluded that the developed curd balls incorporated with 3.0 % guava leaf powder could be successfully stored under aerobic packaging for 25 days at $4 \pm 1^\circ\text{C}$ with an acceptable physico-chemical, antioxidant parameters, lipid oxidation microbiological quality and sensory attributes.

Conflict of interest: None

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Characterization of ultra-heat-treated whole milk in Tehran during their shelf life: Physicochemical changes, microbiological quality and sensory evaluation

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Abstract: In the present study, the physicochemical changes (titratable acidity, fat, density and solids-not-fat), microbiological quality (total count) and sensory properties (odor, flavor, color and overall acceptance) of whole ultra-heat-treated (UHT) milk marketed in Tehran, Iran were assessed during the storage period (0, 90 and 180 days). Five brands of whole UHT milk were selected to determine the quality of samples. The results showed that acidity of UHT milk samples increased during storage, whereas the fat content decreased. However, titratable acidity and density of brands C and B respectively were not in accordance with standard. Total bacteria count tests indicated that there was less than 10 cfu D mL microorganism in UHT milk samples over storage time (0, 90 and 180 days). All brands were similar in respect of color and flavor. Although there was a decrease in sensory characteristics of UHT milk products during storage period (0, 90 and 180 days), they were organoleptically acceptable after six months storage.

Keywords: Quality, Storage, Sensory, Whole UHT milk

Introduction

The worldwide demand for production and consumption of milk and milk products is raising and its increasing trend is expected to continue due to urbanization expansion, population growth and increasing inclination to healthy diet and lifestyle (Krizsan et al. 2021, Zolin et al. 2021). For example, in 1998, the average production of milk in Iran was 4 million tons and it reached 8.8 million tons in 2014 (Abedi et al. 2020). In fact, one of the most nutritious products is milk that contains indispensable macro- and micro-nutrients (amino acids, fatty acids, minerals and

vitamins) for human body (Bordoni and Gabbianelli 2021, Nayik et al. 2021, Verduci et al. 2021). Therefore, its daily consumption is necessary especially by infants and children (Ghaffarian Bahraman et al. 2020, Savarino et al. 2021). The per capita consumption of milk is 30 to 150 kg milk year⁻¹ in Iran based on FAO report, whereas, WHO has recommended a per capita consumption of 200 kg year⁻¹ for milk (Kurajdová et al. 2015, Abedi et al. 2020).

Milk is a suitable medium for growth of numerous pathogenic and spoilage microorganisms due to the presence of essential nutrients and desirable pH (nearly 6.6) (Coolbear et al. 2022). Heat treatment such as pasteurization is a common and old technique to produce a safe product with substantial shelf life (Lindsay et al. 2021). Ultra-high temperature process is applied to inactivate bacteria and enhance the shelf life of milk for up to 9 months in dairy industries. Milk is heated directly or indirectly at around 135-145 °C for around 2-3 s to make it sterile and then quickly cooled (below 32°C) and packaged under aseptic conditions (Akkerman et al. 2021, Krishna et al. 2021). Suzuki et al. (2014) reported that UHT milk produced by direct heating had better quality during long storage time at cold condition compared to indirect heating. Although UHT milk is microbiologically safe for the consumer during storage period but posttreatment contamination and thermoresistant spore-forming bacteria such as *Bacillus sporothermodurans* spores are factors that may present in industrially contaminated UHT milk (Scheldeman et al. 2006). For these reasons, there are still reports of food poisoning owing to UHT milk consumption.

The quality of UHT processed milk is related to the amount of milk fat (fat free, full fat and semi-skim), storage temperature, raw milk properties, seasonal variations, stage of lactation and age of cow that affects the shelf life and acceptance of the product throughout long storage period. The UHT treatment applied to milk has demonstrated desirable results, including increasing the shelf life to several months, reducing consumption of energy and no need storage and distribution in cold conditions (Chavan et al. 2011). However, it has some adverse effects on the quality of milk. UHT processing can induce denaturation of protein, Maillard reactions and oxidation of fat along with the formation of off flavor compounds and the loss of some nutrients during

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thermal processing and storage (Oz et al. 2021, Yun and Imm 2021). One of the causes of cooked off flavor as a common sensory defect in UHT milk is the release of sulfhydryl groups and hydrogen sulfide from lactoglobulin (Ansari et al. 2020, Deeth 2021, Mejares et al. 2022).

Many researches have been published about the quality of whole UHT milk during storage life in several countries (Mudalal et al. 2019, Silva et al. 2021) but there is scarce information about the physicochemical changes, microbial and sensorial characteristics of whole UHT milk in Iran. Furthermore, UHT milk is an important part of people's diet that its consumption has been enhanced in Iran. So, the objective of this work was to survey the physicochemical properties, microbiological quality and sensory evaluation of whole UHT milk from the most-consumed brands in Iran.

Materials and methods

Sampling

5 brands were selected from the most famous and most widely consumed brands of whole UHT milk. Samples were purchased from different supermarkets in Tehran province. All whole UHT milk samples had similar production dates and one -liter size.

Physicochemical analyses

Physicochemical characteristics were performed in this study were titratable acidity, fat content, solids not fat (SNF) and density. Titratable acidity was measured by titration of 10 ml of whole UHT milk with 0.1 N NaOH and phenolphthalein was used as indicator (Dornic method). Fat content was calculated according to the Gerber butyrometer method. SNF was determined gravimetrically according to Iranian National Standard (No. 5272). Also, the density (D) of milk was evaluated at 20! by a thermo-lactodensimeter and it was calculated at a temperature other than 20! as follows:

$$D = D_{\text{read}} + (\text{temperature of milk} - 20^{\circ}\text{C}) \times 0.2$$

Microbial counts

To estimate the total bacteria count, 1 mL of whole UHT milk was inoculated on Petrifilm Aerobic Count Plates and counted using

the pour-plate method in plate count agar and incubated at 30°C for 72 h. After this period, plates with 15-300 colonies were enumerated and the results declared as cfu D mL (colony-forming units per mL whole UHT milk) (Mudalal et al. 2019). The microbial analysis was done on days 0, 90 and 180.

Organoleptic analysis

The sensory properties of whole UHT milk including odor, flavor, color and overall acceptability were analyzed by 30 panelists familiar with evaluation of dairy products. The treatments were compared using a hedonic 5-point structured scale. Therefore, a 5-point rating scale was used for each of sensory characteristics (0 = not consumable; 1 = unacceptable; 2 = acceptable; 3 = satisfactory; and 4 = excellent). After production of whole UHT milk and on day 180, the sensory evaluation was carried out and mineral drinking water was given to assessors to rinse their mouth between tests.

Statistics

Analysis of variance (ANOVA) and Duncan's test ($P < 0.05$) were used to assess the physicochemical and sensorial properties of whole UHT milk during storage (SPSS software). The obtained data were expressed as mean and all measurements were done in triplicate.

Results and Discussion

Physicochemical analysis

According to Table 1, the mean values for titratable acidity of whole UHT milk samples were 0.14-0.17 during 180 days of storage. Table 2 demonstrates titratable acidity of five brands that were in accordance with Iranian National Standard (No. 1528) in the range of 0.14-0.16 (% lactic acid) except brand C at 30! and before incubation. It was observed that by increasing storage time from 90 days to 180 days, titratable acidity increased slightly. In line with the obtained results, acidity of UHT milk samples increased during storage for 4 months at 5 and 30°C and the rate of increase was higher at high temperature. Occurrence of Maillard reaction during processing and storage and conversion of lactose to acids leads to increase of titratable acidity during storage (Ranvir et al. 2021). Similar results have been reported by Ajmal

Table 1 Mean values of physicochemical attributes of the whole UHT milk samples during storage time*.

Days	Solids-not-fat (%)	Fat content (%)	Density (g/mL)	Titratable acidity (%)		
				Before incubation	Incubation at 30°C	Incubation at 55°C
0	8.83 ^a	3.06 ^a	0.98 ^b	0.14 ^a	0.14 ^b	0.16 ^b
90	8.83 ^a	3.06 ^a	0.98 ^b	0.14 ^a	0.14 ^b	0.16 ^b
180	8.81 ^b	3 ^b	1.03 ^a	0.003 ^b	0.17 ^a	0.17 ^a

*Means shown with different small letters represent significant differences ($P < 0.05$) in the same columns.

et al. (2018) by investigation of acidity change in UHT milk during 90 days storage that was associated with the presence of organic acids including lactic acid, acetic acid, citric acid, pyruvic acid, formic acid, succinic acid and oxalic acid. Free fatty acids and changes in calcium phosphate equilibrium have been also proposed to be responsible for increased acidity and reduced pH of stored UHT milk (Swartzel 1983, Schmidt and Renner 1978). Also, increase of acidity during storage of UHT milk and the relation between acidity and storage time has been reported by Kessler and Fink (1986) and Rerkrai et al. (1987), Taw et al. (2014).

Regarding fat content, there was a significant difference ($P < 0.05$) between the values on day 0 and 90 with day 180 and a decreasing trend was observed. According to Table 2, there was a significant difference between fat levels of different brands. However, the values were in accordance with Iranian National standard (No. 1528). Accordingly, Richards et al. (2016) reported an increase of lipolysis in low-fat UHT milk during storage. Similarly, De Longhi et al. (2012) reported reduction in fat level of UHT milk samples that was explained by using milk with a lower-than normal fat level or the fraudulent reduction in the original fat level of the packaged product. Taw et al. (2014) observed no significant changes for fat content in UHT milk samples at different temperatures (4, 22 or 37°C) and time of storage (0-180 days). According to Iranian national standard (No. 1528), the minimum Solid non-fat (SNF) content in UHT milk should be 8% and the results in Table 2 presents that SNF level in UHT milk samples were in the range of 8.51-9.11%. Moreover, Table 1 indicates that the mean values of SNF% were in accordance with the standard limit.

The mean density values in milk samples during 180 days storage were in the range of 0.98-1.03 g/mL. According to Iranian National standard (No. 1528), the minimum density of UHT milk should be 1.029 gr/mL at 15°C. As can be seen in Table 2, except brand B, the density of milk samples was in accordance with the standard. De Longhi et al. (2012) recorded an increase in density of UHT milk samples after 120 days storage reaching to normal values which was attributed to the gelation process that resulted in viscosity increase along with a decrease in the fat level.

Microbial analysis

The total bacterial count of whole UHT milk samples during their shelf life is presented in Table 3. The results indicated the microbial count of all brands analyzed in present study was less than 10 cfu D mL which was below the allowable limit in Iranian National Standard (maximum of 100 cfu D mL; No. 1528) and also storage time had no effect on the microbial population. This result is consistent with the study of Karmaker et al. (2020), Mudala et al. (2019) and Arafat et al. (2015) who reported the microbial counts in UHT milk below the acceptable level in Mymensingh, Gazipur and Toulkarem districts respectively. It seems that UHT treatment has been effective in reducing the microbial load of whole milk, which has led to an increase in storage time. Furthermore, the low microbial load in samples throughout the storage period of 180 days can be attributed to the high quality of raw milk applied for UHT process. In the study of Zhang et al. (2020), the correlation of bacterial count in raw milk and the quality of produced UHT milk was investigated. They mentioned that the shelf life of products made with raw milk containing 10^4 cfu/ mL of *Pseudomonas* reached less than 7 months and the increase of

Table 2 Mean values of physicochemical attributes of the whole UHT milk samples from different brands*.

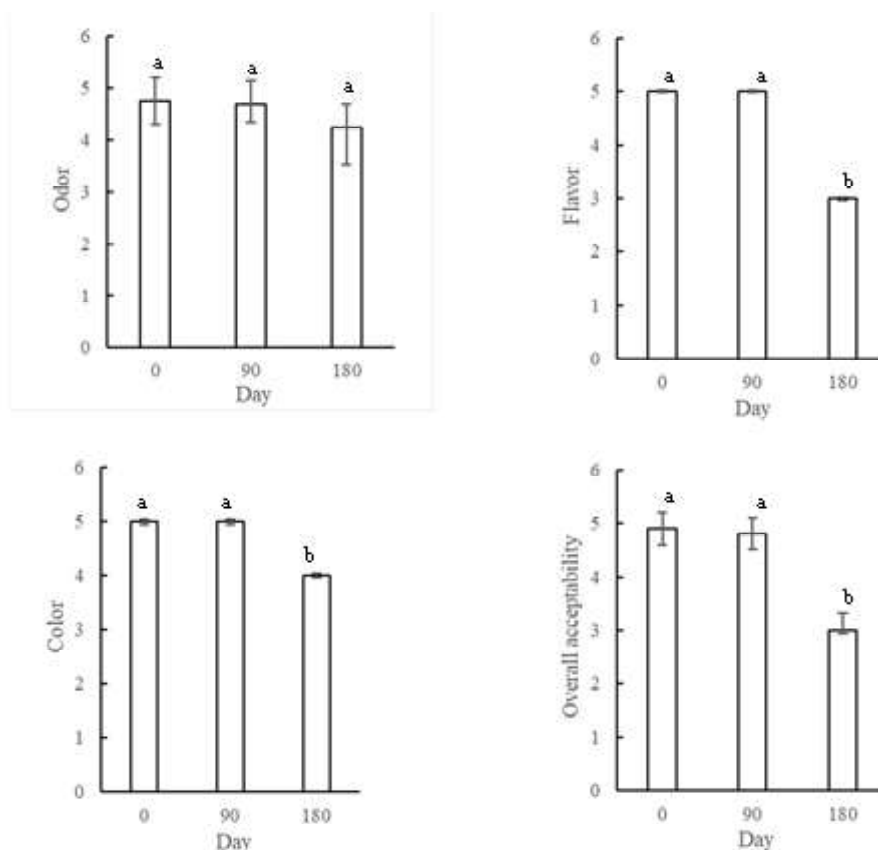
Brands	Solids-not-fat (%)	Fat content (%)	Density (g/mL)	Titratable acidity (%)		
				Before incubation	Incubation at 30°C	Incubation at 55°C
A	9.11 ± 0.1 ^a	3.02±0.04 ^c	1.04±0.01 ^a	0.16±0.01 ^a	0.16±0.01 ^b	0.17±0.01 ^a
B	8.8 ± 0.23 ^b	3.09±0.2 ^a	0.87±0.41 ^b	0.16±0.01 ^a	0.16±0.01 ^b	0.17±0.02 ^a
C	9.10 ± 0.43 ^a	3.02± 0.4 ^c	1.04±0.01 ^a	0.13±0.07 ^b	0.17±0.01 ^a	0.17±0.01 ^a
D	8.63±0.19 ^c	3.04±0.08 ^b	1.04±0.01 ^a	0.16±0.01 ^a	0.14±0.07 ^c	0.17±0.1 ^a
E	8.51±0.004 ^d	3.05±0.12 ^b	1.04±0.01 ^a	0.16±0.01 ^a	0.14±0.07 ^c	0.17±0.01 ^a

*Means shown with different small letters represent significant differences ($P < 0.05$) in the same columns.

Table 3 Total bacterial count (cfu D mL) in whole UHT milk products from different brands during storage periods.

Brands	Incubation at 30°C			Incubation at 55°C		
	0 day	90 days	180 days	0 day	90 days	180 days
A	<10	<10	<10	<10	<10	<10
B	<10	<10	<10	<10	<10	<10
C	<10	<10	<10	<10	<10	<10
D	<10	<10	<10	<10	<10	<10
E	<10	<10	<10	<10	<10	<10

Fig. 1 Sensory evaluation of UHT milk products on days 0, 90 and 180



bacterial population of raw milk led to the production of UHT milk with a shorter shelf life.

Sensory evaluation

The results of the organoleptic test of whole UHT milk samples on days 0 and 180 are displayed in figure 1. It was indicated that there was a significant difference ($P < 0.05$) between the whole UHT milk samples on day 0 and day 180 regarding flavor, color and overall acceptability. Furthermore, the lowest scores were attributed to samples after 180 days of storage for flavor and overall acceptance. In this regard, other researchers also reported that sensorial properties of UHT milk change over the storage time (Hassan et al. 2009, Richards et al. 2016). Alterations in organoleptic attributes of UHT milk can be occurred due to proteolysis and lipolysis of milk- proteins and fat (Chen et al. 2003). Moreover, Maillard reaction may be responsible for color and flavor decrease during storage and also the formation of sulphur containing compounds causes cooked flavor during sterilization process and storage (Mudalal et al. 2019, Arafat et al. 2015). From the Table 4, it is seen that color and flavor scores of UHT milk samples were 4 and 4.5 respectively, which were similar in all brands. In addition, the highest odor scores belonged to brands A and D and brand E had the lowest overall acceptability compared to other brands. However, sensory quality of all brands was acceptable during storage period.

Conclusion

The present study revealed that all of whole UHT milk from the most-consumed brands in Tehran meet the standards in terms of solids-not-fat and fat level. Microbial quality of all brands was good over storage time. In addition, in respect of organoleptic analysis, the highest scores were related to the whole UHT milk samples at the beginning of the storage, because during storage time, decomposition of milk fat and Maillard reaction led to unpleasant changes in sensory parameters. In general, based on the parameters studied, UHT milk samples available in the Iranian market had acceptable quality during shelf life.

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Author contributions

Arameh Shahbaz: Investigation, Conceptualization, Validation, Writing - original draft, Writing – review and editing, Formal analysis, Visualization. **Mohammad Reza Koushki:** Conceptualization, Methodology, Investigation, Validation, Visualization, Writing – review & editing, Supervision, Funding

acquisition. **Elham Khanniri:** Methodology, Investigation, Conceptualization, Validation, Writing – review and editing. **Nasim khorshidian:** Conceptualization, Investigation, Writing – review & editing.

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RESEARCH ARTICLE

Screening of sugar tolerant fast-growing lactic acid bacteria for preparation of *Misti dahi*

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Abstract: *Misti dahi*, a popular fermented milk product in eastern India, is experiencing growing demand across the country. To meet the increasing demand, it is essential to identify sugar-tolerant starter cultures that can ensure more desirable and consistent fermentation outcomes while minimizing the risk of fermentation failure. This study aimed to isolate, screen, and identify sugar-tolerant cultures suitable for *Misti dahi* production. Out of ten isolated cultures, *S. thermophilus* MD3 exhibited remarkable tolerance to high sugar concentrations of up to 20% in milk without compromising cell viability and curdling time. Moreover, it consistently produced *Misti dahi* of superior quality in terms of physicochemical, microbiological, textural, sensory, and rheological attributes. The isolated *S. thermophilus* MD3 culture holds potential for commercialization in *Misti dahi* production.

Keywords: Lactic acid bacteria, Sugar tolerance, *Misti dahi*, Textural attributes, Rheology, Sensory properties,

Introduction

Misti dahi, also known as sweetened curd or Lal dahi, is a popular fermented milk product originating from the eastern region of India (Prajapati and Behare, 2018). Initially, the consumption of *Misti dahi* was limited to the eastern and northern parts of India,

but its demand has been progressively growing throughout the country. The authentic flavour and distinctive taste of *Misti dahi* have garnered significant popularity among the Indian population. Traditionally, it was prepared on a cottage scale to meet the local demand. The customary method includes simmering milk with cane sugar in an open pan at 60-70°C for several hours, leading to moisture evaporation and distinct cooked flavors, a slightly brown color, viscosity, and other physico-chemical changes. After cooling to 30-42°C, a previous day old starter culture was added, and the mixture was then left to curdle overnight in earthenware pots (Chatterjee et al. 2022). However, a significant number of small manufacturers involved in the production of *Misti dahi* neglect the importance of ensuring the reliability and purity of the starter culture. As a result, this lack of attention leads to the production of *Misti dahi* with inconsistent quality and an increased risk of developing off-flavors.

Misti dahi is typically made using sugar-tolerant lactic acid cultures that can effectively produce the desired acid and flavor in sweetened milk, resulting in desirable attributes in the final product. *Misti dahi* starter cultures have traditionally consisted of various species such as *Lactococcus lactis*, *Lactococcus diaceetylactis*, *Lactococcus cremoris*, *Leuconostoc* spp., *Streptococcus thermophilus*, and *Lactobacillus delbreuckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum* (Ghosh and Rajorhia 1990; Gupta et al. 2000; Akter et al. 2010). However, manufacturers now prioritize the use of fast acidifying starters that can produce the desired product in a shorter time. One of the challenges in the production process is the high sugar content, which can cause osmotic shock and negatively affect the viability and activity of the starter cultures (Kashket, 1987). Furthermore, the physico-chemical and sensory properties of *Misti dahi* are influenced by the use of specific starter culture. These properties, such as texture, flavor, and overall quality, are important factors in meeting consumer expectations and preferences. In unorganized sectors, undefined starter cultures are often used for making *Misti dahi*, which can lead to inconsistent quality in the end product. Therefore, it is crucial to employ pure and defined strains of osmo-tolerant cultures that can ensure uniform and predictable fermentative changes, resulting in a consistent quality of *Misti dahi*. Naturally

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fermented products that harbour a diverse range of species and strains offer potential sources for obtaining high sugar-tolerant lactic strains suitable for *Misti dahi* production. By carefully selecting and maintaining a suitable starter culture, the industry can meet the increasing demand for *Misti dahi* while ensuring consistent quality and consumer satisfaction.

In this study, we aim to isolate lactic cultures from traditional *Misti dahi* samples and identify potential strains as fast acidifying starter cultures, impacting the sensory, physico-chemical, and textural characteristics of *Misti dahi*.

Materials and methods

Collection and processing of samples

Nine *Misti dahi* samples (designated as S1 to S9) were collected from the local market of Kolkata, West Bengal. The samples were placed in an ice-box to maintain the freshness and prevent any potential microbial changes and transported to the laboratory for microbiological analysis.

Isolation of lactic acid bacteria strains

To isolate lactic cultures, the samples were diluted serially in a saline solution. Subsequently, 1 ml from each diluted sample (10^{-5} , 10^{-6} , and 10^{-7}) was aseptically transferred to sterile petri plates. Molten MRS and M17 agar media were poured onto the plates separately, and after solidification, the plates were incubated at temperatures of 30°C, 37°C and 42°C for 48 to 72 hrs. The colonies displaying typical characteristics were carefully selected and transferred to MRS or M17 broth tubes. To purify the isolates, a repetitive streaking method was employed and pure isolates were subjected to further analysis.

Primary screening and identification of sugar tolerating cultures

The isolated cultures were evaluated by gram and negative staining and catalase test as per the standard protocol. Activity of the cultures was evaluated by determining titratable acidity (AOAC, 2007), pH, total lactic count in MRS or M17 agar media (ISO19344:2015) and curd setting time in 12% reconstituted skim milk (w/v) containing 15, 18 and 20% (w/v) sugar content. For the study, the sugar-tolerant *Streptococcus thermophilus* NCDC436 and the sensitive *Streptococcus thermophilus* NCDC74 reference strains were obtained from the National Collection of Dairy Cultures (NCDC), ICAR-National Dairy Research Institute, Karnal, Haryana, India, and used as positive and negative controls, respectively. Considering sugar tolerance and curd setting time as the main criteria, promising isolates were selected and subjected for genotypic evaluation.

The selected sugar tolerant isolates were identified using species-specific PCR. After growing the isolates in broth media overnight,

genomic DNA was extracted according to the method of Pospiech and Neumann (1995). The genomic DNA was amplified using conserved sequences of *S. thermophilus* lacZ gene primers, F (5' CACTATGCTCAGAATACA 3') and R (5' CGAACAGCATTGATGTTA3') as suggested by Maheswari et al. (2013) in a Thermal cycler (BIO-RAD S1000 thermal cycler). The species-specific PCR protocol includes the following steps: initial denaturation at 94°C for 3 min, final denaturation at 90°C for 30 sec, annealing at 54°C for 70 sec, extension at 72°C for 30 sec, final extension at 72°C for 10 min, and a total of 35 cycles. The PCR products were separated using a 1.5% agarose gel, and visualized under a UV spectrum after staining with Ethidium bromide.

Preparation of *Misti dahi*

Buffalo milk (6.0 % fat and 9.0% SNF) was collected from Experimental Dairy, ICAR-NDRI, Karnal and *Misti dahi* was prepared according to the method described by Prajapati and Behare, (2018) with some modifications (Fig. 1). Skim milk powder (1.5%, w/v), sugar (15 %, w/v) and caramel (1% v/v) were added during preheating of milk. The mixture was then homogenised at 9000 rpm for 5 min using Ultra Turrax homogenizer. The milk mixture was then heated at 90°C for 10 min followed by cooling up to 42°C. The sugar tolerating strains were inoculated at 2% (v/v) into the milk, distributed into cups and incubated at 42°C until it forms firm coagulum. The set product was immediately transferred to refrigerated storage. The products made by *S. thermophilus* NCDC436 and NCDC 74 were used as control.

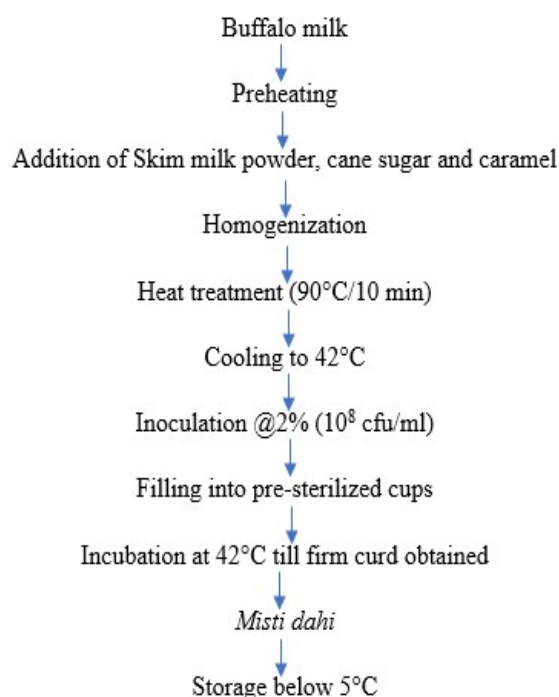


Fig.1 Process flow diagram of preparation of *Misti dahi*

Analysis of *Misti dahi*

The *Misti dahi* samples were analysed for physico-chemical, textural, rheological, Sensory and microbiological parameters. Curd setting time for *Misti dahi* was evaluated by time taken by the starter culture to form the firm curd when incubated at 42°C. Titratable acidity of products was determined by the method described by FSSAI for Dahi (AOAC, 2007) while pH by electronic pH meter.

Texture profile of the *Misti dahi* samples were analyzed by the method described by Hussain et al. (2016). Texture profiling was carried out using compression test by TA.HDplusC texture analyzer (Stable Micro Systems, UK fitted with 5 Kg load cell). *Misti dahi* samples were set in glass beakers of uniform size 150 mL having length and diameter and filled upto 100 mL mark. A cylindrical probe (P25) having diameter of 25 mm was allowed to penetrate the *Misti dahi* samples at temperature of 20°C. The textural parameters such as firmness (g), consistency (g.sec), cohesiveness (g) and work of cohesion (g.sec) were analyzed using Texture Exponent Connect Software. Firmness is defined as the value of peak force recorded during compression. Cohesiveness is regarded as the rate at which the material is disintegrated under mechanical action and it indicates the ability of the product to hold together. Consistency was measured as the area within the curve during the compression. The rheological properties of *Misti dahi* samples were studied at 20°C using rotational rheometer (Model: MCR 52, Anton Paar, Austria) fitted with a cone and plate assembly (CP-75, 75 mm diameter, 1° angle). *Misti dahi* samples were gently sampled from the cup with a spatula and placed on the pre-cooled (20°C) rheometer plate.

Apparent viscosity of *Misti dahi* was measured in the shear rate range of 0.01 to 100 sec⁻¹. The apparent viscosity was recorded as Pa.s. The sensory attributes of *Misti dahi* was performed by 9 points hedonic scale using a panel of six judges having adequate knowledge about dairy products. The nine-point hedonic sensory score card comprised of the following ratings viz. like extremely (9), like very much (8), like moderately (7), like slightly (6), neither like nor dislike (5), dislike slightly (4), dislike moderately (3), dislike very much (2) and dislike extremely (1). In case of microbiological analysis total lactic count (ISO19344, 2015), coliform count (ISO 4832, 2006) and yeast and mold count (ISO 21527-1, 2008) were determined.

Statistical analysis

Three independent trials were carried out and results were expressed as mean ± standard error of mean. One way ANOVA (Analysis of variance) followed by Tukey's test was carried out in IBM-SPSS to determine significant differences among the data (p<0.05). Graphs were prepared in GraphPad Prism (version 8).

Results and discussions

Isolation of lactic cultures

Lactic isolates that formed typical colonies on M17 and MRS agar were randomly picked and transferred to respective broth media. Out of the 40 colonies transferred, ten pure isolates were obtained, including five cocci (MD1 to MD5) and five rods (MD6 to MD10). These isolates appeared as gram-positive, catalase-negative cocci and rods arranged in long and short chains (Table 1, Fig. 2). A total of ten lactic cultures were isolated from the nine

Table 1: Details of isolated cultures from *Misti dahi* samples

Sl. No.	Name of the culture	Source of isolation	Isolation media	Isolation temperature (°C)	Morphological characteristics
1	MD1	S-1	M17	42	Gram positive long chains of cocci
2	MD2	S-2	M17	42	Gram positive long chains of cocci
3	MD3	S-2	M17	37	Gram positive long chains of cocci
4	MD4	S-3	M17	30	Gram positive short chains of cocci
5	MD5	S-5	M17	42	Gram positive long chains of cocci
6	MD6	S-7	MRS	37	Gram positive long chains of rods
7	MD7	S-8	MRS	37	Gram positive long chains of rods
8	MD8	S-8	MRS	42	Gram positive long chains of rods
9	MD9	S-9	MRS	30	Gram positive long chains of rods
10	MD10	S-9	MRS	37	Gram positive short chains of rods

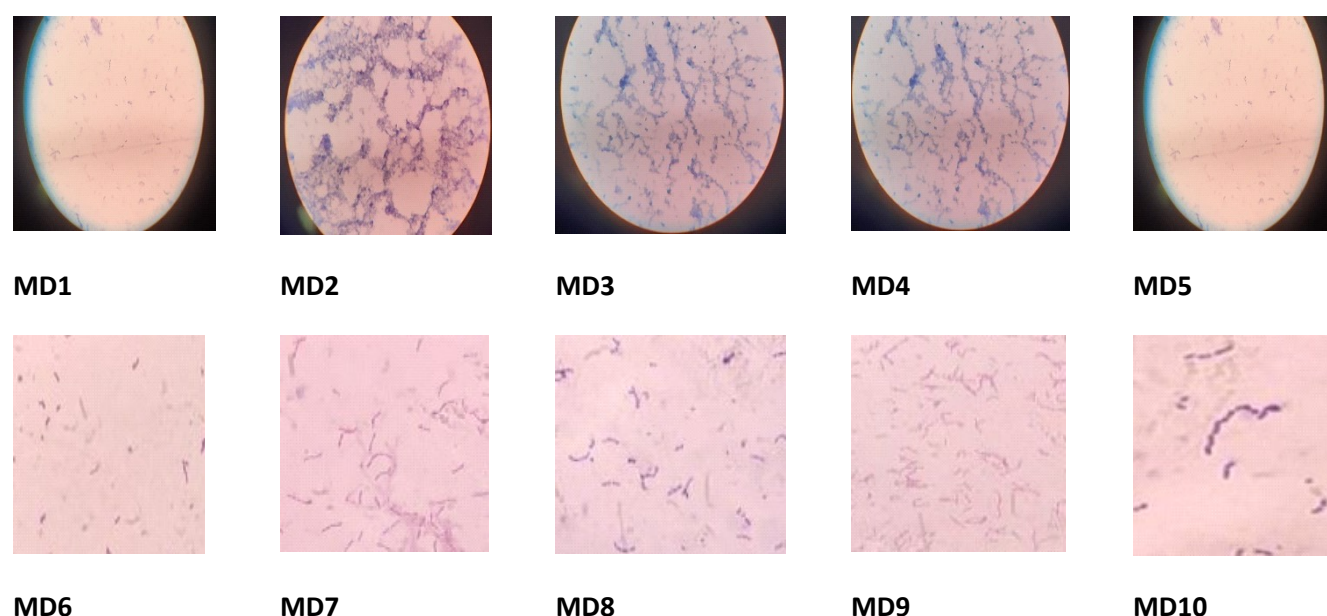


Fig. 2 Morphological evaluation of selected cultures by Gram staining

Misti dahi samples using MRS and M17 media (Table 1). They were tentatively considered as species of *Lactococcus*, *Lactobacillus* and *Streptococcus*. Out of all the isolates, only two were capable of growing at mesophilic temperatures, whereas the majority thrived in thermophilic conditions. The isolation of lactic acid bacteria from different dairy and non-dairy samples varied among the various reports. Goa et al. (2022) obtained twelve LAB isolates from three samples of Ergo collected in Jimma town, Ethiopia. These isolates comprised both mesophilic and thermophilic species such as *Lactococcus lactis* subsp. *lactis*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, and *Leuconostoc lactis*. Maheswari et al. (2013) reported that 74 strains of *Streptococcus thermophilus* isolated from various plant source by polymorphic approach. On the other hands, Rashid et al. (2007) isolated 12 lactic acid bacteria strains from traditional *dahi* samples collected from the stock cultures of Animal Food Function Laboratory, The Graduate School of Natural Science and Technology, Okayama University, Japan.

Sugar tolerance of isolated cultures

In current study, osmo-stability of the isolated strains was evaluated in reconstitute skim milk added with different levels of sugar. The curdling time and metabolic activity associated with the viable cells was lower for high sugar tolerant strains (20%) (Table 2). Similarly, Sameen et al. (2010) isolated four lactic strains as starter culture which had an ability to ferment milk in presence of sucrose and other sugars. Sugar tolerance capability of lactic strains is considered as one of the crucial parameters for selecting starter culture for *Misti dahi*. Many bacteria do not exhibit this behaviour while few osmotolerant lactic strains have been

reported to adapt these environments by displaying specific enzyme activities (Sleator and Hill., 2001). Although, lactic isolates (MD4 and MD9) obtained at mesophilic temperature curdled the milk, they took longer fermentation time as compared to thermophilic isolates. Vandna (2017) prepared mesophilic *Lactococcus* culture for *Misti dahi* which took 12-14 hrs to set the curd. In contrast, the isolated thermophilic culture was able to set the curd within four hours. Based on our experiment, four isolates MD1, MD2, MD3 and MD5 was showing significantly ($p < 0.05$) higher total lactic count and titratable acidity, lower curd setting time and pH in 20% sugar containing milk and hence were further identified by species-specific PCR.

Identification of sugar tolerant lactic cultures

The size of amplified PCR products of four selected isolates was 968 bp (Fig 3.), which was corresponding to *Streptococcus thermophilus* NCDC 074 and sugar tolerating *S. thermophilus* NCDC 436. Maheswari et al. (2013) observed that the isolated *S. thermophilus* strains exhibited a species-specific PCR product size of 968 bp, which provided confirmation that the selected strains were *Streptococcus thermophilus*. This finding aligns with previous studies conducted by Lick et al. (1996) and Schroeder et al. (1991), who also reported the same size of PCR product for *S. thermophilus* strains.

Effect of sugar tolerant cultures on properties of *Misti dahi* Physico-chemical properties

The physico-chemical properties of *Misti dahi* made by promising sugar tolerant cultures are shown in fig. 4. The titratable acidity of the samples ranged from 0.74% to 0.81% LA (Fig. 4A), falling within the limit specified by FSSAI for dahi. The pH values, as

Table 2: Preliminary technological screening of lactic strains in sugar containing milk

Isolate designation	Log of Total lactic count (cfu/ml)						Curd setting time (hr)						pH			Titratable acidity (%LA)								
	0% Sugar		15% Sugar		18% Sugar		20% Sugar		0%		15%		18%		20%		0%		15%		18%		20%	
	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	
MD1	9.684±0.08 ^{ab}	9.629±0.05 ^a	9.509±0.04 ^a	9.203±0.08 ^a	4.25±0.35 ^d	4.5±0.00 ^{ef}	5.1±0.14 ^{bcd}	5.75±0.35 ^f	4.77±0.03 ^d	5.03±0.02 ^{bc}	5.04±0.00 ^{de}	5.10±0.01 ^{cd}	5.19±0.03 ^c	5.04±0.00 ^{de}	5.10±0.01 ^{cd}	5.19±0.03 ^c	5.04±0.00 ^{de}	5.10±0.01 ^{cd}	5.19±0.03 ^c	5.04±0.00 ^{de}	5.10±0.01 ^{cd}	5.19±0.03 ^c	5.04±0.00 ^{de}	
MD2	9.321±0.26 ^{abc}	9.496±0.06 ^{ab}	9.381±0.09 ^a	8.985±0.27 ^a	4.25±0.07 ^d	5.1±0.14 ^{ef}	5.65±0.21 ^{cd}	5.75±0.35 ^f	4.82±0.03 ^d	5.0±0.01 ^c	5.10±0.01 ^{de}	5.19±0.03 ^c	5.19±0.03 ^c	5.10±0.01 ^{de}	5.19±0.03 ^c	5.19±0.03 ^c	5.10±0.01 ^{de}	5.19±0.03 ^c	5.19±0.03 ^c	5.10±0.01 ^{de}	5.19±0.03 ^c	5.19±0.03 ^c	5.19±0.03 ^c	
MD3	9.761±0.07 ^a	9.586±0.09 ^a	9.514±0.08 ^a	9.276±0.03 ^a	3.55±0.07 ^d	3.8±0.28 ^f	4.1±0.14 ^d	4.25±0.35 ^f	4.68±0.02 ^d	4.87±0.03 ^c	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	4.89±0.05 ^e	
MD4	9.716±0.05 ^a	9.462±0.07 ^{ab}	9.066±0.07 ^{ab}	8.915±0.05 ^a	9.1±0.14 ^a	9.25±0.35 ^{cd}	9.35±0.21 ^{bc}	10±0.00 ^{cd}	4.95±0.07 ^{cd}	5.13±0.02 ^{bc}	5.26±0.09 ^{cd}	5.5±0.07 ^b	5.5±0.07 ^b	5.26±0.09 ^{cd}	5.5±0.07 ^b	5.5±0.07 ^b	5.26±0.09 ^{cd}	5.5±0.07 ^b	5.5±0.07 ^b	5.26±0.09 ^{cd}	5.5±0.07 ^b	5.5±0.07 ^b	5.5±0.07 ^b	
MD5	9.286±0.02 ^{abc}	9.298±0.07 ^{abc}	9.268±0.07 ^a	9.037±0.05 ^a	4.5±0.00 ^d	4.65±0.21 ^{ef}	4.55±0.07 ^d	5.25±0.35 ^f	5±0.14 ^d	4.94±0.00 ^c	4.95±0.07 ^{de}	5.21±0.00 ^c	5.21±0.00 ^c	4.95±0.07 ^{de}	5.21±0.00 ^c	5.21±0.00 ^c	4.95±0.07 ^{de}	5.21±0.00 ^c	5.21±0.00 ^c	4.95±0.07 ^{de}	5.21±0.00 ^c	5.21±0.00 ^c	5.21±0.00 ^c	
MD6	9.245±0.03 ^{abcd}	9.062±0.07 ^{abcd}	8.656±0.16 ^{bc}	8.076±0.06 ^b	6.25±0.35 ^{bc}	6.75±0.35 ^{def}	8.1±0.14 ^{bcd}	8.75±0.35 ^{de}	5.26±0.02 ^{abc}	5.4±0.14 ^{ab}	5.63±0.02 ^{ab}	5.66±0.00 ^{ab}	5.66±0.00 ^{ab}	5.63±0.02 ^{ab}	5.66±0.00 ^{ab}	5.66±0.00 ^{ab}	5.63±0.02 ^{ab}	5.66±0.00 ^{ab}	5.66±0.00 ^{ab}	5.63±0.02 ^{ab}	5.66±0.00 ^{ab}	5.66±0.00 ^{ab}	5.66±0.00 ^{ab}	
MD7	8.718±0.07 ^{cd}	8.625±0.16 ^{bcd}	8.062±0.08 ^d	7.101±0.02 ^c	5.5±0.00 ^{bcd}	7.3±0.28 ^{cde}	7.85±0.07 ^{bcd}	8.1±0.14 ^c	5.47±0.03 ^a	5.38±0.02 ^{ab}	5.43±0.04 ^{bc}	5.53±0.02 ^b	5.53±0.02 ^b	5.38±0.02 ^{ab}	5.53±0.02 ^b	5.53±0.02 ^b	5.43±0.04 ^{bc}	5.53±0.02 ^b	5.53±0.02 ^b	5.38±0.02 ^{ab}	5.53±0.02 ^b	5.53±0.02 ^b	5.53±0.02 ^b	
MD8	9.067±0.07 ^{bcd}	8.46±0.33 ^{cd}	8.298±0.21 ^{cd}	7.989±0.04 ^b	9.1±0.14 ^a	10.25±0.35 ^c	10.5±0.70 ^b	11.5±0.70 ^c	5.31±0.02 ^{ab}	5.59±0.12 ^a	5.57±0.12 ^{abc}	5.60±0.07 ^b	5.60±0.07 ^b	5.59±0.12 ^a	5.60±0.07 ^b	5.60±0.07 ^b	5.57±0.12 ^{abc}	5.60±0.07 ^b	5.60±0.07 ^b	5.59±0.12 ^a	5.60±0.07 ^b	5.60±0.07 ^b	5.60±0.07 ^b	
MD9	8.94±0.08 ^{cd}	8.47±0.31 ^{cd}	8.601±0.07 ^{bcd}	7.958±0.09 ^b	10.5±0.70 ^a	19±1.41 ^a	21±1.41 ^a	24±0.00 ^a	5.34±0.01 ^a	5.6±0.07 ^a	5.73±0.02 ^{ab}	5.72±0.06 ^{ab}	5.72±0.06 ^{ab}	5.6±0.07 ^a	5.73±0.02 ^{ab}	5.72±0.06 ^{ab}	5.73±0.02 ^{ab}	5.72±0.06 ^{ab}	5.73±0.02 ^{ab}	5.6±0.07 ^a	5.72±0.06 ^{ab}	5.72±0.06 ^{ab}	5.72±0.06 ^{ab}	
MD10	8.645±0.17 ^d	8.3±0.06 ^d	8.195±0.07 ^{cd}	7.605±0.21 ^{bc}	6.5±0.70 ^b	15.5±0.70 ^b	18±0.00 ^a	18±0.00 ^a	5.55±0.07 ^a	5.7±0.07 ^a	5.83±0.02 ^a	5.83±0.02 ^a	5.83±0.02 ^a	5.7±0.07 ^a	5.83±0.02 ^a	5.83±0.02 ^a	5.83±0.02 ^a	5.7±0.07 ^a	5.83±0.02 ^a	5.7±0.07 ^a	5.83±0.02 ^a	5.83±0.02 ^a	5.83±0.02 ^a	

(Different superscripts represent the significant difference ($p<0.05$) across the cultures (Analysed by one-way ANOVA following Tukey test).

shown in Fig. 4B, ranged from 4.58 to 4.85. The curd setting time, depicted in Fig. 4C, varied between 4 and 6 hrs. Notably, *S. thermophilus* MD3 demonstrated a curd setting time of four hours, which was significantly ($p<0.05$) lower compared to the other cultures. In contrast, the negative control exhibited an extended curd setting time of up to 11 hrs. According to Rashid et al. (2007), the pH of isolated *S. thermophilus* strains reached 3.92 after 24 hrs when incubated at 37°C in unsweetened reconstituted skim milk. Concurrently, the titratable acidity of this culture was 1.26% LA at the same time. Vandna (2017) developed freeze-dried DVS culture which could set the *Misti dahi* at 11.5, 12.5, 14 and 12 hrs by employing *Lactococcus lactis* ssp *lactis* NCDC314, *Lactococcus lactis* ssp *lactis* NCDC94, *Lactococcus* sp NCDC128 and *Lactococcus lactis* ssp *lactis* NCDC97 respectively. The spray-dried DVS cultures, in comparison, did not exhibit any significant deviation from the freeze-dried cultures. The pH of the *Misti dahi* produced using the laboratory-scale DVS culture was determined to be 4.60. In contrast, the lactic strains isolated in our study demonstrated the ability to grow rapidly, even in the presence of high sugar in the media.

Microbiological quality

Misti dahi prepared with *S. thermophilus* MD3 exhibited the highest total lactic count as shown in Fig 4D, which was comparable to the *Misti dahi* prepared using reference sugar tolerating strain *S. thermophilus* NCDC436. However, there were no significant differences ($p<0.05$) observed in *Misti dahi* made from MD1, MD2, and MD5. The absence of coliforms, yeast, and molds indicates that the product was manufactured in a hygienic environment. Vandna (2017) reported a total lactic count of 9.07 log cfu/ml in *Misti dahi* samples prepared using the DVS *Misti dahi* culture. This finding demonstrates that our culture is equally efficient in producing *Misti dahi*.

Rheological properties

Fig. 5 presents the textural properties of *Misti dahi* samples. Among the experimental *Misti dahi* samples, *S. thermophilus* MD3 obtained higher values for all the textural attributes. Textural attributes of *Misti dahi* sample prepared using MD2 were inferior when compared to others. Notably, *Misti dahi* made with reference *S. thermophilus* NCDC 436 strain demonstrated superior firmness, consistency, cohesiveness, and work of cohesion in comparison to the other samples. Textural attributes of dahi or yoghurt samples depends on various parameters viz. milk type, milk composition, heat treatment given to milk, starter culture used, and the incubation conditions. Since all other process parameters were kept similar, the differences in textural attributes of *Misti dahi* samples could be attributed to the

Fig. 3 Agarose gel showing PCR products obtained using *S. thermophilus* species specific primers. Lane L1: 1 kb DNA ladder, A: NCDC 074, B: NCDC 436, C: MD1, D: MD2, E: MD3, F: MD5, L2: 100 bp DNA ladder

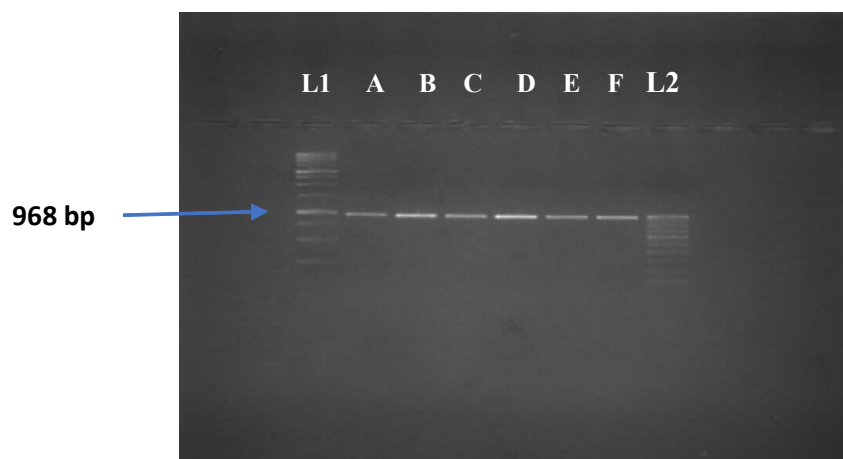
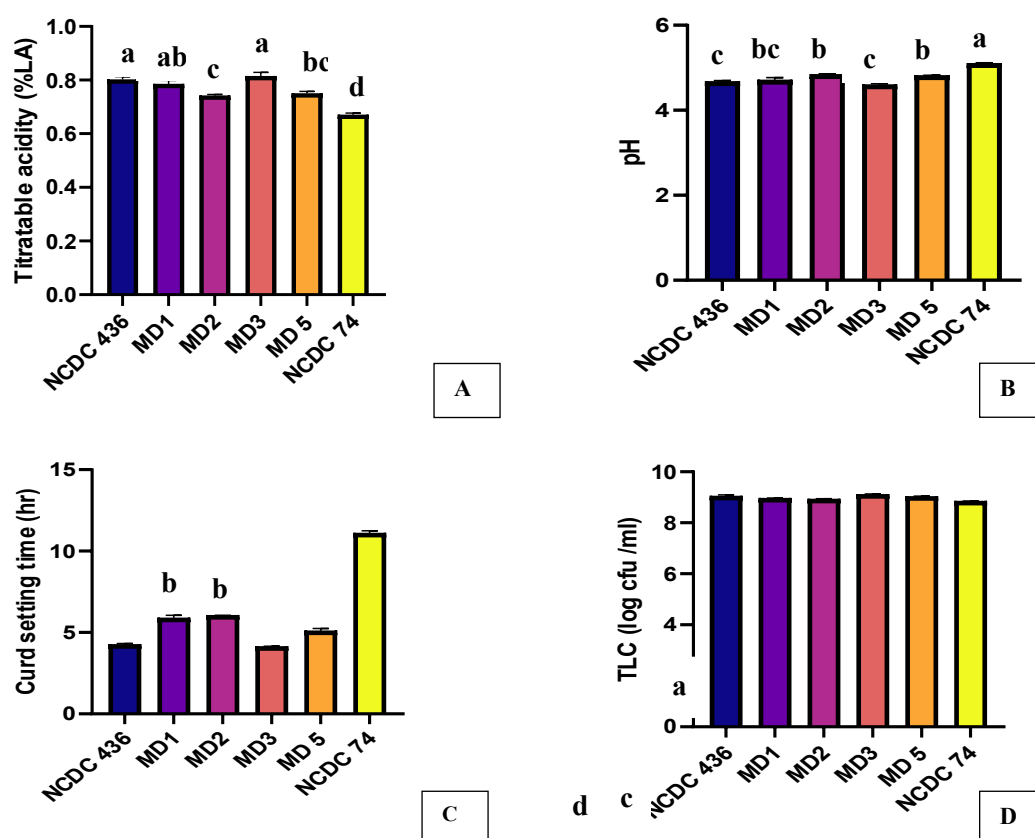


Fig. 4 Physico-chemical and microbiological properties of *Misti dahi*. A. Titratable acidity; B. pH; C. Curd setting time and D. Total lactic count

Different superscripts represent the significant difference ($p < 0.05$) across the cultures (Analysed by one-way ANOVA following Tukey test).



use of different cultures for their preparation. Starter cultures differ in their proteolytic activity and acidification rate. Even the starter cultures belonging to same species of bacteria may have differences in their acidification rate and proteolytic activity. Amani et al. (2017) observed that yoghurts produced with starter cultures possessing distinct proteolytic activities exhibited varying textural attributes. Those starter cultures with higher proteolytic activity resulted in yoghurts with weaker textural characteristics. Similarly, Han et al. (2014) found that LAB isolates from traditional Chinese fermented foods displayed different acidification rates, leading to yoghurts with significantly diverse

textural attributes. Raju and Pal (2009) also reported differences in the textural attributes of *Misti dahi* samples, which were prepared using different starter cultures.

The flow-curves of *Misti dahi* samples are given in Fig. 6. All the *Misti dahi* samples exhibited pseudoplastic (shear-thinning) behavior during the steady-shear rheological measurements. Shear thinning behaviour indicates that the viscosity of *Misti dahi* samples decreased with increasing shear. The apparent viscosity (Pa.s.) of *Misti dahi* samples at 50 s^{-1} shear rates are as follows 0.24, 0.22, 0.79, 0.26, 0.29 and 0.61 for MD1, MD2, MD3,

Fig. 5 Texture profile of *Misti dahi* prepared with lactic strains. A. Firmness (g) B. Consistency (g.sec) C. Cohesiveness (g) D. Work of cohesion (g.sec) Different superscripts represent the significant difference ($p < 0.05$) among the strains (Analysed by one-way ANOVA following Tukey test).

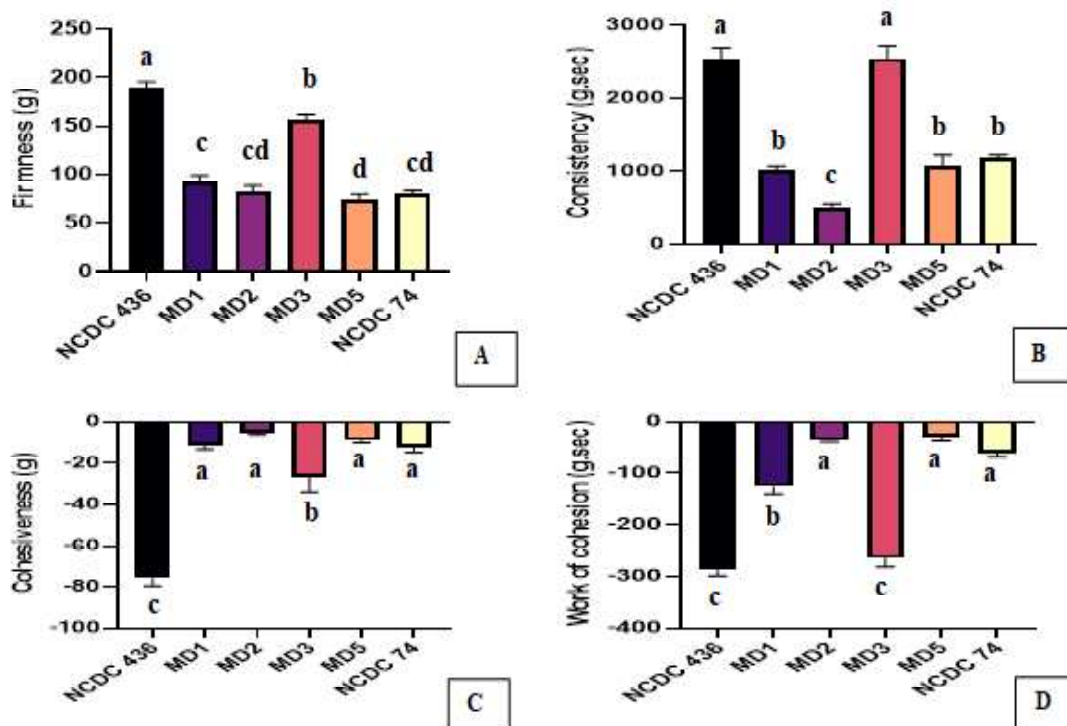
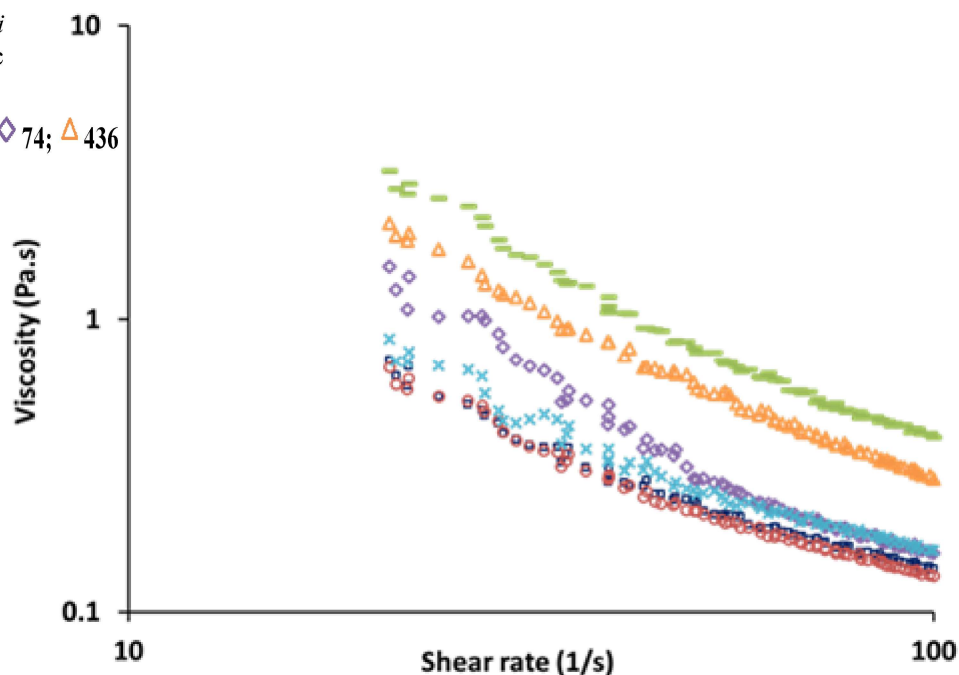


Fig. 6: Flow-curve of *Misti dahi* samples prepared with different lactic strains

□ MD1; ○ MD2; ▬ MD3; × MD5; ◇ 74; △ 436



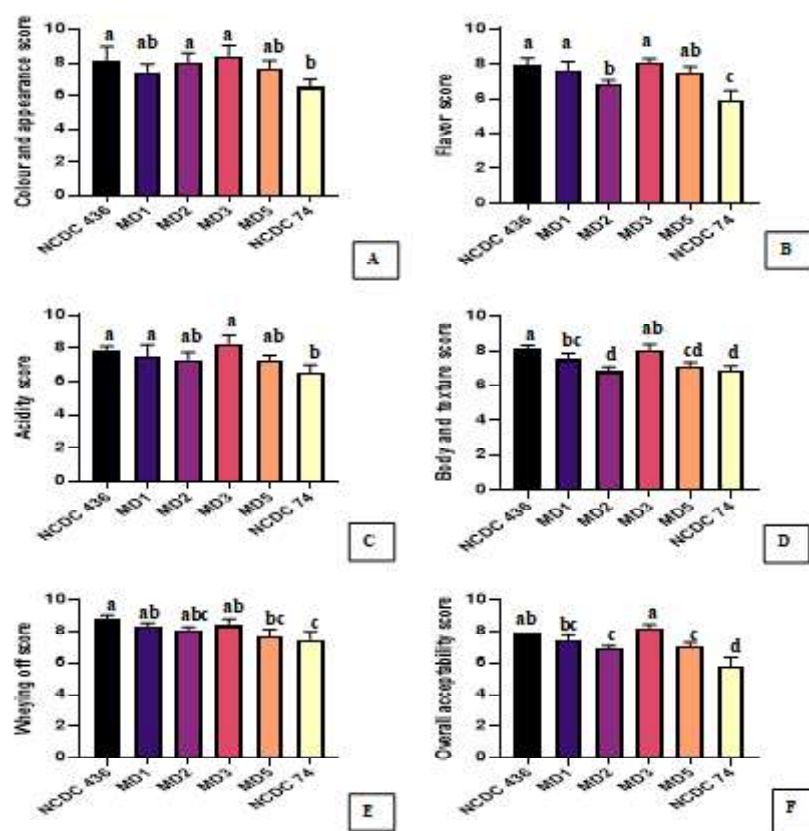
MD5, 74 and 436 respectively. The highest and lowest viscosity values were obtained for MD3 and MD2, respectively. Celik and Temiz (2022) reported that lactobacilli isolate from traditional yoghurts of Turkey exhibited significantly different ($p < 0.05$) viscosities. The authors reported that these LAB isolates had different acidification profiles which could be a possible reason for differences in their viscosity values. Similar variations in

viscosity were reported by Behare et al. (2013) in different dahi samples produced using various starter cultures. Xu et al. (2015) observed that differences in acidifying rates of yoghurt starters resulted in yoghurts with varying viscosities. Starter cultures with slow acidification rates produced yoghurts with higher viscosities. The results obtained in the current investigation were in contradiction to those mentioned by Xu et al. (2015). Our results

Fig. 7 Sensory evaluation of prepared *Misti dahi* samples

A. Colour and appearance score, B. Flavor score, C. Acidity score, D. Body and texture score E. Wheying off score, F. Overall acceptability score

Different superscripts represent the significant difference ($p < 0.05$) across the cultures (Analysed by one-way ANOVA following Tukey test).



were supported by Ruas-Madiedo et al. (2005) who advocated that proteolytic activity of the strains did not seem to play any significant role in rheological attributes.

Sensory properties

Nine-point hedonic scale was used for sensory evaluation, and factors such flavour, body and texture, colour and appearance, acidity, wheying off, and overall acceptability were considered. The outcome depicted in Fig. 7 shows that there are discernible differences between the four distinct *Misti dahi* samples, suggesting that the product prepared with *S. thermophilus* MD3 can provide an end product of comparable quality to commercial culture i.e., *S. thermophilus* NCDC436. Whereas, in case of negative control (NCDC74), the sensory score was significantly ($p < 0.05$) lower than the others. Raju and Pal (2009) found no significant differences in sensory attributes between the prepared *Misti dahi* samples and the control sample. Similarly, Akter et al. (2010) noted no significant difference in the organoleptic scores of the prepared dahi samples.

Conclusion

The present study focused on isolating and characterizing sugar-tolerant cultures to identify suitable strains for *Misti dahi*

production. The selection of an ideal culture is crucial to achieve the desired attributes of the final product. Among the isolated cultures, *S. thermophilus* MD3 exhibited superior results in terms of technological, sensory, and rheological parameters of *Misti dahi*. This study confirms the suitability of *S. thermophilus* MD3 for *Misti dahi* production. Furthermore, the comprehensive evaluation of microbiological quality reaffirms the safety of the final product.

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RESEARCH ARTICLE

Study on incidences, risk factors and bacterial populations involved in subclinical mastitis cases in the conventional *vis-à-vis* organic dairy farming under small holder system in Indian Sundarban region

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Abstract: Organic dairy farming is being promoted in Indian Sundarban region through the ‘Women Milk Producers’ Cooperative Society’ under the aegis of Sundarban Milk Union of West Bengal. The present study was undertaken to investigate the prevalence of sub clinical mastitis (SCM) in dairy herds maintained by small holders in two production system (organic *vis-à-vis* conventional). Total numbers of 34 dairy farmers were selected for the study. Out of the 34 numbers of farmers, 50% farmers (n=17) had adopted organic milk production practices and the remaining (n=17) farmers used to practice conventional dairy farming. California Mastitis Test (CMT) kit was used to evaluate incidences of SCM. A semi-structured questionnaire was used for analysis of associated management risk factors for SCM. Positive milk samples from organic growers were subjected to microbial analysis and antibiogram. Data was analyzed for descriptive statistics by SPSS program v. 20.0. Results revealed that the incidences of SCM is not significantly ($p<0.05$) different in organic (35.3%) and conventional (29.4%) dairy production system, indicating chronic and long standing infection in udders. Besides, no significant ($p<0.05$) difference between the two production system was observed in management risk factors associated with SCM. It was found that *Staphylococcus aureus* is the causative organism responsible for SCM under organic dairy production system in the study area. It can be concluded that to reduce high rate of SCM in the region, regular monitoring of udder health with holistic approach is necessary.

Keywords: Antibiogram, California Mastitis Test, Organic Dairy, Sub Clinical Mastitis

Introduction

Health-conscious metropolitan consumers are increasingly inclining to consume branded organic milks for obvious human health benefits (Butler and Stergiadis, 2020). Driven by consumer demand, organic milk market size in India has already reached INR 6,082 million in 2021 and expected to further expand 5 times by 2027. Organic milk production system in general, based on feeding cows on feed ingredients grown organically (without using synthetic pesticides or chemical fertilizers) and avoiding using synthetic growth promoters, hormones, antibiotics and drugs in husbandry. Apart from the milk production with enhanced health benefits, one of the prime objects of organic dairy farming is the welfare of dairy animals (Chander et al. 2013).

From the production point of view, maintenance of udder health is one of the critically important aspect in dairy cows. Inflammation of udder is termed as ‘mastitis’ and in India alone this disease causes estimated economic losses to the extent of 7000 crore (Bansal and Gupta, 2009). Sub clinical mastitis (SCM), characterized by low level of persistent inflammation and reduced milk yield, is much more prevalent form than clinical mastitis (Birhanu et al. 2017; Sohidullah et al. 2023). SCM like tip of an iceberg, not only can endanger mammary tissue of the lactating cow by progressive fibrosis, but also may impact human health through possible zoonoses in the value chain. Although, incidences of SCM are reported from organic dairy herds in different parts of the globe (Villarand López-Alonso 2015; Hansmann et al. 2019), little information is known regarding its incidences and microbial population involved in SCM cases in local cattle breeds reared under organic practices in coastal saline affected zone of Indian Sundarban. Present study was conducted to explore the prevalence of SCM in dairy cows maintained under organic certification regulations (NPOP 2005) and conventional husbandry practices by small holder dairy farmers in Indian Sundarban region.

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Materials and methods

Study area and sampling

Two villages namely Nafargunj and Masjidbati in Basanti block of Indian Sundarban region were purposively selected for the study during May-June 2022. Map of the studied area was shown in Fig 1 and the precise locations where the samples were collected are indicated by red dots. The map was prepared using QGISv. 2.2 software (QGIS Development Team 2013).

Randomly 34 numbers of dairy farmers who were the members of 'Women Milk Producers' Cooperative Society' under the aegis of Sundarban Milk Union, West Bengal, were selected for milk sampling and door to door survey. Out of the 34 numbers of farmers, 50% farmers (n=17) had adopted organic milk production practices and certified by Rajasthan State Organic Certification Agency (RSCOA) as per National Program for Organic Production (NPOP). Remaining 50% farmers (n=17) were rearing cows under conventional practices. A pre-tested and piloted semi-structured questionnaire was used for analysis of risk factors related to the development of Sub-clinical mastitis in dairy herds. The questionnaire was developed in local language (Bengali) with consultations from subject expert and previous research studies.

Milk Sample Collection

Cow milk samples (n = 34) from apparently healthy quarters were screened for detection of subclinical mastitis by California Mastitis Test Kit (De Laval, India) as per Dingwell et al. (2003). CMT scores (N, Trace, 1, 2 and 3) for the affected udder quarter were based on the degree of gel formation, where 'N' means no reaction and 'Trace', '1', '2' and '3' denote positive samples with increasing reaction.

Isolation and identification of organism in the positive milk samples

The positive milk samples (Trace, 1, 2 and 3) were pooled from all the quarters and transported to ICAR-IVRI (Eastern Regional

Station), Kolkata in an icebox for bacterial isolation and identification (Bhattacharyya et al. 2016). Briefly, 10 µl of each sample was incubated overnight in trypticase soy broth (BD, BBL) at 37°C and inoculated in Baird Parker agar (BD, BBL) with egg yolk emulsion and mannitol salt agar (MSA; HiMedia). Colonies surrounded by bright yellow zone in MSA or black, shiny, and convex colonies surrounded by a clear zone in Baird Parker agar were primarily selected as *Staphylococcus*. Single-isolated colonies were taken in nutrient agar (HiMedia) slant and were further processed for confirmation as *S. aureus* using standard tests such as Gram's staining, catalase, coagulase, oxidase, indole, methyl red, urease, Voges-Proskauer, lecithinase production, mannitol, and glucose fermentation.

Antimicrobial susceptibility

Antibiogram of the isolated organisms was also studied as per Bhattacharyya et al. 2016 by disc agar diffusion technique using commercially available discs (HiMedia) against the following antibiotics— *Chloramphenicol*, *Cefoxitin*, *Tetracycline*, *Penicillin*, *Oxacillin*, *Trimethoprim-sulfamethoxazole*, *Erythromycin*, *Gentamicin*, *Linezolid* and *Ciprofloxacin*. The zone of inhibition was measured after incubation with antibiotic discs at 37°C for 18–24 hours following CLSI (CLSI, 2018) guidelines

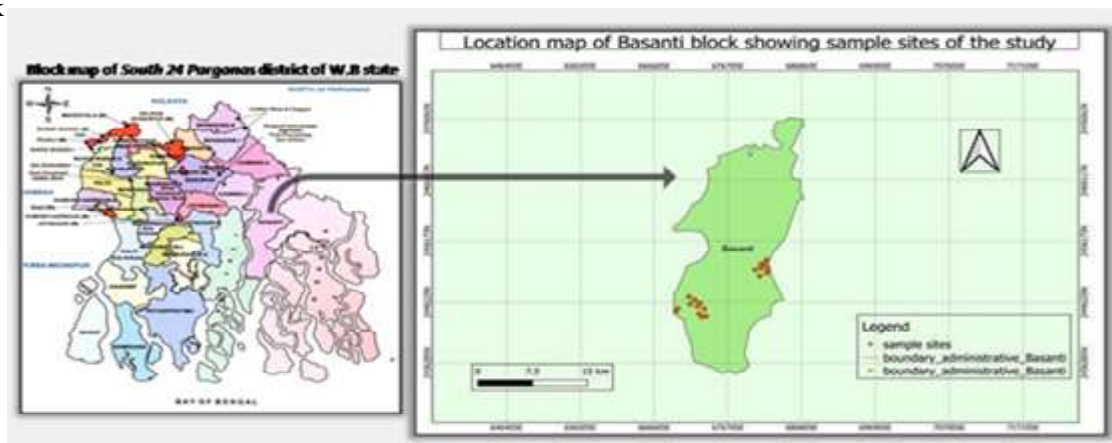
Statistical Analysis

The data was statistically analyzed by using SPSS program v. 20.0 (IBM Corp. NY, USA) as per method described by Snedecor and Cochran (1994). Possible association of udder health with milk production practices (Organic *vis-à-vis* Conventional) was determined by *chi* square test. Values were considered significant at $P < 0.05$.

Results and Discussion

Socio-economic profiles of dairy farmers are presented in table 1. Most of the dairy farmers are middle aged adults with 61.8% belong to more than 35 years of age group. Dairy farming is a

Fig. 1 Location map of milk sample collection



skilled job and thus may require experienced, physically capable and energetic work force. Education level is important for adoption of knowledge and skills. Results revealed that around 73.5% farmers are with secondary education background. This is in contrary to Das et al.(2017) who observed that majority of the dairy farmers under Sundarban Milk Union was only with primary educational background. Present study further revealed that majority (61.8%) of the dairy farmers belonged to middle class economy. Ahmed et al. (2020) similarly reported that majority (42.6%) of the dairy farm workers were from upper lower economic

strata in the vicinity of Southwest Delhi, India. Most of the dairy farmers in our present study belonged to small and marginal farmers and thus their dairy practice level might be influenced by their socio economic status. Under the present study, majority (88.2%) respondents reported that they belonged to agricultural labour and 58.8% of respondents reported that they had 1-3 acre of land. This profile is typical to the Sundarban area and demonstrated that small holder dairy farming in this area is primarily for subsistence.

Table1:Socio-economic profile of dairy farmers

	Characteristics	N	Frequency (%)
Education	Illiterate	4	11.8
	Primary	2	5.9
	Secondary	25	73.5
	College	3	8.8
Family member	<3	4	11.8
	3-5	21	61.8
	>5	9	26.5
Economic status	Upper Middle Class	1	2.9
	Middle class	21	61.8
	Poor	12	35.3
Age	<20	0	0
	20-35	13	38.2
	>35	21	61.8
Occupation	Housewife	1	2.9
	Agricultural labour	30	88.2
	Non-agricultural labour	2	5.9
	Job	1	2.9
Land	<1 acre	11	32.4
	1-3 acre	20	58.8
	>3 acre	3	8.8

Table 2:Udder Health condition as denoted by California Mastitis Test (CMT) scores in two different milk production types

Variables	CMT Scores	N	Frequency (%)
Organic Production	N	11	64.7
	T	4	23.5
	1	1	5.9
	2	1	5.9
	3	0	0
Conventional Production	N	12	70.6
	T	2	11.8
	1	3	17.6
	2	0	0
	3	0	0

(N: No reaction; T: Trace; 1: Distinct precipitate but does not gel; 2: Distinct gel formation; 3: Strong gel formation)

In the present study, 34 cows (local cattle breed) were screened for SCM using CMT kit and 11 cows were detected positive for SCM (Table 2). Around 64.7% and 70.6% cows respectively from organic and conventional dairy system gave negative test in CMT. Only 5.9% of milk samples from organic dairy production were scored 2 in CMT test, whereas only 17.6% samples from conventional dairy production reached only score 1. This indicates incidence of SCM is well under control in local cattle breeds in both the production system.

Table 3 depicts number of udder quarters affected with SCM in organic and conventional production systems. Under the organic production system, two quarters showed higher incidence (50%) of SCM infection followed by single quarter (33.33%) and three quarters (16.67%). Under conventional system of rearing, single quarter infection was mostly prevalent (80%) followed by two quarters infection. Overall, the results indicate that quarter level prevalence is lower in conventional farming system than organic farming system. This may be due to use of antibiotics in conventional farming system.

The association between production type (organic *vis-à-vis* conventional) and incidence of SCM is presented in Table 4. SCM was observed in 35.3% and 29.4% of cows respectively in organic and conventional dairy farming system. No significant ($p>0.05$) difference was found between two farming system in regards to udder health. This shows that SCM is a matter of concern in both organic and conventional dairy practices. In agreement to our findings, Kouoimská et al. (2014) observed that the somatic cell count (SCC) in milk samples did not vary in organic and conventional dairy farms. It is evident that SCM is more about maintaining udder health and hygiene in individual dairy cows than the farming system itself.

Management level risk factors associated with SCM were found to be at par ($p>0.05$) in both conventional and organic production systems (Table 5) in the study area. Present study revealed that 94.1% and 76.5% farmers under conventional and organic regime respectively adopted good practices like '*Use of disinfectants for cleaning of milking utensils and cow shed*' and '*Washing of udder and hands before and after milking*'. However, dairy farmers in both the production system showed the reluctance in following the other critical udder management practices like '*Teat dipping*' or '*Dry cow therapy*'. It is obvious from the present study that small holder dairy farmers irrespective of the production system, needs to be exposed in more intensive training on the importance of maintaining the udder health. Bhakat et al. (2022) indicated that the risk factors associated with udder health are more in the small holder dairy farmers (with 2-3 dairy cows) in tropical climate. Poor sanitation and hygiene is the foremost cause of sub clinical mastitis in dairy animals (Sinha et al. 2014). Our present study clearly showed that the both conventional and organic dairy production system managed by the small holder dairy farmers of the coastal zone of Sundarban faces prominent threats of sub clinical mastitis. The region may become epitome of chronic mastitis infection if potential management risk factors cannot be mitigated to a certain level. Risk factors for SCM are also associated with socio economic status of the dairy farmers (Ahmed et al. 2020). Thus multiple factors are intriguingly related to management risks associated with bovine sub clinical mastitis. Further scientific study needs to be carried out to identify the knowledge and practice gap of small holder dairy farmers in that area.

The result (Table 6) obtained from bacteriological analysis of the organic milk samples revealed that *Staphylococcus aureus* is the only causative organism responsible for SCM (35.3%) in the study area. The milk samples from conventional production system was not analysed for bacteriology. *S. aureus* is the most common

Table 3: Number of quarters affected with SCM in two production types

Organic production			Conventional production		
No. of quarters affected	No. of animals	Prevalence	No. of quarters affected	No. of animals	Prevalence
1	2	33.33%	1	4	80%
2	3	50%	2	1	20%
3	1	16.67%	3	0	0
4	0	0	4	0	0

Table 4: Association between the production type and incidence of SCM

	Responses	Production Type n, (%)		χ^2	p - value
		Organic	Conventional		
Incidence of SCM	Positive	6 (35.3%)	5 (29.4%)	0.134	0.714
	Negative	11 (64.7%)	12 (70.6%)		

Table 5: Management risk factors related to development of SCM

Variables	Production Types	Characteristics	N	Frequency (%)	χ^2	p value
<i>Milking Method</i>	Conventional	Full hand milking	3	17.6	0.234	0.5
		Stripping	14	82.4		
	Organic	Full hand milking	2	11.8		
		Stripping	15	88.2		
<i>Use of disinfectants for cleaning of milking utensils and cow shed</i>	Conventional	Yes	16	94.1	2.110	0.33
		No	1	5.9		
	Organic	Yes	13	76.5		
		No	4	23.5		
<i>Teat dipping</i>	Conventional	Yes	1	5.9	1.030	0.5
		No	16	94.1		
	Organic	Yes	0	0		
		No	17	100		
<i>Dry cow therapy</i>	Conventional	Yes	1	5.9	0.366	0.5
		No	16	94.1		
	Organic	Yes	2	11.8		
		No	15	88.2		
<i>Washing of udder and hands before and after milking</i>	Conventional	Yes	16	94.1	1.030	0.5
		No	1	5.9		
	Organic	Yes	17	100		
		No	0	0		

Table 6: Bacterial species isolated from subclinical mastitis cases observed under organic production

Bacterial Species	Total number of isolates	Prevalence (%)
<i>Staphylococcus aureus</i>	6	35.3%
Negative	11	67.7%
Total	17	100%

pathogen associated with sub clinical mastitis and one of the principal causes of the food borne diseases in humans (Campos et al. 2022). Absence of teat dipping practices during milking, continued adoption of stripping milking methods, poor hygiene in the shed and maintenance of chronically affected cows in the herd might be linked with high proportion of SCM cases with presence of *Staphylococcus aureus*. The antibiogram (Table 7) showed 100% sensitivity to *Chloramphenicol*, *Cefoxitin*, *Tetracycline*, *Oxacillin*, *Trimethoprim-sulfamethoxazole*, *Gentamicin*, *Linezolid* and *Ciprofloxacin*. However, isolates showed intermediately resistant to *erythromycin* (33.33%) and

resistant to *Penicillin* (33.33%). Amofo et al. (2021) indicated that the prevalence of resistant microbial population is higher in the conventional dairy farms than organic dairy farms. In the present research, the bacterial isolates from organic growers were sensitive to most of the common antibiotics and this might be due to the prevalent organic dairy farming practices like eliminating antibiotic usages, natural farming practices for fodder cultivation, use of herbal products to cure diseases etc. The growing emergence of AMR bacteria might be limited by adopting such organic practices in small dairy farms in the coming days.

Conclusion

Table 7:Antibiogram of isolates (n=6)

Name of Antibiotics	Resistant n(%)	Intermediate n(%)	Sensitive n(%)
Chloramphenicol	0 (0%)	0 (0%)	6 (100%)
Erythromycin	0 (0%)	2 (33.33%)	4 (66.67%)
Cefoxitin	0 (0%)	0 (0%)	6 (100%)
Penicillin	2 (33.33%)	0 (0%)	4 (66.67%)
Tetracycline	0 (0%)	0 (0%)	6 (100%)
Oxacillin	0 (0%)	0 (0%)	6 (100%)
Trimethoprim -sulfamethoxazole	0 (0%)	0 (0%)	6 (100%)
Gentamicin	0 (0%)	0 (0%)	6 (100%)
Linezolid	0 (0%)	0 (0%)	6 (100%)
Ciprofloxacin	0 (0%)	0 (0%)	6 (100%)

It was concluded that the prevalence of sub clinical mastitis in dairy herds is high among small holder dairy farmers in Indian Sundarban region. Production type (organic *vis-à-vis* conventional) did not significantly affect the incidences of sub clinical mastitis. Substantial management risks associated with SCM were observed in both the production type. Awareness and training by the milk unions might be the driver of change to reduce the risk factors present in the prevalent management of dairy farming. Microbial analysis from milk samples from organic growers revealed that *S. aureus* is the responsible organism for SCM in the organic dairy herds. In the backdrop of global emergence of AMR bacterial population, the more detailed study on SCM in different production system with more number of animals is the need of the time. The study strongly recommends more strict adherence to regulations of organic dairy farming and regular monitoring of udder health with CMT kit in the field to eliminate the threats of resistant infections.

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RESEARCH ARTICLE

Physico-chemical properties of optimized Kaalan-A traditional dairy product

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Abstract: Kaalan is a buttermilk, coconut and vegetable-based culinary preparation filled with the goodness of spices, famous in the South Indian state of Kerala. The Kaalan available in the market differs widely which can be attributed to a lack of well-defined characterization and standardization, hindering efforts towards industrial production. The complex procedures of preparation and lack of knowledge limit its preparation to caterers and households. The current study aimed to optimize the production process utilizing the Response Surface Methodology for achieving standardized large-scale production within the organized sector. The outcomes from the Response Surface Methodology (RSM) demonstrated that the experimental observations could be suitably accommodated within a second-order polynomial model, exhibiting a satisfactory coefficient of determination ($R^2 > 90\%$). The proposed formulation for the Kaalan was a combination containing 194.97g vegetables, 748.09 ml buttermilk, and 126.78g coconut. The optimized Kaalan had 3.49 ± 0.005 per cent protein, 7.12 ± 0.07 per cent fat, 9.95 ± 0.08 per cent carbohydrate, 4.84 ± 0.22 per cent dietary fiber, 2.17 ± 0.03 per cent ash and 28.36 ± 0.04 per cent total solids. The curcumin content was 13.63 ± 0.03 ppm and the energy value was 117.77 ± 0.42 kcalories per 100 g. The data shows that the optimized Kaalan can be established as a nutritionally valuable, low-calorie functional food.

Keywords: Kaalan, traditional, dairy, response surface, optimization, curcumin

Introduction

Kerala, a state in southern India, has a diverse and amusing food culture deeply rooted in its traditions and way of life. The cuisine of Kerala is renowned for its abundant use of coconut in various forms and aromatic spices in its flavourful dishes. Sadya is a multi-course meal with a wide variety of vegetarian dishes, typically served on a banana leaf on special occasions and festivals. Kaalan is a traditional culinary preparation native to Kerala and is a significant component of the Sadya. It is a buttermilk-based curry prepared using unripe plantains, elephant foot yam (EFY), and coconut as its primary ingredients. Despite the delightful taste of Kaalan, the younger generation seldom prepares it due to its time-consuming process and limited knowledge about this dish (Aneena, 2009). The significant obstacles faced by the Indian food processing sector are lack of standardization, the absence of proper quality measures, and the high cost for establishing cold chain infrastructure (Singh et al. 2022). These challenges are particularly relevant to the production of Kaalan, because of the lack of information regarding its quality and characteristics. It was found in the study conducted by the same authors that the Kaalan available in the market vary widely in the quality attributes making it difficult to authenticate this traditional product.

The constituents employed in the preparation of Kaalan demonstrate high nutritional properties and present an array of well-established functional characteristics. The elephant foot yam (*Amorphophallus paeoniifolius*) and unripe plantain (*Nendran* variety from *Musa* spp-AAB group) exhibit elevated nutritional, and functional qualities. The coconut (*Cocos nucifera*) serves as a source of medium chain fatty acids renowned for their cardioprotective effects (DebMandal and Mandal, 2011). The tangy flavor and nutrient rich composition of sour buttermilk make it a versatile ingredient in culinary preparations, while its abundant phospholipids and potential health benefits underscore its significance in both traditional and modern contexts. Turmeric (*Curcuma longa*) contains curcumin, a

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compound possessing anti-inflammatory, antioxidant, anti-bacterial, antifungal, antithrombotic, anti-carcinogenic, neuroprotective, and cardio-protective properties (Khajehdehi, 2012). Remarkably, the inclusion of pepper (*Piper nigrum*) enhances the bioavailability of curcumin present in turmeric (Patil et al. 2016; Shoba et al. 1998). Furthermore, other spices aid in improving digestion. While the functional properties of individual ingredients are well-documented, the synergistic effects in dishes like Kaalan require further validation. In order to preserve the culinary heritage of Kaalan and ensure its endurance for future generations, it is crucial to establish proper standardization, validate its functional properties, and document its properties. Achieving this would also support its industrial production and facilitate its entry into the global market.

Optimization means identifying the optimal quality standards for both the product and process efficiency, all while minimizing time and cost (Bas and Boyaci, 2007). In food preparation, many factors affect the final quality such as quantity and quality of ingredients, their interactions, processing conditions, etc. Response surface methodology (RSM) has emerged as an effective and powerful approach for food product modelling and optimization, offering substantial advantages over traditional single-factor studies. By considering multiple factors together, RSM enables us to thoroughly investigate how different ingredients interact. This approach provides a clearer understanding of the best conditions to achieve desired outcomes in developing and optimizing food products.

In the present study, RSM was used for optimizing the formulation of Kaalan. Further, the physico-chemical characteristics and nutritional value of Kaalan were also analysed. Optimizing the method of preparation and assessing the nutritional and functional properties are of utmost importance to position Kaalan as a high-quality food in the international culinary repertoire and qualify it as a functional food.

Materials and methods

Cow milk was purchased from Kerala Veterinary and Animal Sciences University Dairy Plant, Mannuthy and used for buttermilk preparation. Various starter cultures were procured from the Revolving Fund Project at the Department of Dairy

Microbiology, Verghese Kurien Institute of Dairy and Food Technology (VKIDFT), Mannuthy, Thrissur, Kerala. These cultures were screened for their flavour and acid production capabilities. Five starter cultures (Table 1) exhibiting favourable functional properties were chosen for curd preparation. Cow milk was heated to 90°C for 10 minutes, cooled and inoculated with one per cent culture. It was incubated for 12 hours at 37 °C, the curd was cooled and churned to remove butter. The buttermilk samples thus obtained were subjected to sensory evaluation using a 9-point hedonic scale. EFY, plantain (*Nedunenthran* variety), coconut and spices such as pepper, turmeric, fenugreek, cumin and mustard were purchased from the local market. Pepper, turmeric, and fenugreek were washed, dried and milled in a domestic mixer grinder separately. Fenugreek was roasted before milling. Prepared spice powders were stored in air tight containers in the refrigerator.

Consumer preferences and methodology for the preparation of Kaalan employed across the state were gathered through a survey conducted among both caterers and households. Kaalan samples were collected from the market and physico-chemical and sensory analyses were carried out on these samples. The samples were ranked based on their sensory scores using a fuzzy logic technique and identified the top/best five samples, details of which can be found elsewhere (Divya et al. 2023). The levels of ingredients utilized in the preparation of these top/best five samples as obtained from the questionnaire served as a guide to fix the minimum and maximum levels of factors to be considered in the Response Surface Methodology (RSM) experimental design.

Experimental design

The Central Composite Rotatable Design (CCRD) of Response Surface Methodology (RSM) was utilized to optimize the quantity of major ingredients. The factors considered were the quantity of vegetable blend (EFY and Plantain in 1:1), buttermilk and coconut. The statistical analysis, including Analysis of Variance (ANOVA) and multiple regression tests, was conducted using Design Expert® software (version 13.0.13 of Stat-Ease, Inc, 1300, Godward Street Northeast, Minneapolis, USA). The range of the independent factors viz., vegetables, buttermilk, and coconut, was set from 100g to 350g, 500 ml to 1000ml, and 60g to 200g,

Table 1: Starter cultures tested and corresponding sensory scores

Name of starter culture	Accession Number	Flavour score	Overall Acceptability score
<i>Lactococcus lactis ssp. lactis</i>	NCDC-091/ UD 708	7.33±0.17 ^b	7.17±0.16 ^{ab}
<i>Lactobacillus delbrueckii ssp. bulgaricus</i>	NCDC-304/ NCIMB 702395	7.50±0.29 ^b	7.5±0.28 ^c
<i>Lactobacillus rhamnosus</i>	NCBI-MT 491095	6.50±0.29 ^a	6.67±0.17 ^b
<i>Lactobacillus helveticus</i> -DM 053	NCBI-MH 191154	6.10±0.26 ^a	6.00±0.00 ^a
<i>Lactobacillus fermentum</i> - DM 013	NCBI-KY 379153	8.67±0.17 ^c	8.83±0.17 ^d

respectively. The second-order Central Composite Rotatable Design (CCRD) suggested 20 runs as shown in Table 2. Kaalan was prepared as per traditional procedure (Fig. 1) except for the quantity of buttermilk, coconut, and vegetables. These three ingredients were added according to Table 2. The obtained responses were fed into the software, and the sensory levels of the factors were optimized. The data was fitted to a basic model equation as given in the equation (1).

$$Y = \hat{\alpha}_0 + \hat{\alpha}_1 X_1 + \hat{\alpha}_2 X_2 + \hat{\alpha}_3 X_3 + \hat{\alpha}_{11} X_1^2 + \hat{\alpha}_{22} X_2^2 + \hat{\alpha}_{33} X_3^2 + \hat{\alpha}_{12} X_1 X_2 + \hat{\alpha}_{23} X_2 X_3 + \hat{\alpha}_{31} X_3 X_1 + \text{Error term} \quad (1)$$

Where Y is the predicted response, β_0 is the constant coefficient, β_1 , β_2 , and β_3 represent linear coefficients, β_{11} , β_{22} , and β_{33} denote quadratic coefficients, β_{12} , β_{23} and β_{31} show interaction coefficients.

The model adequacy was evaluated by the coefficient of determination (R^2), Adequate Precision Value (APV) and the F value (at a 5% level of significance). APV measures the signal-to-noise ratio. The predicted sensory responses corresponding to the optimum levels of the factors were verified using actual responses in a one-sample t-test of SPSS 24 software.

Analysis of optimized product

Total moisture content was determined by the gravimetric method. Fat in the sample was measured using Mojonnier method (AOAC,

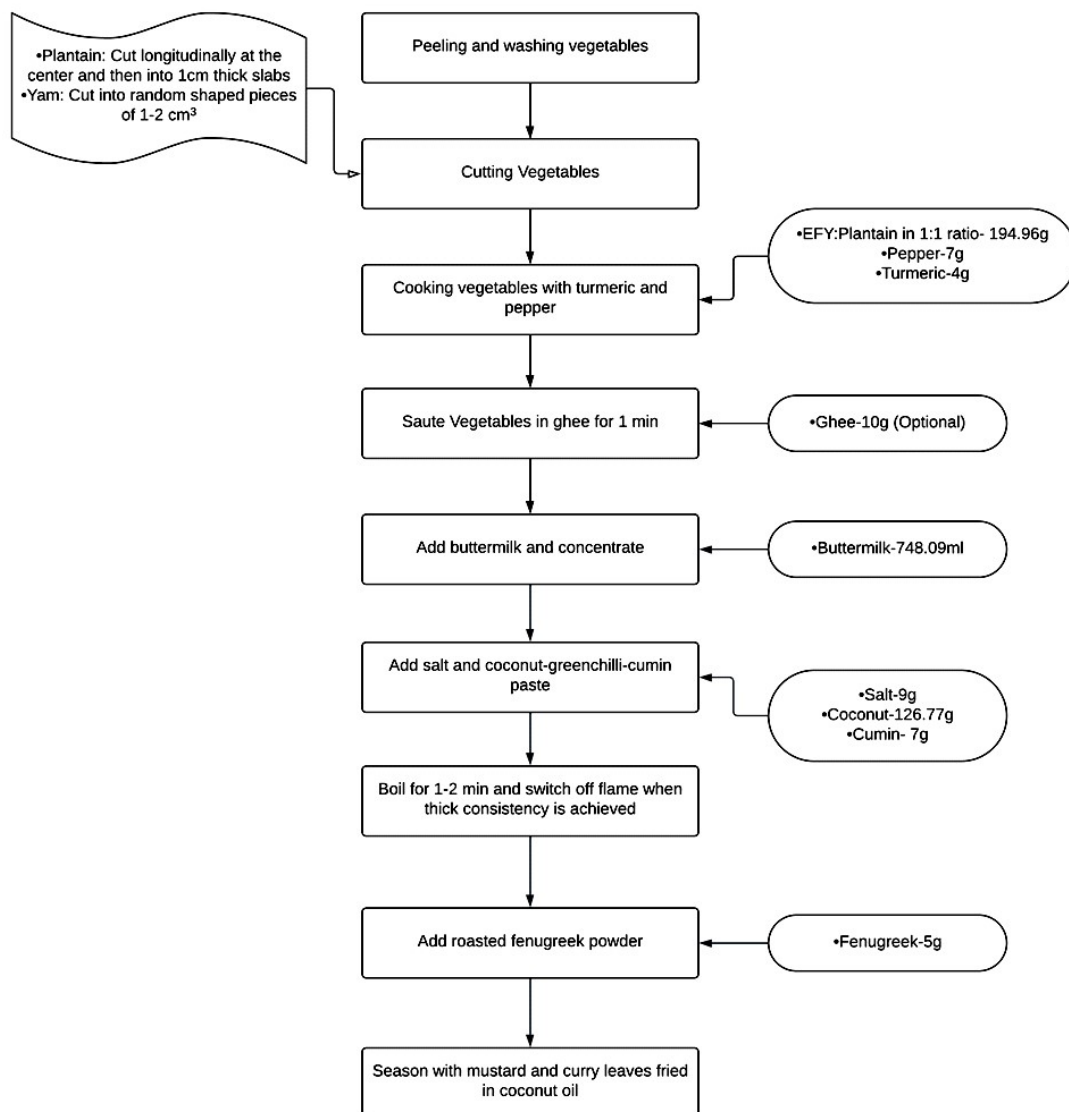


Fig. 1 Flow chart for the preparation of optimized Kaalan

1990) with modifications in sample preparation. The sample was hydrolysed using conc. HCl. Total nitrogen was estimated using Micro Kjeldahl method given in AOAC (2016). Total protein was calculated using a conversion factor of 6.25. The total ash content and total carbohydrate present in the Kaalan samples were also determined (AOAC, 2016). The total dietary fiber content in the product was determined using the 'Total Dietary Fiber Assay Kit' supplied by Sigma Aldrich Inc. (TDF-100A). The analysis uses a combination of enzymatic and gravimetric methods based on AOAC (1997).

The pH of samples was determined immediately after preparation using a portable food pH meter (Model No: HI99161, Hanna Instruments Inc, USA) by immersing the electrode directly into the sample. The titratable acidity was also determined by the titration method recommended by FSSA (2016) and expressed the results as per cent lactic acid. Colour characteristics were measured by reflectance spectroscopy technique employing a reflectance meter, colour flex (Mini Scan EZ 4500 portable

spectrophotometer, Virginia, USA). Data were received from the software in terms of 'L' [Lightness, ranges 0 (black) to 100 (White)], 'a' [Redness, ranges from +60 (red) to -60 (green)], and 'b' [Yellowness, ranges from +60 (yellow) to -60 (blue)] values of the international colour system. The water activity of the samples was measured using AQUALAB 4TE water activity meter (Decagon Devices, USA) which is equipped with chilled mirror dew point sensor. Curcumin content in the samples were also determined (Maurya et al. 2020). A calibration curve, $y = 0.1667x + 0.05.72$ ($R^2 = 0.997$) was prepared using curcumin (Sigma Aldrich, USA) as reference and calculated values were expressed in ppm. The energy content of the samples on a wet weight basis was determined using a conversion rate of 4 kilo calories per gram for protein and carbohydrates and 9 kilo calories per gram for fats.

Sensory evaluation

Table 2: Design matrix showing factors and their responses for optimization of Kaalan

Standard Order	Factors			Responses			
	Factor 1 A: Vegetable blend (g)	Factor 2 B: Buttermilk (ml)	Factor 3 C: Coconut (g)	Response 1: Flavour	Response 2: Colour and Appearance	Response 3: Body and Texture	Response 4: Overall acceptability
1	100	500	60	7	7.07	7.43	7.1
2	350	500	60	7.6	7.64	7.5	7.69
3	100	1000	60	7.81	7.56	7.56	7.56
4	350	1000	60	7.29	7.67	7.43	7.5
5	100	500	200	7.06	7.61	7.56	7.44
6	350	500	200	7.36	7.65	7.43	7.36
7	100	1000	200	7.71	7.57	7.43	7.68
8	350	1000	200	6.61	7.45	7.22	7
9	14.78	750	130	7.78	7.67	7.83	7.63
10	435.22	750	130	7.17	7.83	7.4	7.51
11	225	329.55	130	7.68	7.56	7.67	7.5
12	225	1170.45	130	7.45	7.71	7.57	7.32
13	225	750	12.27	6.94	6.89	7.28	7.06
14	225	750	247.73	6.64	7.07	7.14	6.75
15	225	750	130	8	8.1	8	8
16	225	750	130	7.8	7.9	7.83	7.98
17	225	750	130	8.04	8	8	8.11
18	225	750	130	8	7.9	7.92	7.83
19	225	750	130	8.1	7.94	7.92	7.94
20	225	750	130	8.07	8.07	8	8.21

The faculty members of VKIDFT, located in Mannuthy, Kerala, India, underwent a screening process to assess their physical well-being, concentration abilities, and preferences for fermented/sour dairy products. A total of ten panel members were selected and trained in the specific sensory attributes associated with Kaalan. During the evaluation process, four samples were concurrently presented to the panellists in glass bowls, each identified by a unique three-digit code number. The panellists were provided with scorecards and instructed to assign scores in a 9-point hedonic scale. This scale allowed judges to provide numerical ratings within the range of 1 to 9, for four key quality attributes: flavour, colour and appearance, body and texture, and overall acceptability. The number '1' on the scale represented 'dislike extremely' and the number '9' 'like extremely' (Stone and Sidel, 2004).

Results and Discussion

Selection of starter culture

The cultures of Lactic acid bacteria from the stock of the Department of Dairy Microbiology, VKIDFT were evaluated by looking into skim milk coagulating properties and sensory characteristics. The culture (*Lactobacillus fermentum*) (*Limosilactobacillus fermentum* as per new taxonomy) employed in the preparation of the buttermilk obtained the highest flavour and overall acceptability score and was chosen for product preparation (Table 1). Naghmouchi et al. (2019) conducted a comprehensive review of the biomedical and food preservation attributes of *Lb. fermentum* summarizing it as the cell factories that enhance the effectiveness and nutritional value of functional foods.

Preparation of Kaalan

The principal steps involved in the preparation of Kaalan were cooking EFY and plantain along with turmeric and pepper powder, adding sour buttermilk (*moru*) and concentrating it, adding grated coconut-green chilli-cumin paste followed by final seasoning with fenugreek powder, mustard, and curry leaves fried in coconut oil (Fig. 1). The resultant Kaalan exhibited a creamy texture and a mild, tangy taste with the infusion of coconut and spices.

Effect of different levels of the factors on the sensory attributes of Kaalan

The optimization of the ingredients of Kaalan formulation was done by CCRD of RSM. The factors considered were "vegetable blend", "buttermilk" and "coconut". These three independent factors were selected and set to their levels coded as -1, 0 and +1. The Table 2 illustrates the various treatment combinations for the three independent factors and their sensory responses. The trials are listed in standard order. The aim was to maximize the sensory scores, such as flavour, colour and appearance, body and texture, and overall acceptability. The partial regression coefficients of linear, quadratic and interactive terms for the regression model for each response with their R^2 and Adequate Precision Values (APV) are shown in Table 3. A quadratic regression model was fitted to the experimental data for each response (Table 4). The non-significant lack-of-fit, significant F-test value, R^2 value of more than 0.8, and adequate precision (which indicates signal to noise ratio) of more than four for all the sensory attributes indicate that the model is fit and can give a

Table 3: Regression coefficients of quadratic model and their statistical significance for sensory characteristics of Kaalan

Coefficients	Sensory Responses			
	Flavour	Colour and Appearance	Body and Texture	Overall Acceptability
Intercept	8.00	7.98	7.95	8.01
A-Vegetables	-0.127**	0.064*	-0.082**	-0.0316 ^{ns}
B-Buttermilk	0.001 ^{ns}	0.039 ^{ns}	-0.033 ^{ns}	-0.0112 ^{ns}
C-Coconut	-0.107**	0.047 ^{ns}	-0.038 ^{ns}	-0.0653 ^{ns}
AB	-0.315**	-0.078*	-0.035 ^{ns}	-0.1563**
AC	-0.11*	-0.095*	-0.035 ^{ns}	-0.1613**
BC	-0.075 ^{ns}	-0.095*	-0.050 ^{ns}	-0.0488 ^{ns}
A ²	-0.171**	-0.061*	-0.119**	-0.1214**
B ²	-0.1391**	-0.102**	-0.117**	-0.1780**
C ²	-0.413**	-0.333**	-0.262**	-0.3565**
Model Fit Statistics				
Lack-of-fit	1.84 ^{ns}	1.33 ^{ns}	0.95 ^{ns}	1.26 ^{ns}
Model F-value	28.65**	25.34*	33.33*	15.03*
R ²	0.96	0.96	0.97	0.93
PRESS Value	0.88	0.44	0.207	0.97
APV	16.28	15.53	16.77	11.31

** - Highly significant ($p < 0.01$), * - Significant ($p < 0.05$), ns- non-significant ($p > 0.05$).

good prediction of the parameters under study (Anderson et al. 2017).

A previous study using fuzzy logic by Divya et al. (2024), reported that taste was the primary attribute influencing the acceptability of Kaalan in general, with mouthfeel being the subsequent significant factor. Aroma and colour were ranked lower in terms of their impact on acceptability. A lack of prior research exists pertaining to the sensory attributes of Kaalan or analogous products, thereby impeding comparative analysis. Nevertheless, research studies that employed RSM in fermented dairy foods (Ghosh and Kulkarni, 1991; Manohar, 2005; Manohar and Balasubramanyam, 2007; Modha and Pal, 2011) were referred to for elucidating the influence of ingredients on sensory attributes.

Effect on Flavour

Flavour comprises the taste, aroma, and any discernible physical characteristics of the substance (Bartoshuk et al. 2019). Flavour scores ranged from 6.61 to 8.1 and the maximum score was observed for a combination of central points of experimental design. The coefficient of determination (R^2) was found to be 0.96, with an APV of 16.28. The model F value noted was 28.65 ($p < 0.01$) and with a non-significant lack of fit, an adequate model

was obtained. The 3-D response plots in Fig. 2a- 2c depict the above data. Considering various factors that impact flavour of Kaalan, it was observed that the quantity of vegetables and coconut had a negative and statistically significant influence ($p < 0.01$), while the levels of buttermilk chosen for testing did not exhibit any apparent impact. Moreover, an increase in coconut content was associated with a reduction in the flavour rating. Yaakob et al. (2012) reported that the scent of coconut was generally disliked by most panellists in coconut yogurt, which may be applicable in this context as well. Decrease in flavour score of Kaalan at linear level could be due to the bulkiness formed by higher quantity of the vegetables. The interaction of vegetables with both buttermilk ($p < 0.01$) and coconut ($p < 0.05$) had a significant negative impact on the flavour of the product. However, the interaction effect of buttermilk and coconut was non-significant. From this, it can be concluded that when the quantity of vegetables and coconut are increased, the flavour of Kaalan get negatively affected as it reduces the tangy flavour of buttermilk and masked the zesty flavour of spices. Similar trend was reflected in Kadhi, where the addition of Bengal gram flour substantially diminished the flavour score (Manohar, 2005). Numerous variables associated with aroma significantly impact the overall food quality. Ensuring the stability of flavours is a

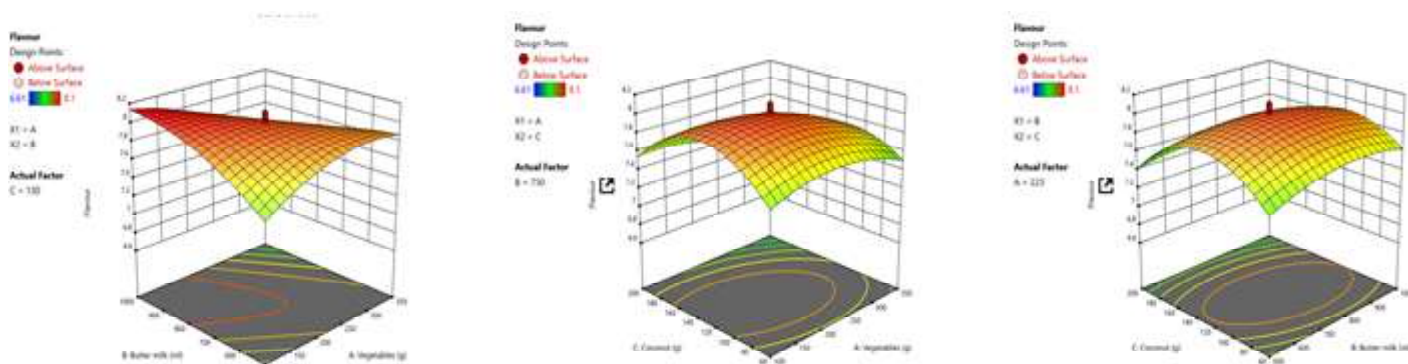


Fig. 2 (a-c) Response surface plot of the effects ingredients on flavour of Kaalan

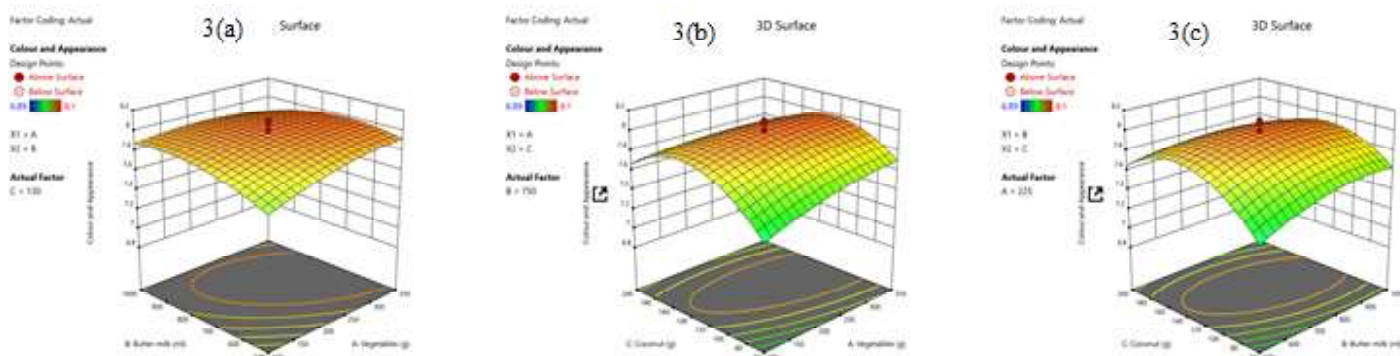


Fig. 3 (a-c) Response surface plot of the effects ingredients on colour and appearance of Kaalan

critical for maintaining the inherent properties of food products (Al Saqqa, 2022). Different steps in food processing, cooking, storage, and packaging, as well as the inherent characteristics of its ingredients, may change the overall flavour profile of food including reductions in intensity or the generation of undesirable flavour compounds. The flavour of fermented milk products is highly affected by the type of starter culture used and the extent of fermentation. In the preparation of optimized Kaalan the milk added with *Lim. fermentum* was incubated till the acidity reached one per cent lactic acid. In a similar study, dahi with one per cent titratable acidity yielded the high flavour score in Kadhi, a traditional fermented milk-based curry in India (Manohar and Balasubramanyam, 2007).

Effect on color and appearance

Judges initially assess colour and appearance as the primary sensory attributes. Colour holds a significant role in marketing of a product. Research has demonstrated that the visual presentation of a meal exerts influence on appetite modulation, ranging from appetite stimulation to suppression, thereby impacting the overall sensory experience and resulting in either pleasure or aversion (Mavrommatis et al. 2011). Consequently, the visual aesthetics of a food or beverage significantly affect its

desirability and acceptance, shaping the anticipatory craving and receptiveness prior to any direct sensory contact. This principle underscores the adage that “we eat first with our eyes”. The colour and appearance scores of the Kaalan ranged between 6.89 and 8.1 (Table 2). The response surface equation to predict the change in colour and appearance with different levels of the vegetable, buttermilk, and coconut is represented in Table 4. The maximum score was observed for standard run 15 which had a vegetable blend, buttermilk, and coconut content of 225, 750, and 130 grams respectively which had a bright mustard yellow colour. The samples with minimum scores were criticized for ‘very dark’ or ‘very light’ yellow colour. The regression coefficients and their significant levels for the model are given in Table 3. With R² value of 0.96, non-significant lack-of-fit and model F-value of 25.34, a significant (p<0.05) model was obtained. Adequate Precision (APV) was 15.53 which was highly desirable. Graphs 3a-3c illustrate the three-dimensional response plots obtained for the response. Among the various factors impacting colour and appearance, only the quantity of vegetables had a significant (p<0.05) influence, while the levels of buttermilk and coconut selected for testing showed no significant impact. However, interaction of different factors had significant negative influence on colour and appearance. Increasing the quantity of vegetables improve the colour and appearance of Kaalan. The

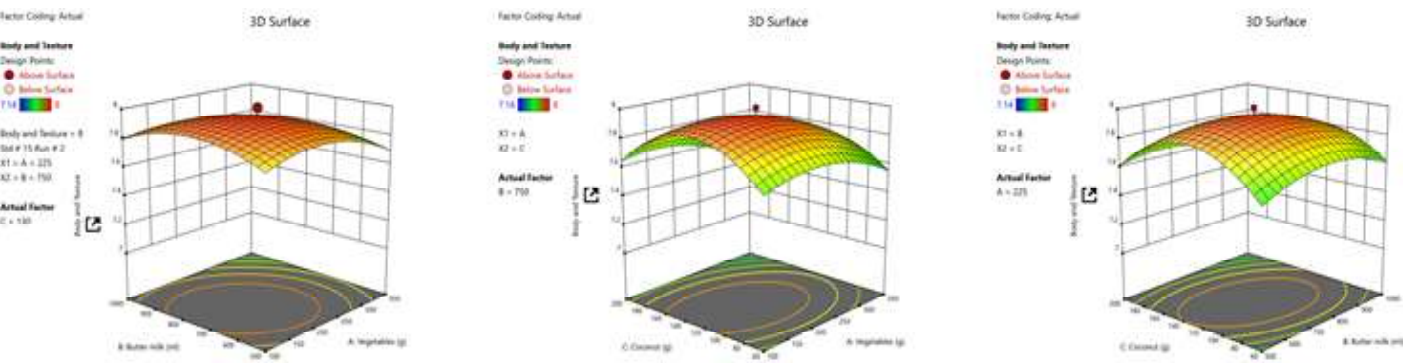


Fig. 4 (a-c) Response surface plot of the effects of ingredients on body and texture of Kaalan

Table 4. Regression equation to predict the changes in the sensory attribute at different levels of the independent factors in CCDR

Attribute	Response surface Equation	R ²
Flavour	8.00 – 0.1278*A + 0.0010*B – 0.1072*C – 0.3150*AB – 0.1100*AC – 0.0750*BC – 0.1709*A ² – 0.1391*B ² – 0.4131*C ²	0.96
Colour and Appearance	7.98 + 0.0636*A + 0.0390*B + 0.0471*C - 0.0775*AB - 0.0950*AC - 0.0950*BC - 0.0610*A ² - 0.1017*B ² - 0.3332*C ²	0.96
Body and Texture	7.95 - 0.0822*A - 0.0328*B - 0.0377*C - 0.0350*AB - 0.0350*AC - 0.0500*BC - 0.1185*A ² - 0.1167*B ² - 0.2617*C ²	0.97
Overall acceptability	8.01 - 0.0316*A - 0.0112*B - 0.0653*C- 0.1563*AB - 0.1613*AC - 0.0488*BC - 0.1214*A ² - 0.1780*B ² - 0.3565*C ²	0.93

A-Vegetable blend, B-Buttermilk, C-Coconut

preferred colour of Kaalan is a bright mustard yellow and the vegetables used also contribute to the yellow colour. It negates the black/grey trace contributed by pepper. However, the white/creamy colour contributed by coconut and buttermilk reduced the brightness of Kaalan. Modha and Pal (2011) also reported that addition of pearl millet flour negatively affected the colour and appearance of the Rabadi-like fermented milk beverage. Our results align with this as the addition of ingredients affected the colour characteristics Kaalan.

Effect on body and texture

The sensory scores for body and texture ranged from 7.14 to 8. With 0.97 R^2 , non-significant lack-of-fit and 33.33 model F-value a significant ($p < 0.05$) model was obtained. APV was 16.77 which is highly desirable. The 3-D response graph and the regression equation are cited in Fig. 4a- 4c and in Table 4 respectively. The texture of Kaalan is defined by its thick body, which consists of firm yet well-cooked vegetable pieces enveloped in a concentrated, finely ground gravy. In sensory evaluation, the body and texture of a dish play a crucial role as they greatly influence the overall perception of quality and palatability, which is a fundamental consideration in assessing food products in general. Looking at the different factors affecting body and texture, only the quantity of vegetables had a highly significant ($p < 0.01$) negative influence whereas the buttermilk and coconut had no influence at the levels selected for testing. Other researchers also reported similar trends. High levels of bengal gram increased the viscosity of kadhi and

reduced the sensory score of the body and texture (Ghosh and Kulkarni, 1991; Manohar, 2005). Our results also align with this as increasing the quantity of vegetable increased the viscosity which had a negative effect on mouthfeel. However, in Rabdi-like fermented milk beverage, addition of pearl millet flour increased the consistency (Modha and Pal, 2011). The interaction of various ingredients seemed not to influence the body and texture of Kaalan.

Effect on overall acceptability

The aim of evaluating overall acceptability is to gauge how well a product meets consumer expectations and preferences. It serves as a valuable indicator of a product's market potential and consumer appeal. The overall acceptability assessment of Kaalan across various ingredient combinations is detailed in Table 2. Examination of model fit parameters revealed a coefficient of determination (R^2) of 0.93 and a model F-value ($p < 0.05$) of 15.03, signifying the model's robustness. Furthermore, a high adequate precision value (11.31) and the absence of a significant lack of fit indicate the model's capability to predict response changes across factor levels. The 3-D graphical representation of the observations can be visualized from Fig. 5a - 5c. It is noteworthy that none of the individual ingredients exert a significant effect on the overall acceptability of Kaalan. However, the interactions between vegetable blend with both buttermilk and coconut exhibited a highly significant, negative influence on the overall acceptability of Kaalan.

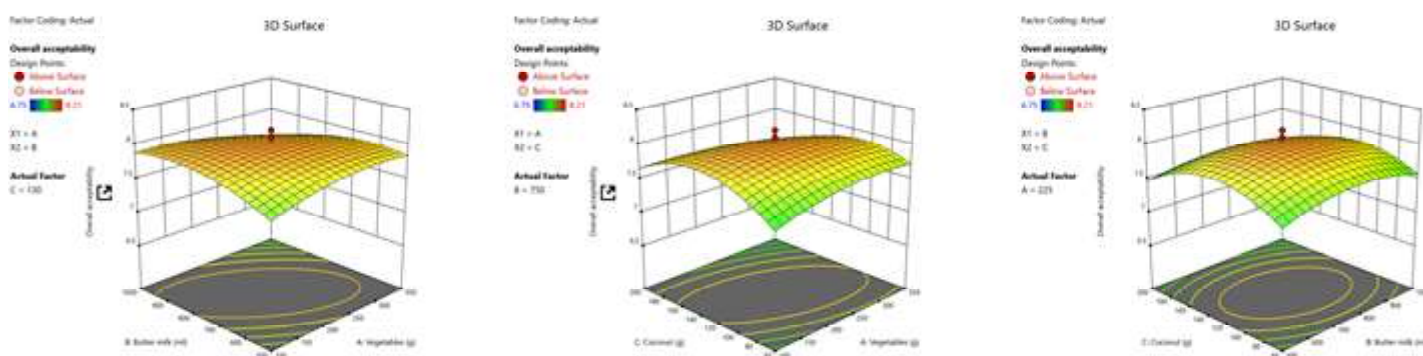


Fig. 5 (a-c) Response surface plot of the effects of ingredients on overall acceptability of Kaalan

Table 5: Constraints and criteria for the optimization of Kaalan

Constraints	Goal	Lower limit	Upper limit
A: Vegetable blend	In range	100	350
B: Buttermilk	In range	500	1000
C: Coconut	In range	60	200
Flavour	Maximize	6.61	8.1
Colour and Appearance	Maximize	6.89	8.1
Body and Texture	Maximize	7.14	8
Overall acceptability	Maximize	6.75	8.21

Optimization of selected variables for Kaalan preparation

Optimization was performed to achieve the optimal combination of vegetable blend, buttermilk and coconut to be used for the preparation of Kaalan. Specific objectives and responses were identified for each factor, and diverse weights were assigned to refine the shape of their individual desirability functions. The sensory analysis scores were targeted for maximization and other factors were maintained within defined ranges throughout the optimization process (Table 5). RSM suggested levels of vegetables, buttermilk, and coconut as 194.97g, 748.09ml and 126.78g respectively. Additionally, the solution demonstrated a significantly high desirability value of 0.91. The optimal formulation, as determined by the software, underwent validation, revealing that the observed values did not exhibit statistically significant differences ($p > 0.05$) in comparison to the predicted values across all assessed attributes. The proposed formulation for the development of Kaalan was prepared and evaluated for sensory attributes. The sensory data obtained were compared with predicted values given by the software. Statistical verification was done using the t-test assuming equal variance and is represented in Table 6. The difference between the predicted scores (RSM) and the observed sensory scores were insignificant. Hence, the predicted level of factors for the optimum Kaalan formulation obtained holds good.

Table 6: Comparison of predicted values and observed values for optimized Kaalan

Attributes	Predicted value	Observed value	t-value
Flavour	7.83±0.08	8.12±0.08	1.981 ^{ns}
Colour and Appearance	7.98±0.06	8.15±0.07	1.614 ^{ns}
Body and Texture	7.75±0.04	8.11±0.12	2.411 ^{ns}
Overall Acceptability	7.9±0.09	8.17±0.05	2.364 ^{ns}

All observations are mean values of four replications with SE,
ns- non-significant ($p \geq 0.05$)

Table 7: Comparison of physico chemical properties of optimized Kaalan with market samples of Kaalan (on wet basis)

Attributes	Optimized Kaalan	Market samples of Kaalan	t-value
Protein (per cent)	3.49±0.005	6.34±0.59	4.856*
Fat (per cent)	7.12±0.07	9.31±1.42	1.538 ^{ns}
Carbohydrate (per cent)	9.95±0.08	11.28±0.75 [#]	ND
Dietary Fiber (per cent)	4.84±0.22	ND	ND
Ash (per cent)	2.17±0.03	2.41±0.09	2.514 ^{ns}
Total solids (per cent)	28.36±0.04	29.36±0.10	1.001 ^{ns}
Energy value (kcal/100g)	117.77±0.34	154.32±10.84	3.371*
pH	4.23±0.08	4.32±0.11	0.711 ^{ns}
Acidity (per cent lactic acid)	0.77±0.05	0.84±0.008	1.245 ^{ns}
Curcumin (ppm)	13.63±0.03	2.54±0.37	29.751**
L	60.67±0.19	61.95±0.14	5.374*
a	-0.19±0.09	2.88±1.66	1.839 ^{ns}
b	53.85±0.27	45.89±1.67	4.703*
Water activity	0.99±0.001	0.99±0.001	ND

Figures are mean ± standard error, *significantly different at $p < 0.05$, ** significantly different at $p < 0.01$, ND=not determined, [#] Carbohydrate + dietary fiber

content rather than full fat curd/yoghurt. It may also be due the use of high quantity of vegetables compared to that of most of the market samples. The curcumin content in the optimized Kaalan was significantly higher ($p < 0.01$) compared to market samples. This distinction could be attributed to the incorporation of freshly powdered turmeric in the preparation of optimized Kaalan. Yewle et al. (2021) reported that prolonged storage of turmeric can lead to a reduction in curcumin content. The lower values in the market samples may be due to the use of packaged turmeric powders available in the market, which often undergo extended storage periods. The discrepancy may also be influenced by the moderated usage of turmeric powder in the preparation of market samples. In the examination of colour characteristics, it becomes evident that the 'L' value was significantly higher for market Kaalan whereas the 'b' value was significantly higher for optimized Kaalan ($p < 0.05$). Herein, the 'L' value is indicative of lightness, and the 'b' value denotes yellowness. The optimized Kaalan had high 'b' value and high curcumin content. The 'b' value is positively correlated ($r = 0.943$) to the curcumin content in Kaalan. Furthermore, the presence of a negative 'a' value suggests a subtle greenish undertone in the optimized Kaalan, whereas a positive 'a' value in the market sample signifies redness, likely arising from the inclusion of red chilli powder in its preparation (Divya et al. 2023).

Conclusion

Kaalan holds cultural and gastronomic significance, symbolizing the region's culinary heritage and fostering a sense of communal harmony during festive gatherings. Its preparation and consumption reflect the traditional culinary wisdom and cultural practices that have been passed down through generations in Kerala. The production of Kaalan was optimized using response surface methodology. The optimized Kaalan had 3.49 per cent protein, 7.12 per cent fat, 9.95 per cent carbohydrate, 4.84 per cent dietary fibre and 13.63 ppm curcumin. It exhibited 31 per cent reduction in energy value when compared to the Market sample. It had a high dietary fiber content due to the use of elevated levels of vegetables. This demonstrates that Kaalan is a functional food with low calories and lots of dietary fiber. The optimization and characterisation of Kaalan could lead to its large-scale industrial production and widespread acceptance. It is imperative to conduct both *in vitro* and *in vivo* digestion studies, to ascertain the bio-functionality of Kaalan,

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Studies on suitability to incorporate *Piper betel* leave extract in flavored milk

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Abstract: The present study was planned and executed to evaluate suitability to incorporate *Piper betel* leaves (PBL) in flavor milk and to investigate the antioxidant and phenolic potential of -fortified flavored milk (FM) during its storage. The FM was prepared by fortifying it with aqueous extract of PBL, sugar and carboxy methyl cellulose (CMC) stabilizer @ 5.15, 10.30, and 0.1%, respectively followed by its pasteurization (FM₁). The bottles were stored at 5±1°C and compared with control (FM₂) i.e. milk without PBL extract. Sensory evaluation was conducted at an interval of 4 days along with proximate composition, physicochemical properties, antioxidant and phenolic potential. It was observed that on 20th day of storage the flavor score for sample FM₂ was <6.00 and that for the sample FM₁ >7.00. The storage period, treatment, and their interaction effect was significantly different (P<0.05) on various quality attributes under study. The viscosity of the FM during storage was altered from 9.62±0.17 to 12.04±0.04 and from 10.85±0.70 to 12.42±0.90 cP in FM₁ and FM₂ sample(s), respectively. The change in antioxidant and total phenolic content were also decreased during storage, the values on 0 day of storage for FM₁ was 5.56±0.53 µM/mL and 5.14±0.32 GAE/mL. A significant increase was observed in microbial counts from 2.61±0.06 to 5.00±0.04 log₁₀ cfu/ml during storage for 24 days in FM₁ as compared to the control sample

from 2.61±0.03 to 4.75±0.03 log₁₀ cfu/ml. No any coliforms observed in any of the samples during the storage period of 24 days.

Keywords: Antioxidant and phenolic potential; Flavored milk; *Piper betel* leaves; sensory qualities; Storage stability,

Introduction

A trend has been predominantly observed that the children prefer the flavored milk (FM) as compared to the milk, thereby fulfilling the recommended intakes of dairy products. A recent market survey (Theresa et al. 2022) also substantiated these observations. The FM is a sweetened dairy product made from milk, sugar, colorings, and synthetic or natural flavorings. The FM has a remarkable growth @ 27% per annum in the Indian market (Baisya 2005). In India, the flavoured milk market is dominated by several brands (Ravindra et al. 2014). Bisig (2011) defined FM as the ready-to-drink products made from milk of varying fat contents fortified with sweetener, cocoa powder, fruit juice, coffee, aromatics, additives and flavors. Dairy industries developing innovative milk-based beverages and products to meet consumer demand by addressing health concerns through natural ingredients (Keshtkaran et al. 2013).

Natural colors and flavors are the food additives intend to make products more appealing with improved taste. The FM is routinely flavored and colored with synthetic ingredients, may lead to hyperactivity and behavioral problems in children (Weiss, 2012). Therefore the global thrust gaining momentum in the food and beverage industry towards the natural additives owing to their growing demand. Natural flavors and colors are preferable through clean label declarations and these could be easily mixed in milk (Kamble et al. 2019). Various plant-based materials like bark, leaves, seeds, flowers, etc. could be utilized in FM.

The deep green heart-shaped leaves of *Piper betel* leaves (PBL) are popularly known as *Paan* in India. It is also known as *Nagaballi*, *Nagurvel*, *Saptaseera*, *Sompatra*, *Tamalapaku*, *Tambul*, *Tambuli*, *Vaksha patra*, *Vettilai*, *Voojangalata*, etc. in different parts of the country. It's chlorophyll is beneficial in maintaining healthy teeth, refreshes the mouth and throat. It also helps in digestion by enhanced salivation and neutralizing excess

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acid. It is also used as a mouth freshener after the meals (Kumar, 1999). It is rich in essential oils (0.7-2.6 %), carbohydrate (0.5-6.1%), fat (0.4-1.0%) protein (3.0-3.5), fiber (2.3%), minerals (2.3-3.3 %), with good source of water and oil soluble vitamins (Guha, 2006 and Ramamurthi and Usha Rani, 2012). Further, the PBL is a rich source of vitamins and minerals (Kruawan and Kangsadalampai, 2006). It is reported that PBL contained a significant amount of antioxidants like hydroxyl chavicol, eugenol, ascorbic acid, and beta carotene (Chakraborty and Shah, 2011). It also contains phenolic compounds, which are bioactive substances that act mostly as radical scavengers (Hodzic et al. 2009) and some act as metal chelators. The presence of an antioxidant is one of the fastest ways to reduce lipid peroxidation to maintain overall product quality (Abdullahi, 2011).

Pasteurized FM gets spoiled at refrigerated temperatures due to the activities of psychotropic bacteria, particularly *Pseudomonas* spp. (Datta and Wallace 2002). The average shelf life of stored FM is approximately 7 days at refrigerated temperature (Khusniati et al. 2008). It is attributed to the extracellular enzymes of the putrefactive organisms (Datta and Wallace 2002). By incorporating the PBL food matrix, it is expected that it can increase the biochemical properties as well storage stability of flavored milk by suppressing the psychotropics. In view of this the present investigation was planned and executed.

Materials and methods

Preparation of ethanolic extract

The ethanolic extract of PBL of Culcatta cultivar was prepared as per the method suggested by Chakarabarty and Shah (2011) with some modifications. About 1 g of PBL was mixed in 10 ml ethyl acetate followed by crushing for a minute and transferred in a centrifuge tube (10 ml capacity). The tube was placed in a dark place for 2 h followed by its centrifugation at 22000 rpm for 5 min. The supernatant was taken (as a sample) for estimation of antioxidant properties.

Preparation of methanolic extract

The methanolic extract of PBL was prepared as above by replacing ethyl acetate with methanol as solvent. The extraction was carried out for 3 h and used for antioxidants estimation.

Preparation of aqueous extract

The aqueous extract of PBL was prepared by mixing 1 g of PBL in 10 ml distilled water followed by crushing in mortar and pastel for a min. The solution was filtered through a muslin cloth. The filtrate was transferred in a centrifuge tube and it was placed in a dark place for 2 h and centrifuged at 22,000 rpm for 10 min. The supernatant was collected (as a sample) for the estimation of antioxidant properties.

Preparation of PBL extract for milk enrichment

Fresh PBL were washed under running tap water and a 10 g leaf sample was crushed by mixing 100 ml of distilled water for 2-3 min. Then leaves extract was allowed to filter through four folded muslin cloths. The filtered extract (10 %) was termed as PBL Extract and was used for flavoring and coloring the milk.

Preparation of flavored milk using (PBLE)

The BLE added FM was prepared as per protocol developed by Kamble et al. (2019) as shown in **Fig. 1**. The product was fill in sterilized glass bottles (200 ml) and sealed by crown cork. These sealed milk bottles were pasteurized at 68°C for 30 min and stored at 5±1°C (FM₁) and compared with control (FM₂) i.e. milk without added PBLE. In both the samples sugar and stabilizer were mixed @ 10 and 0.1%, respectively. Each sample of FM was evaluated at an interval of 4 days for sensory, physico-chemical, and microbial qualities along with change in antioxidant and phenolic properties.

Estimation of antioxidant and phenolic properties PBL and of FM

Total antioxidant activity was measured by FRAP assay as per Benzie and Strain (1999). Total phenolic content expressed as Oxygen Radical Absorbance Capacity (ORAC) of flavored milk was analyzed by the Folin-Ciocalteu method (Kahkonen et al. 1999).

Physico-chemical analysis of FM

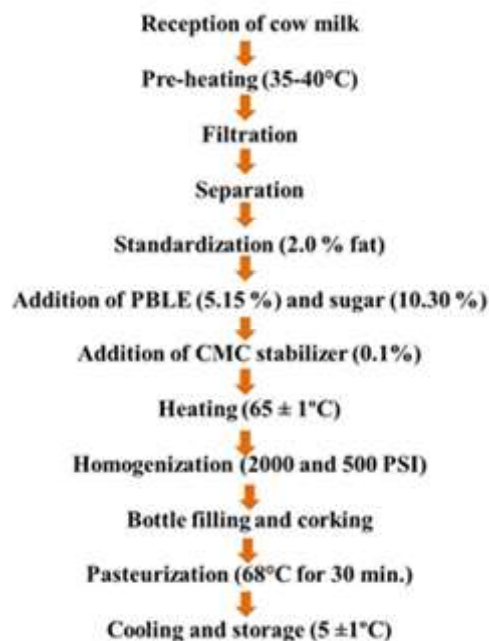


Fig. 1 Flow diagram for preparation of flavored milk added with PBLE (Piper betel leaves extract)

The proximate analysis, as well as pH, acidity, specific gravity, and viscosity of both the milk, were analyzed as per the method of AOAC (2005). The method described by Keeney and Basette (1959) was used for the measurement of HMF (Hydroxy methyl furfural) value of milk during storage. The TBA value of milk measured as per the method developed by King (1962) with suitable modifications.

Sensory evaluation

Sensory evaluation of PBLE FM was carried out by a semi-trained panel of judges from the Institute using a 9-point Hedonic scale as described by Amerine et al. (1965). Samples were served in the coded number flasks.

Microbial quality analysis

The samples were evaluated for commercial sterility. They were evaluated for total viable counts, Coliform, Yeast and Mould counts by the method described by FSSAI (2016).

Statistical analysis

The data obtained were analyzed statistically in SPSS software (Version 20.0) as per the standard

Results and Discussion

Antioxidants and phenolic properties of *Piper betel* leaves

The methanolic extract of PBL was found to possess a higher concentration of antioxidants ($41.22 \pm 0.87 \mu\text{g/ml}$) than ethanolic ($34.08 \pm 15.9 \mu\text{g/ml}$) and aqueous extract ($14.27 \pm 2.77 \mu\text{g/ml}$). These findings are in close conformity with those reported earlier (Dasgupta and De, 2004 and Chakarabarty and Shah, 2011). Along with PBL, the antioxidant properties of milk were also determined and it had low antioxidant activity compared to PBL (Fig. 2a and 2b).

The phenolic contents in terms of gallic acid equivalent (GAE)/ml and the ethanolic extract of PBL showed the highest 41.22 ± 0.87 phenolic activity whereas in methanolic and aqueous extract it was 12.82 ± 0.06 and 13.49 ± 0.41 GAE/ml, respectively. As expected the milk sample had lower phenolic content as was expected. Chakarabarty and Shah (2011) monitored the phenols of PBL and reported that the methanolic extract possesses a high concentration of phenolic and flavonoids in moderate concentration and tannins in limited concentrations.

Changes in sensory qualities of FM During Storage

Changes in color and appearance

The color and appearance are most important for the consumers. As it create the first impression towards dairy products. It is clear from Fig. 3a that the color and appearance score was decreased significantly ($p < 0.05$) from 8.34 ± 0.10 to 7.06 ± 0.48 in milk added with PBLE (FM₁) whereas, the score of control samples (FM₀) had changed from 8.01 ± 0.16 to 7.38 ± 0.11 . It was observed that the storage period, the addition of PBLE, and their interaction exerted a significant effect ($P < 0.05$) on the color and appearance score during storage. The judges reported that as the storage period progressed the color of the FM₁ sample was significantly decreased from the 16th day onwards. It might be because of progress in the Maillard reaction and subsequent increase in HMF (Hydroxy methyl furfural) level as earlier reported by Singh and Patil (1989).

Change in consistency score

Consistency is an important parameter in evaluating fluid products like the FM. The length of the storage period, type of sample, and its interaction with treatment showed a significant effect on consistency score (Fig. 3a). The lower consistency score of FM₁ sample may be because of the addition of PBLE, as was supported by Kumar et al. (2017) and Rejesh et al. (2017).

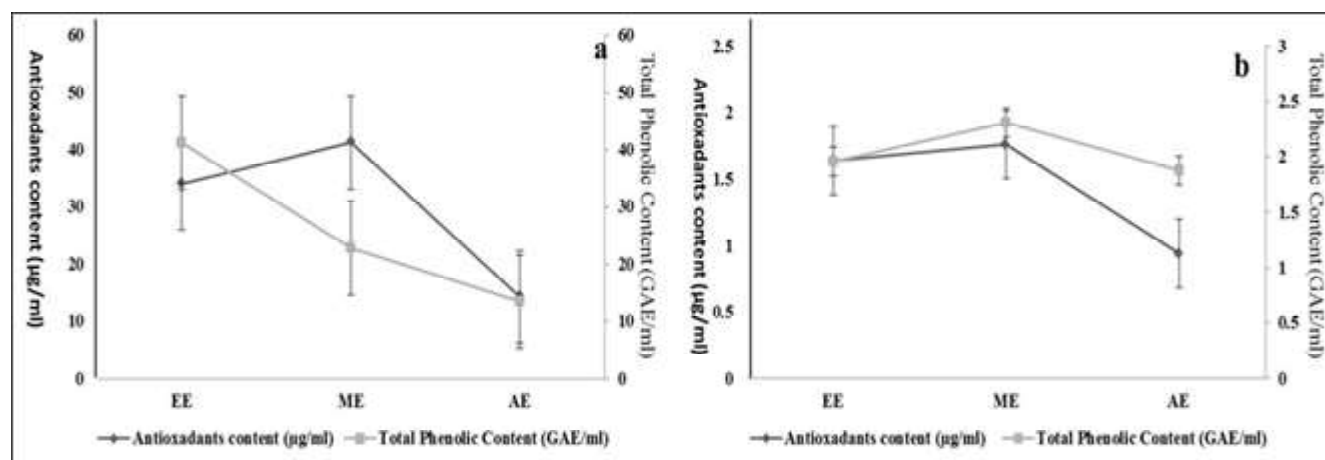


Fig.2: Antioxidants ($\mu\text{g/ml}$) and Total phenolic content (GAE/ml sample) of (a) *Piper betel* leaves and (b) milk (EE-Ehtanolic extract, ME-Methanolic extract, and AE- Aqueous extract)

The initial consistency score was comparatively lower than the middle period of storage (score 8.06 on 12th days of storage). It may be because of age gelation in the product or aggregation of protein particles. However at the end of the storage period in both the samples the score was significantly lowered.

Effect on sweetness score

The sweetness score for the FM₁ sample was decreased from 8.22 ± 0.10 to 7.04 ± 0.46 during the entire storage period whereas, for the control sample it was decreased from 8.30 ± 0.04 to 7.60 ± 0.53 (Fig. 3a). The storage period had a significant ($P < 0.05$) effect on sweetness score ($P > 0.05$). Tamilarasi (2001) reported that inverse relationship between sweetness score and storage period might be because of bacterial decomposition of the products.

Changes in flavour score

The mean flavour score of the treated (FM₁) sample, as depicted in Fig. 3b, indicates that the storage period and treatment have significant effect ($P < 0.05$). The sample without PBLE fetched scores less than 6, while those with PBLE recorded a score of 7 in the Hedonic scale. It was observed that there was development of a slight staleness at the end of storage period. The flavor score of FM₁ was not changed significantly up to 16th day of storage, thereafter it was significantly ($p < 0.05$) decreased. Our findings are in agreement with a study by Ravindra et al. (2014) who reported that the flavored milk showed decrease in flavor score with the increase in storage period.

Changes in overall acceptability

It could be seen from the data presented in Fig. 3b that the maximum overall acceptance score was 8.36 ± 0.01 and 8.08 ± 0.07 for FM₁ and FM₀ sample on 0 day and 6.17 ± 0.23 and 6.00 ± 0.12 on 24 and 20th day of storage for respective sample. Hassan et al. (2015) recorded the decreasing trend in overall acceptability score during refrigerated storage. Perez and Sanz (2001) described the

slight changes in flavor, taste and overall acceptability of FM. It was stated that it may be due to the degradation of ascorbic acid and furfural during storage.

Kumar et al. (2017) found that storage period did not affect significantly ($P < 0.05$) the quality characteristics and sensory scores till 15 days of storage during which the control sample recorded overall acceptability score 5.45 ± 0.12 on 20th days, which was less than '6' on '9' point i.e. neither like nor disliked hence it was discontinued from the study at this point. Similar action was taken on FM₁ sample on 24th days of storage.

Changes in physico-chemical properties of flavored milk during storage

Changes in specific gravity

It could be seen from Table 1 that the specific gravity of sample F1 was decreased. The rate of decrease was higher in control sample than that of the treated one. It was found that the effect of storage period and treatment was significant during the storage. Palthur et al. (2014a) also reported the change in specific gravity of the ginger extract added FM as compared to its normal variant. Anandh et al. (2014) observed that the specific gravity of the rose flavored milk (1.035-1.037) showed a slight rising tendency over the control during storage.

Changes in viscosity

It could be seen from the data on viscosity presented in Table 1 that it was increased from 9.62 ± 0.17 to 12.04 ± 0.04 P and from 10.85 ± 0.70 to 12.42 ± 0.90 P in FM₁ and FM₀ samples, respectively. The rate of increase in viscosity was higher in control samples than that of PBL flavored sample. Effect of storage period, treatment and their interactions were significantly ($P < 0.05$) affected the viscosity of the product. It is attributed to proteolysis, aggregation enzymatic action and interaction of milk fat and protein. Our findings are supported by Dey and Karin (2013).

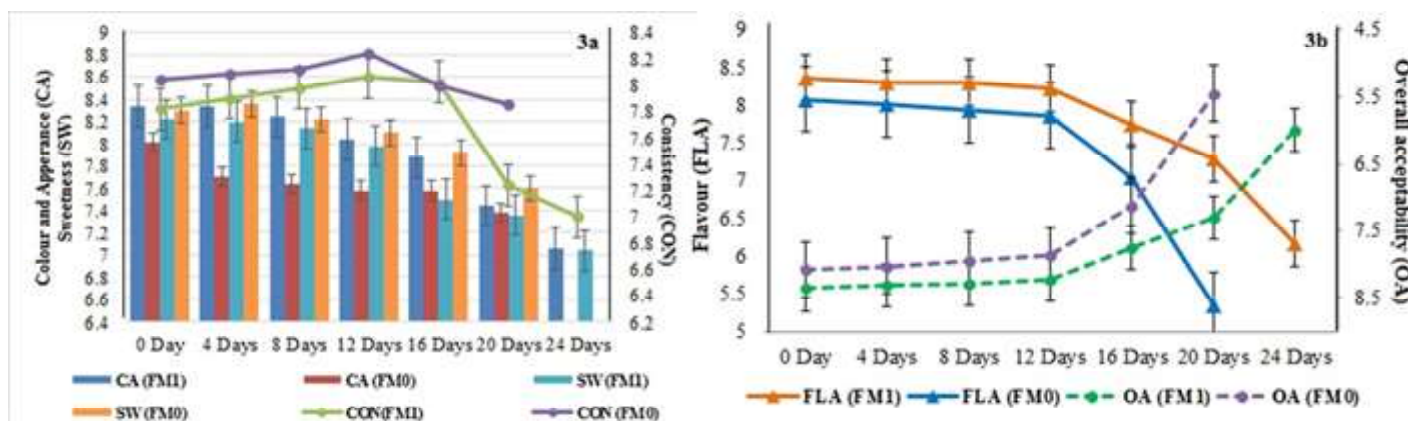


Fig. 3: Changes in sensory attributes (score) of flavored milk during storage at 5±1°C

(CA-Color and Appearance, SW- Sweetness, CON- Consistency, FLA- Flavour, and OA- Overall acceptability)

Table 1: Changes in proximate composition of flavored milk during storage at 5±1°C

Parameter (Score)	Sample	Storage period (Day)						
		0	4	8	12	16	20	24
Protein *(%)	FM ₁	3.52 ±0.19	3.50 ±0.27	3.44 ±0.27	3.42 ±0.20	3.40 ±0.24	3.36 ±0.16	3.30 ±0.09
	FM ₀	3.54 ±0.29	3.52±0.18	3.50 ±0.15	3.46 ±0.21	3.40 ±0.19	3.30 ±0.12	--
Fat *(%)	CD (P<0.05)	Storage period NS		Treatment NS		Storage period x Treatment NS		
	FM ₁	1.68 ±0.02	1.68 ±0.04	1.66 ±0.02	1.65 ±0.02	1.63 ±0.03	1.60 ±0.03	1.50 ±0.02
Lactose *(%)	FM ₀	1.72 ±0.05	1.69 ±0.02	1.60 ±0.02	1.58 ±0.01	1.56 ±0.03	1.53 ±0.02	--
	CD (P<0.05)	Storage period 0.32		Treatment 0.17		Storage period x Treatment 0.46		
Sucrose *(%)	FM ₁	4.60 ±0.09	4.60 ±0.16	4.40 ±0.22	4.22 ±0.15	4.00 ±0.05	3.92 ±0.07	3.88 ±0.19
	FM ₀	4.64 ±0.17	4.62 ±0.24	4.42 ±0.21	4.24 ±0.19	4.14 ±0.10	3.98 ±0.18	--
Ash *(%)	CD (P<0.05)	Storage period NS		Treatment NS		Storage period x Treatment NS		
	FM ₁	10.74 ±0.05	10.74 ±0.17	10.72 ±0.15	10.69 ±0.14	10.69 ±0.25	10.68 ±0.16	10.65 ±0.16
Total solids *(%)	FM ₀	10.80 ±0.06	10.80 ±0.10	10.79 ±0.24	10.80 ±0.46	10.78 ±0.25	10.72 ±0.16	--
	CD (P<0.05)	Storage period 0.18		Treatment 0.09		Storage period x Treatment 0.25		
Specific gravity	FM ₁	21.67 ±0.05	21.61 ±0.07	21.34 ±0.03	21.06 ±0.25	20.77 ±0.54	20.57 ±0.52	20.20 ±0.69
	FM ₀	21.64 ±0.29	21.58 ±0.18	21.25 ±0.18	20.93 ±0.06	20.74 ±0.39	20.31 ±0.50	--
Viscosity *(cP)	CD (P<0.05)	Storage period 0.69		Treatment 0.37		Storage period x Treatment 0.97		
	Sample (FM ₁)	1.064 ±0.012	1.059 ±0.042	1.054 ±0.039	1.052 ±0.028	1.050 ±0.019	1.050 ±0.059	1.050 ±0.059
	Control (FM ₀)	1.071 ±0.031	1.068 ±0.044	1.068 ±0.035	1.057 ±0.022	1.054 ±0.016	1.052 ±0.033	--
	CD (P<0.05)	Storage period NS		Treatment NS		Storage period x Treatment NS		
	Sample (FM ₁)	9.62 ±0.17	10.80 ±0.49	11.00 ±0.32	11.50 ±0.55	11.88 ±0.56	11.92 ±0.29	12.04 ±0.04
	Control (FM ₀)	10.85 ±0.70	11.00 ±0.55	11.40 ±0.40	11.80 ±0.58	12.06 ±0.92	12.42 ±0.90	--
	CD (P<0.05)	Storage period 1.15		Treatment 0.61		Storage period x Treatment 1.63		

pH	Sample	6.30	6.30	6.16	6.11	6.01	6.00	5.98
	(FM ₁)	±0.04	±0.05	±0.05	±0.11	±0.12	±0.17	±0.17
	Control	6.32	6.30	6.10	6.00	5.95	5.70	--
	(FM ₀)	±0.07	±0.07	±0.12	±0.12	±0.17	±0.21	
	CD	Storage period		Treatment		Storage period x Treatment		
	(P<0.05)	0.27		0.14		0.38		
Acidity	Sample	0.157	0.159	0.165	0.168	0.179	0.186	0.193
*(% LA)	(FM ₁)	±0.004	±0.004	±0.007	±0.006	±0.005	±0.003	±0.003
	Control	0.154	0.159	0.166	0.172	0.180	0.189	
	(FM ₀)	±0.005	±0.005	±0.003	±0.005	±0.003	±0.003	--
	CD	Storage period		Treatment		Storage period x Treatment		
	(P<0.05)	0.009		0.004		0.012		

CD: Critical difference NS: Non-significant *: Significant difference

Table2. Changes in Antioxidant*($\mu\text{M/mL}$) and Phenolic content *(GAE/ml) of flavoured milk during storage at $5\pm 1^\circ\text{C}$

Parameter	Treatment	Storage period (Days)						
		0	4	8	12	16	20	24
Antioxidant* ($\mu\text{M/mL}$)	Sample	5.56	5.49	5.42	5.39	5.33	5.22	5.18
	(FM ₁)	±0.53	±0.59	±0.44	±0.29	±0.25	±0.30	±0.89
	Control	4.15	4.06	4.07	4.00	4.00	3.91	--
	(FM ₂)	±0.22	±0.28	±0.04	±0.48	±0.55	±0.44	--
	CD P<0.05)	Storage period		Treatment		Storage period x treatment		
		0.49		0.46		0.63		
Phenolics* (GAE/ml)	Sample	5.14±	5.08±	5.02±	5.00±	4.98±	4.90±	4.88±
	(FM ₁)	0.32	0.35	0.22	0.56	0.14	0.23	0.24
	Control	4.34±	4.29±	0.98±	4.00±	3.96±	3.89±	--
	(FM ₂)	0.39	0.19	1.79	0.34	0.04	0.58	--
	CD P<0.05)	Storage period		Treatment		Storage period x Treatment		
		0.65		0.66		0.77		

The possible increase in viscosity of PBLE added milk may be related to interaction of herb component with milk constituents and its destabilizing effect.

Changes in pH

The average pH value was found higher in control sample than that fortified with PBLE. A highly significant ($p < 0.05$) decrease in pH value was noticed from 16th and 8th days of storage period in flavored and control sample, respectively (Table 1). Average pH value was found lower and statistically different in PBLE added flavored milk i.e., 6.17 than control. Gupta et al. (2017) and Sawale et al. (2017) observed decreasing pH of functional dairy drinks during storage. Wegrzyn et al. (2008) found that the pH of pasteurized apple flavor milk decreased with storage period of 12th week. These workers also reported a drop in pH of herb-fortified flavor milk.

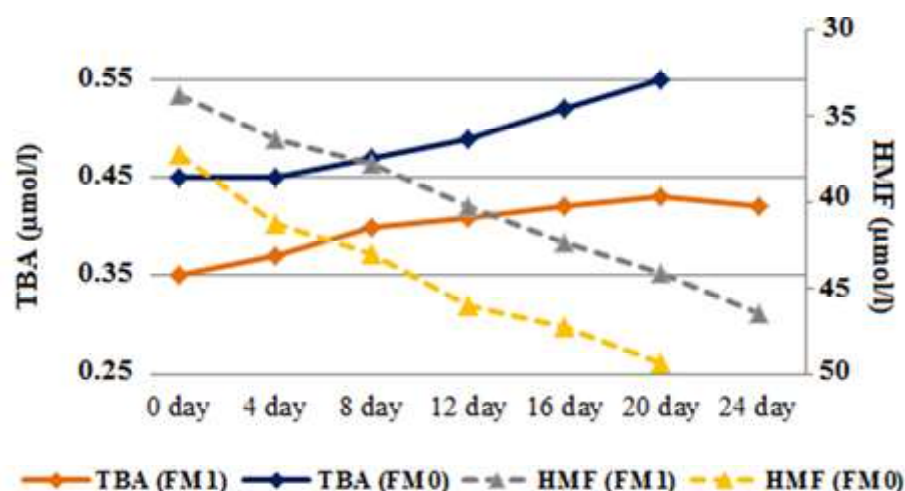
Changes in proximate composition of milk during storage

The protein content of treated sample (FM₁) was significantly decreased as compared to the control sample (FM₀). The changes in protein content were not significant during storage period

(Table 1). These findings are in close conformity with the report of Hassan et al. (2015). These workers observed that during refrigerated storage the mean protein content of beverages showed slight decrease in protein content, but the difference was nonsignificant ($p > 0.05$). The fat content of treated sample 0, 4, 8, 12, 16, 20 and 24 days were 1.68 ± 0.02 , 1.68 ± 0.04 , 1.66 ± 0.02 , 1.65 ± 0.02 , 1.63 ± 0.03 , 1.60 ± 0.03 and $1.50\pm 0.02\%$, respectively. The lactose content of treated (FM₁) milk sample was $4.60\pm 0.09\%$ on the 0 day storage and in control milk (FM₀) sample lactose content was 4.64 ± 0.17 . Slight decline in lactose content during storage might be due to utilization of lactose by bacteria and its conversion into lactic acid, such type of effect of slightly observed in milk treated with PBLE. Similar results were reported by Shukla et al. (2018). The sucrose content of the treated and control sample milk were ranges from 10.74 to 10.80%, respectively.

Sucrose content of milk sample was decreased with increased storage period. It was observed that the ash content of the sample was slightly increased in treated sample (FM₁) as compared to control (FM₀). The initial average values of TS of treated samples and control samples are shown in Table 1 which shows that the TS of the control sample is slightly higher than the treated sample due low moisture content. Ammra et al. (2009) reported a non-

Fig.4 Changes in TBA*($\mu\text{mol/l}$), HMF*($\mu\text{mol/l}$), Antioxidant*($\mu\text{M/mL}$), Phenolics* (GAE/ml) milk during storage at $5\pm 1^\circ\text{C}$



significant decline in TS content of UHT milk during the storage period. Singh et al. (2014) also observed same trends.

Effect on chemical parameters

Lipid oxidation

The extent of lipid oxidation during storage was measured in terms of thio-barbituric acid (TBA) value. The changes in TBA value (in terms of absorbance) of samples stored at refrigeration are presented in Fig. 4. In all samples, a steady and significant increase in TBA value was observed during storage. The effect of storage period, treatment and interaction showed significant changes in TBA values. Results are in well agreement with earlier report (Bandyopadhyay et al. 2007) who incorporated herbs such as turmeric (*Curcuma longa* L.), coriander (*Coriandrum sativum* L.), curry leaf (*Murraya koenigii* L.), spinach (*Spinacia oleracea*) and Aonla (*Emblica officinalis*) at 10% levels in Sandesh. In the present study, a decreased rate of oxidation in case of herb supplemented samples might be related to antioxidant compounds of herbs which inhibited the lipid oxidation. Dasgupta and De (2004) reported similar results.

Effect on HMF

It was observed that the HMF content of the products increased significantly ($p < 0.05$) during storage (Fig.4) in treated and control samples. The interaction between treatment and days of storage was found to be significant ($P < 0.05$) in case of HMF. Similar findings are reported by Richards et al. (2016) and Shukla et al. (2018) in case of storage of UHT milk and flavored dairy drinks.

Changes in antioxidant and phenolic properties during storage

Changes in antioxidant activity

The antioxidant activity was 5.56 ± 0.53 and $4.15 \pm 0.22 \mu\text{M/mL}$ in FM_1 and FM_0 , respectively on 0 day of storage (Table 2). During

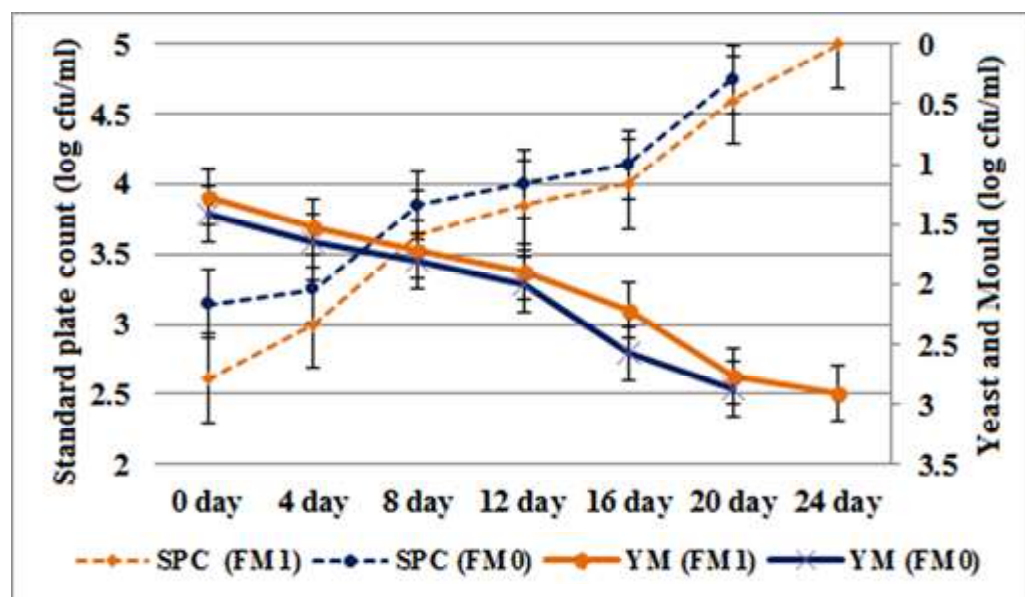
storage period their rate of ferric reducing antioxidant power of both the milk was decreased. The storage period, treatment and their interaction had exerted a significant ($P < 0.05$) effect on antioxidant content of the product. The herb PBLE contains bioactive compounds such as Chavicol, Chavibetol, Chavibetol acetate, and Eugenol, etc. (Swapna et al. 2012) which could be responsible for the higher radical scavenging activity of PBLE added sample as compared to their controls. In the present study, higher antioxidant activity could be due to the interaction of herb or milk constituents with oxygen or any other component that might have quenched radicals during heating leading to increased antioxidant activity. The antioxidant properties of the PBLE leaves were also reported by Kruawan and Kangsadalampai, (2006); Niranjana et al. (2002), Dasgupta and De, (2004) and Chakraborty and Shah (2011).

Changes in phenolic content

Phenolic compounds in herbs and spices act mostly as radical scavengers and metal chelators are considered as potential protectors against lipid oxidation (Abdullahi, 2011). In the present study, on the day first higher phenol content (5.14 ± 0.32 GAE/mL) was found in PBLE added sample (FM_1), than in the control (FM_0). In both, the sample the total phenols were decreased and the rate of degradation of total phenol content was significantly (< 0.05) lower in a treated sample over control (Table 2). Changchub and Maisuthisakul (2011) reported that encapsulation of mango seed kernel using maltodextrin, gum arabic and tween-80 by spray drying technique had obtained significantly ($p > 0.05$) low phenol content compared to non-encapsulated form.

Chang et al. (2010) observed a rise in phenolic content of the *Ginseng jungkwa* extract after heat treatment (100°C for 3, 6 and 12 h). It was attributed to phenolic content, which leads to increase the free and conjugated phenolic acid and maltol content during heat treatment. Increase in phenolic content of sample could be ascribed to the release of the bound phenolic compounds from PBLE during its pasteurization at $68^\circ\text{C}/30$ min. In case of

Fig. 5: Changes in microbial quality of flavored milk during Storage at $5\pm 1^\circ\text{C}$



encapsulated dairy drink, maltodextrin-gum arabic matrix might have resisted the release of phenolic compounds into outer medium thus resulted in lower amount of phenolic content during the analysis.

Changes in microbial quality of flavored milk during storage at $5\pm 1^\circ\text{C}$

It could be seen from the data presented in Fig. 5 that there was a sharp increase in standard plate count from an initial count of 2.61 ± 0.06 cfu/ml to 5.00 ± 0.04 log cfu/ml at the end of shelf life (24 days) in case of FM₁ and in case of FM₀ count were increased (Fig. 5). There was no any coliform in both treated and control sample up to 20th and 24th day of storage period. There was sharp increase in yeast and mould count from initial of 1.28 ± 0.07 logcfu/ml to 2.91 ± 0.02 log cfu/ml at the end of storage period which is attributed to an increase in acidity during fermentation process which possibly might have provided suitable conditions for growth (Sengupta, et al. 2013 and Nagarajappa and Butulla, 2017). Datta et al. (2011) and Sripradha (2014) studied various properties of PBL include antioxidant, antifungal, and antimicrobial properties, it was reported that the *Piper betel* appears to be a promising and valuable source of antimicrobial compounds which acts against undesirable microorganisms in milk.

Conclusions

The antioxidant properties and phenolic activity of aqueous extract of PBL were 4.27 ± 2.77 $\mu\text{g/ml}$ and 3.49 ± 0.41 GAE/ml, respectively. Based on sensory evaluation, the milk added with PBL aqueous extract could be stored up to 20th days as against the plain sweet milk up to 16th days of storage at $5\pm 1^\circ\text{C}$. During storage there were increased in the values of acidity, viscosity, TBA and HMF content, whereas, the values of specific gravity, pH, protein, fat, lactose, sucrose and total solid content were

decreased. The antioxidant and phenolic properties of milk were decreased as storage period progress. The standard plate count and yeast and mould counts in both the samples were increased during storage.

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Characterisation of effective antifungal *Lactobacillus* strain isolated from Chilika curd

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Abstract: In this study different strains of Lactobacilli isolated from *Chilika curd*, (fermented milk product native to the Chilika region of Odisha, India) were assessed for their antifungal activity. *Limosilactobacillus fermentum* C-14 was shown to have the strongest inhibitory activity against tested yeast and mold cultures. This antifungal activity of cell free supernatant (CFS) Lb-C-14 was found to be stable at high temperature and up to pH 6. Antifungal activity has been observed in the bacterial cell-free supernatant, and the compounds have been identified as Phenyllactic acid (PLA), acetic acid and lactic acid. *L. fermentum* Lb-C-14 has the potential antifungal activity and could be suggested as a protective culture for the biopreservation of fermented foods.

Keywords: Antifungal; *Chilika curd*; Phenyllactic acid; *Limosilactobacillus fermentum*

Introduction

All foods, including cereals, meat, fruits, nuts, milk, and dairy products can support growth of fungi. Food spoilage caused by fungi results in significant financial losses for the food industry and may pose a health risk due to their toxicity and pathogenicity (Cheong et al. 2014). Several chemical and physical preservation techniques are used to increase the shelf-life of milk products.

Drying, freeze drying, cold storage, modified atmospheric packaging and heat treatment constitute physical methods; these methods are costly and tend to change the texture of products. Salts of benzoic acid, sorbic acid, propionate, and methyl, ethyl, and propylesters of p-hydroxybenzoic acids are used as antifungal chemical preservatives. Their incorporation at specific concentrations can increase the shelf-life of food products. Nevertheless, there remain some complications associated with the use of these chemicals (Cosentino et al. 2018).

Lactic acid bacteria (LAB) are a prominent group of microbes involved in fermented products as starter cultures to initiate fermentation. In addition to fermentation, their antibacterial and antifungal activities have received substantial research. There are many substances synthesized by LAB with antimicrobial activity such as bacteriocin, H₂O₂, diacetyl, organic acids, carbon dioxide, oleamide, trans-cinnamic acid, and citric acid etc. (Voulgari et al. 2010, Barrios-Roblero et al. 2019, Ramos et al. 202, Peng et al. 2023). Recently there has been research interest into the phenyllactic acid synthesis by LAB due to its effective antimicrobial activity (Bustos et al. 2018). It is produced as a by-product of phenylalanine metabolism in LAB identified in fermented dairy products (Valerio et al. 2016, Jung et al. 2019). The lactate dehydrogenase (LDH) is the main responsible enzyme for the production of PLA in LAB which catalyzes PLA release from the direct precursor Phenylpyruvate. PLA production has been documented in a wide range of LAB such as *L. plantarum*, *L. fermentum* and *L. casei* (Muhialdin et al. 2011, Cortés-Zavaleta et al. 2014, Jung et al. 2019, Xu et al. 2021). However, its production has been reported as species and strain dependent (Wang et al. 2012, Li et al. 2014, Xu et al. 2021).

One of the oldest and most well-known fermented milk products in the Indian subcontinent is dahi or curd, which is similar to yogurt. The Chilika curd is made by the local ethnic community in and around Chilika Lake region in Odisha, India, typically made from the milk of Chilika breed of buffalo (Singh et al. 2017). The Chilika dahi is extremely distinctive in terms of having an incredibly long shelf life (Nanda et al. 2013, Sahoo et al. 2020). Therefore, it is crucial to understand the wide diversity of LAB in Chilika curd for the nature of such properties that gives its extended shelf life. Previously some of the *Lactobacillus* sp.

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were isolated and screened for antifungal activity but additional studies are required focusing on characterisation of those isolates and acquisition of data regarding antifungal compounds they produce (Nanda et al. 2013). In this study, we have investigated the antifungal activity of *Lactobacillus* strain that was isolated from Chilika curd and conducted preliminary studies on their ability to produce PLA as a compound of antifungal properties.

Materials and methods

Microbial cultures

The six *Lactobacillus* cultures previously isolated from Chilika dahi were submitted to National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal under the numbers Lb-C-4 (NCDC-847), Lb-C-5 (NCDC-848), Lb-C-6 (NCDC-849), Lb-C-8 (NCDC-850), Lb-C-9 (NCDC-851), and Lb-C-14 (NCDC-852) were used for investigation (Nanda et al. 2013). The freeze-dried cultures of *Lactobacillus* were propagated in de Man, Rogosa and Sharpe broth (MRS, Hi-media, India) and incubated at 37°C for 48 hrs. The stock cultures were stored at -20°C in 15% (v/v) glycerol stocks and cultures were regularly activated in MRS broth.

The fungal cultures used as test microorganisms were *Aspergillus flavus* NCDC 226, *Aspergillus niger* NCDC 315, *Aspergillus niger* NCDC 267, *Aspergillus parasiticus* NCDC 54, *Aspergillus oryzae* NCDC 301, *Penicillium roqueforti* NCDC 170, *Rhizopus oryzae* NCDC 52, *Candida butyri* NCDC 280, and *Rhodotorula glutinis* NCDC 51 were procured from National Collection of Dairy Cultures, ICAR-NDRI, Karnal. All the yeast cultures were grown in Yeast extract Peptone Dextrose (YPD) broth and mold cultures were cultivated on Potato Dextrose agar (PDA) pH 4 adjusted and incubated at 30°C for 5 days.

The mold spores were harvested from 5 days old cultures by pouring sterilized peptone water containing 0.05% Tween 80 onto the plates. The number of conidia in the obtained suspension was adjusted to 10⁶ conidia/ml using a hemocytometer.

Screening for antifungal activity

Using agar overlay assay described by (Rouse et al. (2008)) was followed with minor modifications. Briefly, 20 ml MRS agar was poured into 90 mm petri dish and allowed solidification. Then 4 µL of active inoculum containing 1 × 10⁸ CFU/ml was spotted on the agar surface and plates were incubated at optimum temperature for 48 h. Following the incubation, the surface was covered by 7 ml soft agar seeded with fungal spores or yeast cells at 1 × 10⁴ CFU/ml. After the solidification of the soft agar layer, plates were incubated at 30 °C for 48 h. After incubation, each zone of inhibition was measured. The inhibition level of indicator fungal culture by *Lactobacillus* sp. was graded as follows: (-) no suppression, (+) least inhibition with inhibitory zone diameter < 12 mm, (++) moderate inhibition with inhibitory

zone diameter 12-18 mm, (+++) Strong inhibition with inhibitory zone diameter >18 mm.

Molecular identification of *Lactobacillus* sp.

The species of Lb-C-14 strain was identified based on the fermentation profile using API 50CHL test kit (Biomérieux, France) and molecular characterization by partial sequencing of 16S rRNA gene using the primer pairs 27F (5'AGAGTTTGAT(C/T)(A/C)TGGCTCAG3') and S-G-Lab-0677-a-A-17 reverse primer (52 - CACCGCTACACATGGAG-32) was performed as per the method described by Heilig et al. (2002). The 750 bp size amplicon of 16S rRNA gene was sequenced from Automated DNA Sequencing Services provided by Xcleris Lab Ltd. (Ahmadabad, India) and analysed using the BioEdit sequence alignment editor version 7.0.9.0. Basic local alignment search tool (BLAST) was used to check the identity of DNA sequence in the database and for species identification. The obtained nucleotide sequence was submitted to the NCBI Genbank database.

Preparation of cell-free supernatant (CFS)

Overnight active *Lactobacillus* culture suspension was adjusted to 0.5 McFarland standard. The culture was inoculated at 1% into 50 ml sterile MRS broth without sodium acetate and incubated for 48 h at 30 °C. After incubation, cells were removed by centrifugation at 10,000 RPM for 15 min at 4 °C and the supernatant was filter sterilized (0.45 µm pore size) to get CFS. The obtained CFS was freeze dried and reconstituted with Milli-Q water to 10 X concentration was screened for antifungal activity.

Influence of heat and pH on antifungal activity

To evaluate the antifungal compounds in CFS (10X concentrated) for heat and pH, the CFS was subjected to heating (121 °C/15 min/15 psi) and different pH range (4, 5, 6 and 7) and then antifungal activity was determined by agar well diffusion method as described by Roy et al. (1996).

Determination of antifungal components

The PLA and organic acids (lactic and acetic acid) in the CFS of *Lactobacillus* strain was determined using HPLC method as described in Mu et al. (2012) and Kishore et al. (2013), respectively, with slight modifications. All culture was grown in MRS broth and incubated at 30 °C for 48 h. The CFS obtained was treated with acetone to remove protein content, one volume of CFS was treated with three volumes of cooled acetone (acetone stored at -20 °C overnight) and then vigorously shaken for 2 min, after that, it was kept at -20°C for overnight). The precipitated protein was removed by centrifugation at 12000 RPM for 15 min at 4°C and the rest of the supernatant was dried. The dried samples were reconstituted in HPLC grade 50 mM phosphate buffer (6.5 pH) and filter sterilized (0.22 µm) before injection in the UFLC

system (Schimadzu) equipped with equipped with Phenomenex C18 (250 X 4.6 mm) column.

SEM analysis of fungal hyphae

The hyphae samples harvested after treated with CFS for 24 h in YPD broth were fixed on a glass coverslip with 2.5% glutaraldehyde for 4 h at 4°C. The samples were subsequently washed with 0.2 mol/L sodium phosphate buffer for 20 min and incubated in 1% osmic acid solution at 4°C overnight. The samples were first dehydrated for 20 minutes in 50% ethanol with two repeats, then for 20 minutes in 50, 60, 70, 80, 90, and 100% ethanol, respectively. The fixed samples were soaked in 100% isoamyl acetate twice for 20 min each and finally dried. Then the samples were sputter coated with palladium gold in Emitech K550 and the results were observed using SEM (Zeiss DSM 940 A) (Ahmad Rather et al. 2013).

Statistical Analysis

The experiments were performed in triplicates and the data are expressed as mean $\pm$ standard deviation. The obtained data was analysed for analysis of variance and significance was determined at confidence level of 95% using SAS Software.

Results and discussion

Fig. 2 The antifungal activity of CFS of *L. fermentum* C-14 against the selected fungal species. Data are mean $\pm$ standard deviation (n=3). The bars with the same lowercase letter are significantly different ($P < 0.05$).

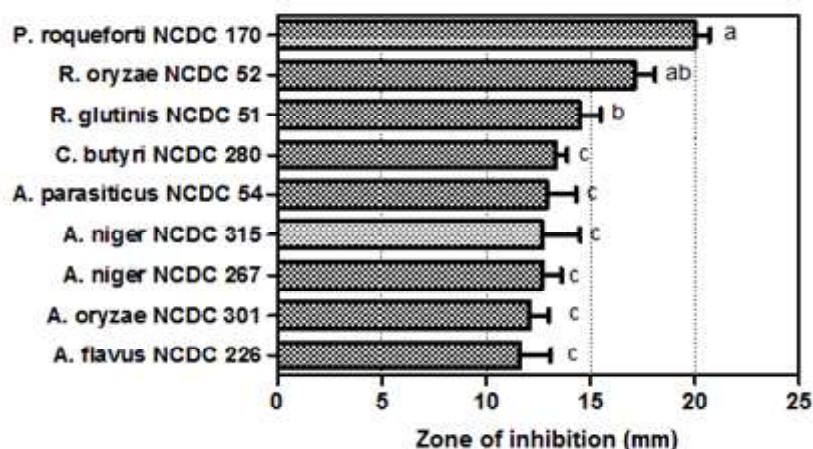


Table 1 Inhibition spectrum of *Lactobacillus* species against different fungi

<i>Lactobacillus</i> cultures	Fungal species		
	<i>C. butyri</i> NCDC 280	<i>G. candidum</i> NCDC 228	<i>A. niger</i> NCDC 315
<i>Lb</i> - C4	++	+	++
<i>Lb</i> - C5	++	+	++
<i>Lb</i> - C6	++	+	++
<i>Lb</i> - C9	++	+	+++
<i>Lb</i> - C10	++	+	++
<i>Lb</i> - C14	+++	+	+++

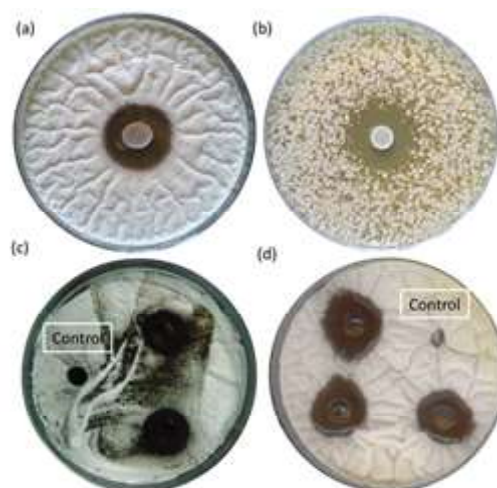


Fig. 1 Antifungal activity of CFS of *Lactobacillus* C-14 against a) *A. niger* NCDC 315 b) *C. butyri* by overlay assay and against c) *A. flavus* d) *A. niger* NCDC 267 by well assay

The preliminary screening of the antifungal activity of among the strains revealed growth of all fungi was inhibited in the presence of lactobacilli strains (table 1). The strains C9 and C14 demonstrated the strongest activity against *A. niger* mold. *G. candidum* was less sensitive toward all the studied strains. The

strain Lb C-14 had the highest antifungal activity against *A. niger*, *C. butyri*, and *G. candidum*. The study of antifungal activity of Lb C-14 was deepened on different fungi by well diffusion assay and results showed a consistent inhibition of growth (Fig 1).

Table 2 Fermentation profile of *Lactobacillus* sp. C-14 strains as per API test

Sugar	<i>Lactobacillus</i> sp. C-14
Control	-
Glycerol	-
Erythritol	-
D-Arabinose	-
L-Arabinose	-
D-Ribose	+
D-Xylose	-
L-Xylose	-
D-Adonitol	-
Methyl- α -D-Xylopyranoside	-
D-Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	-
L-Sorbose	-
L-Rhamnose	-
Dulcitol	-
Inositol	-
D-Mannitol	-
D-Sorbitol	-
Methyl- α -D-Mannopyranoside	-
Methyl- α -D Glucopyranoside	-
N-AcetylGlucosamine	-
Amygdalin	-
Arbutin	-
Esculin ferric citrate	-
Salicin	-
D-Cellobiose	-
D-Maltose	-
D-Lactose(bovine origin)	+
D-Mellibiose	+
D-Saccharose	+
D-Trehalose	-
Inulin	-
D-Melezitose	-
D-Raffinose	+
Amidon (starch)	-
Glycogen	-
Xylitol	-
Gentibiose	-
D-Turanose	-
D-Lyxose	-
D-Tagatose	-
D-Fucose	-
L-Fucose	-
D-Arabitol	-
L-Arabitol	-
Potassium Gluconate	-
Potassium 2-KetoGluconate	-
Potassium 5-KetoGluconate	-

Among the fungal cultures tested better inhibition was recorded against *P. roqueforti* with the zone of inhibition 20 ± 0.7 mm and thus can be considered as the most sensitive mold towards *L. fermentum* C 14 (Fig 2). Therefore *L. fermentum* C 14 isolated from Chilika dahi shown a high inhibitory activity against different fungi growth and was used for further study of antifungal properties.

Muhialdin et al. (2011) studied the antifungal activity of 137 LAB isolated from Malaysian fruits and fermented foods by using agar overlay method against *A. oryzae*, reported that *L. fermentum* Te007, *P. pentosaceus* Te010, *L. pentosus* G004, and *L. paracasei* D5 were the most effective. Bazukyan et al. (2018) screened eight strains of *Lactobacillus* sp. isolated from Armenian dairy products to assess their antifungal activity in MRS media. The authors reported that *L. rhamnosus* MDC 961 was effective against *G. candidum*, *T. viride* and *A. flavus* and recognized the activity of proteinaceous compounds present in CFS. Recently, out of 351 LAB strains isolated from naturally fermented Chinese vegetable samples only *L. crustorum* NWAUFU 1060 strain was reported to produce the highest producer of PLA as an antifungal compound (Xu et al. 2021).

The sugar fermentation pattern of *Lactobacillus* C-14 strain is shown in Table 2. The 16s rRNA gene sequence obtained in this study were analysed Insilco using BLAST of NCBI, the cultures *Lactobacillus* C-14 showed 98% similarity to *L. fermentum* (Fig 3). The analysed sequence of *Limosilactobacillus fermentum* C-14 has been submitted to the GenBank database under accession number KC713956.

To assess the characteristics of antifungal compounds, the CFS from *L. fermentum* C-14 was subjected to high temperature (121°C for 15 min) and pH modifications (pH from 4 to 7). Overall the CFS has retained its activity against *P. roqueforti* NCDC 170 after heat treatment ($P > 0.05$). But the pH modifications revealed that pH above 6 has significantly caused the reduction in antimold activity. These results suggest that antifungal compounds in CFS can tolerate heat denaturation and are likely to be acidic compounds. In previous studies (Zaiton et al. 2011, Bazukyan et al. 2018, Ramos et al. 2021) similar approach has been reported the use of heat and pH stability to assess the antifungal characteristics of CFS of *L. fermentum* te007, *L. pentosus* g004, *L. rhamnosus* MDC 9661 and *P. pentosaceus* te010 against *A. niger*, *A. oryzae*, *P. aurantioviolaceum* and *M. plumbeus* and attributed their antifungal activity to the production of organic acids. In this study, the MRS broth medium fermented by *L. fermentum* C-14 was acidified to a pH of 4 to 4.5. When the pH was increased to neutral the antifungal property of CFS was lost. Similarly, Cortés-Zavaleta et al. (2014) observed that the antifungal activity of culture filtrate of *L. acidophilus* ATCC 4495 was stable at low pH and was drastically reduced at pH 6.5. The possible reason behind the antifungal activity at low pH could be the involvement of organic acids in the antifungal activity.

Fig. 3 Phylogenetic analysis of *L. fermentum* C14**Table 3:** Organic acids present in CFS of *L. fermentum* C-14

Compound	Concentration
Lactic acid	16.3 ± 0.32 mM
Phenyl lactic acid	0.324 ± 0.01 mM
Acetic acid	5.7 ± 0.81 mM

Fig 4 is the UFLC chromatogram of CFS from *L. fermentum* C14 grown in MRS broth at 30 °C for 48 h. The chromatogram revealed that retention time of PLA (15.1 min) was similar for the retention time of compound in CFS of *L. fermentum* C14. Previous studies (Valerio et al. 2004, Gerez et al. 2013, Zhao et al. 2023) have reported PLA production as a fermentation product by *Lactobacillus* sp and major compound responsible for inhibitory effects against a wide range of fungi. Cortés-Zavaleta et al. (2014) reported PLA production 20 and 21.5 mg/L in *L. fermentum* NRRL B-1932 and *L. fermentum* ATCC 11976, respectively. Likewise, in the present study, *L. fermentum* C-14 was able to produce PLA at a relatively higher level up to 0.32 ± 0.01 mM (54.7 mg/L) (Table 3). In addition, UFLC chromatogram of CFS of *L. fermentum* C-14 showed a sharp peak at a retention time 6.31 min, the same retention as it recorded for pure acetic acid with a retention time of 6.31 min. About 5.7 ± 0.81 mM of Acetate was quantified in the processed CFS of *L. fermentum* C-14. The yield of L-Lactate was 16.3 ± 0.32 mM. Thus PLA need not be the only metabolite in CFS responsible for antifungal property as reported by previous researchers. Bian et al. (2016) identified the inhibitory activity of cheese isolate *L. helveticus* KLDS 1.8701 against *Penicillium* species, the responsible antifungal compound was identified as Lactic and acetic acid. Other researchers (Cortés-Zavaleta et al. 2014, Jung et al. 2019, Riolo et al. 2023) identified organic acids and ethanol etc along with lactic acid as fermentation products of LAB having antimicrobial activity and highlighted the possibility of a synergistic effect between the compounds. Thus, knowing the chemical structure and properties of metabolites of LAB is necessary for a complete understanding of possible interactions.

The scanning electron microscope (SEM) was used to reveal the morphological changes on the hyphae of *P. roqueforti* NCDC 170 (Fig 5). In control group, the hyphae was smooth and had tubular morphology. Whereas the hyphae treated with CFS had a rough and disrupted surface and there was cytoplasmic leakage around the hyphae. Previous literature on the investigation of antifungal mechanism has also shown damaged and distorted hyphae with shrivelled and crinkled cell walls, flattened hyphae and reduced hyphae in mold hyphae treated with CFS of *L. plantarum* strains (Sangmanee and Hongpattarakere, 2014). However, the mechanism behind the disruption of hyphae is not fully understood. Only a few mechanisms have been proposed by researchers, *L. plantarum* 29 first attaches to *Penicillium* species hyphae and then colonizes on it, their colonization results in the formation of depressions on hyphae as results damage of hyphae that leads to the inhibition of mould (Sorrentino et al. 2013). Our results also indicate the antifungal compounds in CFS of *L. fermentum* C-14 damage the structural integrity of *P. roqueforti* NCDC 170 and significantly affect the development of mold mycelia.

The fact that LAB produces a significant quantity of acidic metabolites from sugar in the medium that varying antimicrobial activity is well recognized. *Lactobacillus* sp. can produce organic acids that are antifungal such as lactate, acetate, PLA, succinic acids etc. The low pH of 3 to 5 pH favours the undissociated state of organic acids to enter the fungal cells and dissociate within higher pH cytosol causing acidification of the cytoplasm. It can cause the fungal cell to be suppressed or die (Batish et al. 1997, Li et al. 2014, Jung et al. 2019). However many of them are active against bacteria and some organic acid compounds are active against yeast and molds (Wang et al. 2012, Valerio et al. 2016). For many decades the extended shelf life of fermented milk products was attributed to lactic acid and acetic acid produced by LAB. Studies have shown that lactic acid is less effective

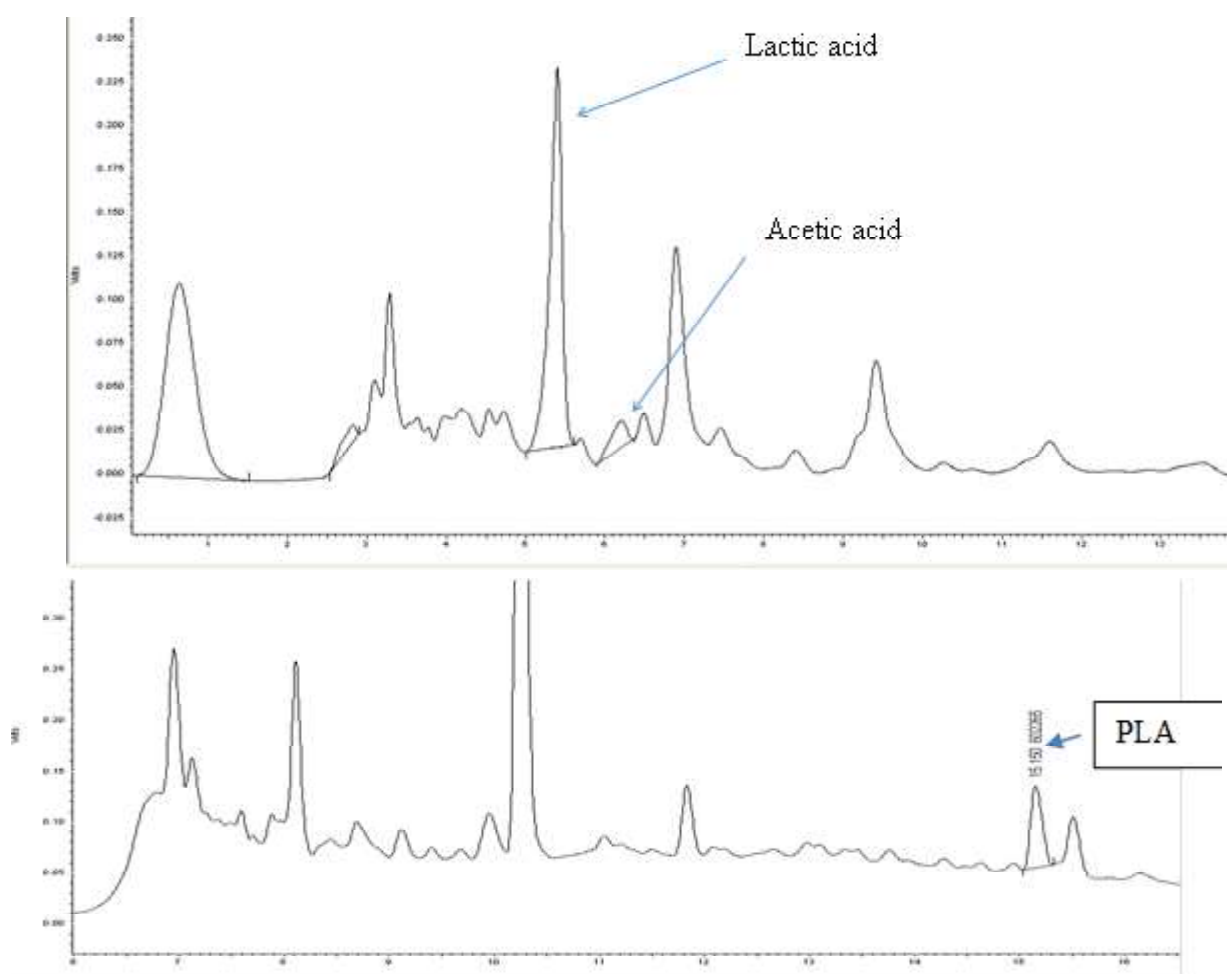


Fig. 4 UFLC chromatograms of lactic acid and acetic acid (A) and PLA (B) in CFS of *Lactobacillus* sp. C-14

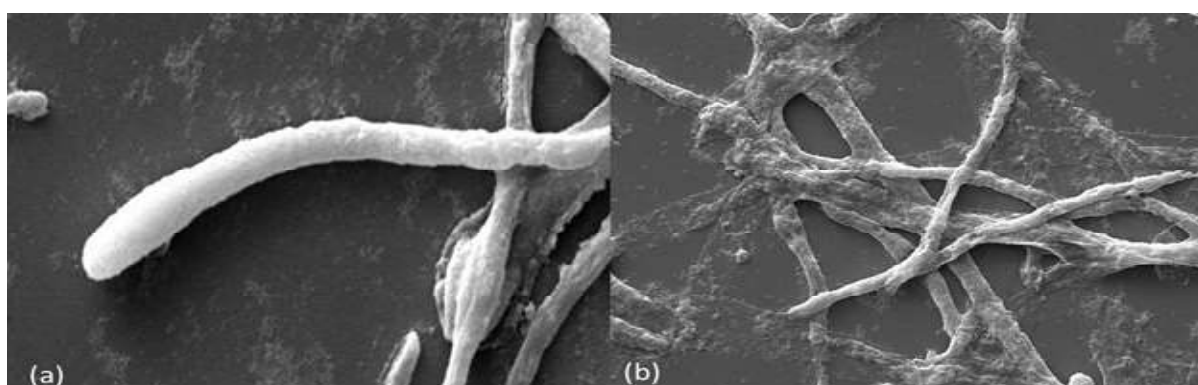


Fig. 5 Scanning electron microscope images of effect of *L. fermentum* C-14 on hyphae of *P. roqueforti* 170. a) Control group b) Experimental group

against fungi while acetic acid and phenyl lactic acid are strongly inhibitory to fungi (Jung et al. 2019, Xu et al. 2021). This study also highlights the importance of studies to discover new LAB strains that have better antifungal activity and the nature of metabolism products.

Conclusions

The *Lactobacilli* sp. isolated from Chilika dahi have shown antifungal activity especially *Limosilactobacillus fermentum* C-14 shown the highest activity. The antifungal activity of this strain remained stable to high temperature and at low pH levels

confirmed the acidic nature of antifungal compounds. The results confirmed that mixture of different organic acids produced are responsible for inhibition of molds. The SEM images showed distorting of the morphology of mold hyphae as a result of treatment by CFS. A further investigation into the structure and interaction between the compounds in CFS on antifungal activity is required. *L. fermentum* C-14 with high antifungal activity has the potential to be exploited as a promising bio-preservative for acidic food products.

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RESEARCH ARTICLE

Development and quality characteristics of functional Kulfi enriched with malted quinoa flour

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Abstract: Kulfi is a frozen traditional indigenous dessert which is liked by consumers of all age groups. The main objective of this study was to optimize and develop a sugar free, high protein and fiber rich kulfi using malted quinoa flour. Pseudo cereal quinoa was incorporated in kulfi at the rate of 10%, 15% and 20% in order to optimize the functional kulfi. The optimized sample K₂ contained moisture (56.60±0.06 %), fat (10.90±0.12 %), protein (4.45±0.07 %), carbohydrate (25.76±0.18 %), total solids (42.31±0.02 %), energy (218.75±0.81 Kcal), crude fiber (1.92±0.01 %), and ash (0.71±0.03 %), respectively. Storage period of 35 days was taken into consideration and changes in physico-chemical properties, antioxidant activity, melting resistance as well as microbial count was observed during this period at an interval of 7 days. The antioxidant activity was reported higher in optimized product then the control sample. Slight change in the acidity was recorded and the microbial activity decreased significantly over storage period.

Keywords: Antioxidant; Functional kulfi; Quinoa; physico-chemical; Melting resistance; Optimization

Introduction

Quinoa is a pseudo-cereal native to the Andes region of the South America. It belongs to the family Chenopodiaceae, class Dicotyledoneae, species quinoa and genus *Chenopodium* (Maricorena and Quezada, 1985). Quinoa is known as super food

due to its massive health benefits. It has gained importance in day- to- day lives in the past few years due to its nutritional benefits. Quinoa is gluten free, high in antioxidants, has low glycemic index and also possess some anti-cancerous properties (Tichy et al. 2020). It is refer as a complete food due to its fiber content and protein quality. Starch present in quinoa has physico-chemical properties such as freeze stability and viscosity which makes it a functional crop for novel uses. In the past decade it has also been recognized as an oil seed crop with an ample amount of omega-6 fatty acids as well as vitamin E content (Lilian and Abugoch, 2009). In order to enhance the nutritional properties and eliminate the anti-nutritional factors such as saponins from the quinoa seeds it is subjected to germination. Germination is a basically a cost effective process and also beneficial in many ways (Lan et al. 2023). Lintschinger et al. (1997) reported that various nutritive factors such as vitamin concentrations and bioavailability of trace elements and minerals increase during germination of wheat, buckwheat and quinoa.

Kulfi is one of the most liked traditional indigenous frozen dessert prepared by concentration of milk to a specific content. The texture of the kulfi is harder when compared to ice cream due to lack of air incorporation. It is also rich in total solids per unit volume. It is manufactured and marketed mainly in the unorganized sector in different flavors during summer season (Dutta et al. 2021). The method of production of kulfi often varies from producer to producer. Addition of some functional compounds namely pistachio powder, banana as well as other fruits and nuts increases the value and nutrient content of kulfi and makes it a functional product for consumption (Singh and David, 2018). This study reports the effect of addition of Malted Quinoa Flour (MQF) on physico-chemical, antioxidant and microbial properties of functional kulfi.

Materials and Methods

Raw materials

Fresh cream with 25 % milk fat, full cream milk and butter (Amul) was obtained from the local market of Varanasi, Uttar Pradesh. Skim milk powder (Amul), sodium alginate (Akshar chem.), glycerol mono stearate, stevia (Stevi0cal, by Rigil Biotech. Pvt.

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Ltd.) and quinoa were procured from online source.

Preparation of malted quinoa flour (MQF)

The process of development of malted quinoa flour is shown in (Figure.1). Sandberg and Svanberg,(1991) & Gustafsson and Sandberg, (1995) reported that in legumes and cereals the techniques such as soaking, germination and fermentation helps in the removal of the anti-nutritional factors such as endogenous phytates, saponins etc., Quinoa was soaked for about 7 to 8 hours in water. Germination of quinoa seeds was carried out in an incubator at $27\pm 2^{\circ}\text{C}$ for a period of 24 hrs. After incubation, the germinated quinoa seeds were tray dried at 55°C for 2 hrs. Drying for a short period of time resulted in retaining its maximum nutritional qualities. Dried quinoa seeds was then ground in a blender to make fine flour. Vacuum packaging of flour was done in Low Density Poly Ethylene (LDPE) and was stored in a cool dry place.

Process optimization of MQF enriched kulfi

Formulation of kulfi was done with some modification in traditional method proposed by (Salooja and Balachandran, 1982). Full cream milk was condensed to half of its volume and other essential ingredients i.e. skim milk powder, stabilizer, emulsifier as well as stevia was added based on laboratory trials. Vacuum packed malted quinoa flour was used in three different proportions for the primary trials @ 10, 15 and 20 % of the mix. Quinoa flour was gelatinized before adding to the mix. All the three samples along with a control sample (without malted quinoa powder) were aged for 3 to 4 hrs at $4\pm 1^{\circ}\text{C}$. The mix was further transfer to a kulfi

mould and freezed at $-4 \pm 1^{\circ}\text{C}$ for 25 ± 5 min. Further hardening was done at $-23 \pm 2^{\circ}\text{C}$ for 10 to 12 hrs as per modified method.

Treatment details used during the study were:

$K = 0\%$ MQF (Control)

$K_1 = 10\%$ MQF (Treatment 1)

$K_2 = 15\%$ MQF (Treatment 2)

$K_3 = 20\%$ MQF (Treatment 3)

Sensory analysis

Sensory analysis of different samples of MQF enriched kulfi was done by a panel of 15 semi- trained judges from Centre of Food Science and Technology, Banaras Hindu University on the basis of 9-point hedonic scale (Stone and Sidel, 2004). The samples were placed in a random order with unique codes. The attributes analyzed were color & appearance, body and texture, flavor and taste and overall acceptability.

Physico-chemical analysis of MQF enriched kulfi

The physico-chemical analysis namely acidity was determined by taking 1.0 g of sample and dissolving it in 100 ml of distilled water and then taken 10 ml from the solution for estimation purpose. Melting resistance (g/min. of kulfi melted at room temperature for 30 min.) was determined by using 5 g of sample and pH was determined by using 5 g of sample dissolved in 100 ml of the distilled water was estimated by using the methods of

Fig. 1 Pictorial representation of preparation of malted quinoa flour



(AOAC, 2000). Moisture content, ash content, crude fiber and fat of moisture free sample was determined by Soxhlet apparatus. Protein (%) was estimated by the macro-Kjeldahl method in which the percentage total nitrogen present in the sample was calculated and then it was multiplied by a factor of 6.38 in order to get the final protein content.

Total phenolic content and DPPH radical scavenging activity

Total phenol content (mg/g GAE) was determined using the Folin-Ciocalteu reagent and gallic acid as a standard (Slinkard and Singleton, 1977). The antioxidant activity was calculated as % anti-radical activity by the DPPH assay (2, 2-diphenyl 1-picryl hydrazyl assay) by the method of (Li et al. 2009).

Mineral analysis

The mineral content such as Ca, Mg, Mn, Fe and Zn was estimated by AAS (atomic absorption spectroscopy): Model-Thermo Fisher Scientist- IN at λ 422.7, 285.2, 457.4, 248.3, 213.9, respectively by wet digestion method (Chiş et al. 2020).

Microbiological analysis

The microbial analysis of the samples was carried out by pour plate method, by pouring approximately 10 ml media using plate count agar media (Kim & Cheigh, 2022).

Shelf-life study

The optimized product was stored for 35 days and was analyzed for certain physico-chemical properties, antioxidants activity and microbial load at an interval of 7 days.

Statistical analysis

Analysis of data was done by using different statistical tools. For testing significant difference test such as two tailed t-test, ANOVA was used with Microsoft excel and NCSS 19 software.

Results and Discussion

Sensory evaluation of MQF incorporated kulfi

For preparation of kulfi, malted quinoa flour was added at the rate of 10%, 15% and 20%. The sensory analysis results showed

that the concentration of the MQF had significant effect on the sensory attributes ($p < 0.05$) of treatment 2 (Table 1). The overall acceptability score for the MQF enriched kulfi (K_2) prepared with 15% MQF was found to be most satisfactory by the sensory panelists. Treatment 3 containing 20% MQF was not liked by the sensory panelists due to the dominant flavor of quinoa. The overall acceptability of (K_2) was 8.13 ± 0.13 , (K_1) 7.33 ± 0.19 and (K_3) 6.50 ± 0.23 , respectively. The sample containing 15% MQF had a rich flavor and mouthfeel and most liked by the sensory panelists. Based on the sensory report treatment (K_2) was selected for further study.

Physico-chemical analysis of MQF enriched kulfi

The physico-chemical properties of optimized kulfi (K_2) and control (K) are listed in (Table 2). Moisture (%) of the best variant (K_2) was less than the control sample due to the incorporation of malted quinoa flour. Wang and Zhu, (2016) reported similar results while studying the formulation of different quinoa products. The total solids (%) of the optimized kulfi significantly increased as compare to control from 42.31% to 40.30%, respectively. Similar type of study was conducted by (Cody et al. 2007) prepared ice cream with rice flour. The protein and fat content of optimized kulfi tend to increase due to incorporation MQF as quinoa is rich in protein. The results showed significantly higher protein content than the control sample. The results are similar to that reported by Ali et al. (2016); Ayar and Gurlin, (2014). The authors reported the functional, antioxidants and sensory qualities of ice-cream from pomegranate seed powder. The viscosity of K_2 was towards higher side than the control sample. The value increased from 25.57 (mPa.s) in control to 26.77 (mPa.s) in optimized kulfi. Arbuckle, (1977) discovered that acidity in the ice cream affects the viscosity. pH of control sample was higher that is 6.41 than the optimized one that is 6.19. Similar results have been reported by (Patel et al. 2020). They develop kulfi incorporated with amaranthus (*Rajgara*) which showed a significant decrease in the pH. The carbohydrate content in optimized product increased eventually after the addition of MQF. The results of this study were accepted because carbohydrate-based fat replacers exhibit a very thick and viscous behavior and have a capability to imbibe water, which would increase the viscosity of the mixes (Hafids et al. 2019). Carbohydrate has good water binding capacity, sometimes even better than proteins (Clark, 1994; Akoh, 1998). Melting resistance is one of the most important factors of frozen

Table 1 Sensory score of MQF enriched kulfi

Amount of MQF (%)	Body and texture	Color and appearance	Flavor and taste	Overall acceptability
K_0 (0)	7.70 ± 0.21	7.60 ± 0.26	7.50 ± 0.26	7.60 ± 0.12
K_1 (10)	7.30 ± 0.36	7.80 ± 0.20	$6.90^* \pm 0.31$	7.33 ± 0.19
K_2 (15)	8.20 ± 0.20	8.00 ± 0.25	8.20 ± 0.24	8.13 ± 0.13
K_3 (20)	$6.60^* \pm 0.40$	$7.30^* \pm 0.30$	$5.60^* \pm 0.26$	$6.50^* \pm 0.23$

Values mentioned as Mean $\pm$ Standard deviation (n=3)

*Attributes with different scores shows significant difference ($p < 0.05$)

Table 2 Physico-chemical analysis of MQF enriched kulfi

Parameters	Control (K)	Optimized (K ₂)	t-value
Fat (%)	10.01 ± 0.11	10.90 ± 0.12	2.21
Moisture (%)	57.35* ± 0.08	56.30* ± 0.06	0.09
Total solids (%)	40.30* ± 0.02	42.31* ± 0.02	0.24
Crude fiber (%)	0.23* ± 0.00	1.92* ± 0.01	122.55
Ash (%)	0.58 ± 0.03	0.71 ± 0.03	2.44
Protein (%)	3.57* ± 0.02	4.45* ± 0.07	11.48
Carbohydrate (g)	24.99* ± 0.17	25.76* ± 0.18	12.33
pH	6.41 ± 0.00	6.19 ± 0.02	1.80
Acidity (%)	0.17 ± 0.00	0.21 ± 0.00	3.46
Melting resistance (g/min.)	6.06 ± 0.01	6.41 ± 0.01	18.82
Viscosity (mPa.s)	25.57* ± 0.03	26.77* ± 0.66	9.38
Energy (Kcal)	213.23* ± 0.67	218.75* ± 0.81	4.26

Values mentioned as Mean ± Standard deviation (n=3)

*Attributes with different scores shows significant difference (p<0.05)

Table 3 Mineral content, antioxidant activity and total phenolic compounds of MQF enriched kulfi

Minerals (mg/100g)	Control (K)	Optimized kulfi (K ₂)	t- value
Calcium	304.84* ± 0.29	345.14* ± 0.35	83.73
Magnesium	35.19* ± 0.11	51.31* ± 0.47	33.24
Iron	0.61* ± 0.00	1.93* ± 0.02	46.70
Zinc	0.12* ± 0.01	1.61* ± 0.12	11.85
Manganese	0.63* ± 0.18	1.11* ± 0.05	2.47
Total phenolic content (mg/g GAE)	Nil	16.71 ± 0.61	NA
Antioxidants (%)	Nil	68.81 ± 1.30	NA

Values mentioned as Mean ± Standard deviation (n=3)

*Attributes with different scores shows significant difference (p<0.05)

dairy products such as kulfi. Kulfi should always melt into a smooth viscous liquid. The increased melting resistance of K₂ might be due to the highly viscous mix.

Mineral analysis

The mineral content which tends to increase after incorporation of MQF is depicted in (Table 3). The Ca, Mg, Zn, Fe and Mn increased to 345.14 mg/100g, 345.14 mg/100g, 1.61 mg/100g, 1.93 mg/100g, 1.11 mg/100g, respectively in optimized kulfi when compared to the control sample. The increase in the mineral content is attributed to the presence of high amount of minerals such as Ca, Mg, S, P and K in the quinoa seeds. Quinoa seeds are rich in total phenol content (TPC) and antioxidants. It has α -tocopherol, γ -tocopherol and phytoestrogens. Degradation of the phenolic compounds tends to decrease the level of TPC at the time of storage due to the fact that the polyphenols are not stable in long term, because they are affected by factors such as temperature, light, metallic ions, pH, etc., (Bukowska et al. 2003). But in this study TPC and the antioxidant content of the test sample increased significantly. A decrease in the antioxidant value occurred during the storage period which is depicted in (Table 4). Thus it might be concluded that a decline in the antioxidant value may be due to the decreased TPC. Karaaslan et al. (2011)

observed similar results while studying phenolic fortification of yoghurt using grape and callus extracts. The major disadvantage of the quinoa seed is the presence of phytic acid and saponins but is can be completely removed by giving certain treatments (Valencia, 2003).

Effect of storage on physico- chemical properties of kulfi

Acidity plays a very important role in flavor of the product as well as spoilage (Gupta et al. 2020). The acidity of the optimized kulfi sample showed very slight change from 0.22 % on 0 day to 0.20% on 35th day of storage (Table 4). Gokhale et al. (2017) reported that acidity content increased possibly due to the action of the lactic acid bacteria during storage condition. pH also showed decreasing trend from 6.19±0.00 to 6.12±0.00 from zero day to 5th week of storage. Melting resistance of K₂ showed more resistance than the control and was 66.41(g/min.) on 0th day and 66.30(g/min.) on 35th day. Siva et al. (2019) developed functional kulfi and concluded that melting resistance is influenced by many important factors such as additives used in it, fat globules and formation of the crystals.

Effect of storage on antioxidant activity and microbial load of developed kulfi

Table 4 Effect of storage on some physico-chemical properties of MQF enriched kulfi

Attributes		Antioxidant activity (%)	Acidity (%)	pH (%)	Melting resistance (g/min.)
0	No. of days				
	Control sample (K)	Nil	0.17 ± 0.10	*6.41 ± 0.02	*6.06 ± 0.02
	Accepted sample (K ₂)	68.61 ± 0.10	0.21 ± 0.04	*6.19 ± 0.01	*6.41 ± 0.03
7 th	Control sample (K)	Nil	0.16 ± 0.01	*6.40 ± 0.00	*6.06 ± 0.03
	Accepted sample (K ₂)	68.59 ± 0.03	0.22 ± 0.05	*6.18 ± 0.00	*6.38 ± 0.02
14 th	Control sample (K)	Nil	0.16 ± 0.02	*6.38 ± 0.02	*6.05 ± 0.01
	Accepted sample (K ₂)	67.90 ± 0.01	0.21 ± 0.02	*6.13 ± 0.01	*6.35 ± 0.03
21 st	Control sample (K)	Nil	0.17 ± 0.06	*6.35 ± 0.02	*6.04 ± 0.05
	Accepted sample (K ₂)	67.57 ± 0.00	0.20 ± 0.01	*6.13 ± 0.03	*6.32 ± 0.03
28 th	Control sample (K)	Nil	0.16 ± 0.02	*6.35 ± 0.04	*6.04 ± 0.11
	Accepted sample (K ₂)	67.32 ± 0.00	0.22 ± 0.01	*6.12 ± 0.05	*6.31 ± 0.06
35 th	Control sample (K)	Nil	0.16 ± 0.02	*6.32 ± 0.07	*6.03 ± 0.02
	Accepted sample (K ₂)	67.01 ± 0.10	0.21 ± 0.01	*6.12 ± 0.05	*6.30 ± 0.04

Values mentioned as Mean ± Standard deviation (n=3)

*Attributes with different scores shows significant difference (p<0.05)

The antioxidant content tends to decrease slightly from 68.61% radical activity at 0th day to 67.01% radical activity on 35th day as shown in (Figure 2). Similar observations has been recorded by (Fernandes et al. 2020). They observed that freezing can affect the antioxidant content which can tends to decrease during storage conditions. It may get lost during storage. A decreasing trend of microbial growth varies from 4.99 cfu/ml on the 0 day to 4.12 cfu/ml on 35th day as shown in (Figure 3). Lee and White (1991) demonstrated that the storage condition plays an important role in the reduction of the microbial content due to the low temperature. The formation of the ice crystals during the storage leads to the disruption of the cell wall of the microbes which kills the microbes during storage (Davidson et al. 2000).

Conclusions

Malted quinoa flour enriched kulfi can be prepared commercially and stored at freezing temperature. It has high amount of

polyphenols as well as antioxidants. MQF enriched kulfi has a unique flavor and color and hence does not require any additional artificial coloring or flavoring agents. Quinoa can be a core ingredient of great importance in many other value added products. High fiber and gluten free property of quinoa makes it valuable product for those having celiac disease and digestive problems. With low glycemic index it serves as a promising food for diabetic patients.

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Fig. 2 Effect of storage on the antioxidant content of the MQF enriched kulfi

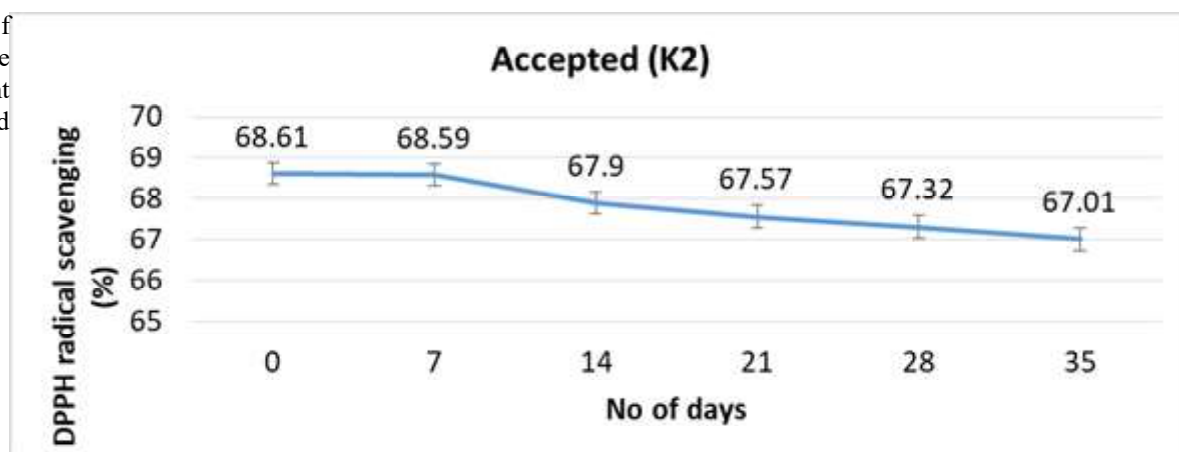
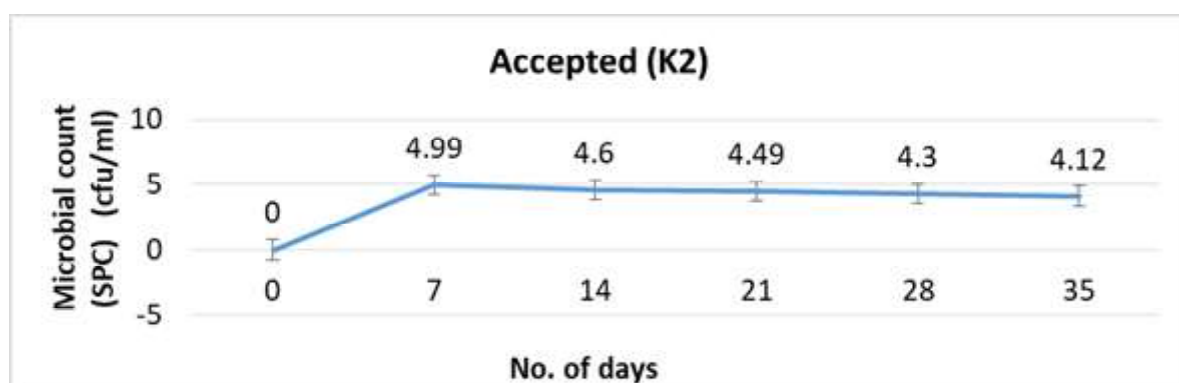


Fig. 3 Effect of storage on standard plate count of MQF enriched kulfi



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RESEARCH ARTICLE

Development of lateral flow enzyme substrate assay strip for qualitative estimation of coliforms

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Abstract: FSSAI 2011 has adopted a conventional IS-5887 (Part-I) 1976 and IS-5401 Part-1 (2012) protocol for monitoring of *E. coli* and coliforms in dairy products respectively. These methods are time consuming and sometimes requires further isolation and confirmation to finalize the true contaminant. The current investigation was carried out to minimize these limitations in detection of coliforms and *Escherichia coli* and to give a real time test to the industry that meets legal limits on day to day basis. The present investigation comprised of development of protocol for qualitative detection of coliforms in spiked samples using sonication technique and enzyme substrate assay. A protocol developed for sample preparation by extracting β -galactosidase (β -gal) using ultra sonicator (amplitude 80% and time 15 minutes) to give a positive ONPG test results within 20 minutes. After finalizing the sample preparation protocol, specific amount of the extract was used to check the presence of coliforms by using X-gal impregnated absorbent strip. Sensitivity of X-gal test was very good as it could detected minimum 10 cells/10 ml of spiked coliforms sample. Spiked cells (10 cells/10 ml of broth) need maximum 10 hours of incubation time and 30 mins for sample preparation and processing (sonication and centrifugation) before testing with ONPG and X-gal assay. It turned white strip in to blue colour indicating qualitative presence of coliforms within 20 minutes at 37 °C. This developed protocol could be able to reduce the actual detection time from 24 hrs by conventional Indian Standards method to nearly 11 hours with accuracy of 1 cells/ml.

Keywords: Coliforms, Enzyme substrate assay, Spiking, qualitative detection, Sonication

Introduction

There are few limitations in selection of commercially available bacteriological media in detection and enumeration of coliform mainly for testing water and food products. Hence, there is a scope for the easier technique which would help to recover the maximum genera of coliforms group and inhibit rest all *i.e.* lactose non-fermenters including *Salmonella* and *Shigella* (Gawai, 2022a, b). The biochemical tests used for bacterial identification and enumeration in classical cultural methods are generally based on metabolic reactions (Tavakoli et al. 2008). For this reason, these are not completely specific and requires many additional tests to obtain precise confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme targeted. Moreover, reactions are rapid and sensitive. Thus, the possibility of detecting and enumerating coliforms through specific enzymatic activities has been under investigations since long time.

Enzyme based methods (*e.g.* chromogenic and fluorogenic media) have been developed and certified. These methods have rendered rapid and much easier measurement of *E. coli* and coliforms than the methods approved in the past, hence these are attracting greater interest from researchers and industries (Kanangire, 2013). These methods concurrently detect the total coliforms and *E. coli* which increasingly make possible the quantification of *E. coli*, rather than simply 'thermotolerant coliforms' (Siegrist, 2020). In the past decade, diverse methods using chromogenic and/or fluorogenic substrates to reveal β -d-glucuronidase and β -d-galactosidase activity on culture media have been reported to determine whether a strain belongs to the coliform group and/or *E. coli* (Siegrist, 2020; Gawai, 2022c). Various commercial media containing chromogenic substrates for the identification and enumeration of *E. coli* and Total Coliforms (TC) are available. Among them, Chromo-agars (Alonso et al. 1999) and Chromocults (Geissler et al. 2000) are able to give results in less than 24 hrs.

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Some reviews on the use of fluorogenic and chromogenic substrates for bacterial qualitative detection are available (Chrost, 1991). These indicate that the use of these substrates has led to improved accuracy and faster detection. Methods for detection or enumeration may be performed in a single medium, thus bypassing the need for a time-consuming isolation procedure prior to identification. Regarding enzyme substrate assays, ISO compiled and recommended a set of such test methods, including β -D-glucuronidase activity for *E. coli* detection (Anon, 2016).

Way back in 1988, Edberg and Edberg proposed a combined substrate technology where in ONPG used as a substrate for the constitutive enzyme β -galactosidase present in all coliforms and the substrate MUGlu was used for the specific detection of *E. coli*. The defined substrate method was basically constituted as a presence or absence test. The tubes, which are colourless after samples addition, are incubated at 35 °C. Development of yellow colour in the test tube (indicating the hydrolysis of ONPG) was taken as a positive for TC. Yellow tube is then exposed to long wave UV light, and blue white fluorescence demonstrates the presence of *E. coli*. No additional confirmatory test were needed (Anon, 2020a).

Chromogens and fluorogens substrates produce color and fluorescence respectively and compounds such as o-nitrophenyl-3-D-galactopyranoside (ONPG), p-nitrophenyl-3-D-galactopyranoside (PNPG), and 4-methylumbelliferyl-p-D-galactopyranoside (MUGal) have been included in a variety of media to demonstrate the presence of β -galactosidase, an enzyme produced by coliforms and 4-methylumbelliferyl-13-D-glucuronide (MUG) as a substrate to detect the presence of *E. coli* in milk and dairy products (Brenner et al. 1993).

Several commercially available liquid presence-absence or most-probable-number media Colilert [EnviroNetics, Inc., Branford, Conn.], Colisure [Millipore Corp., Bedford, Mass.] and ColiQuick [Hach, Loveland, CO, USA] have been developed to detect TC and *E. coli* in water samples within 24 to 28 h. Among them Colilert, Modified Colitag and ReadyCult test are few who have been approved for drinking water analysis (Rompre et al. 2002, Anon, 2020).

Table 1: Optimized final formulation for preparation of selective broth for Coliforms

Ingredients	Quantity per 100 ml
Bile salt	0.25 g
Sodium chloride	0.25 g
Di-sodium phosphate	0.24 g
Mono Sodium Phosphate	0.15 g
Tergitol	0.01 g
Yeast extract	0.30 g
Lactose	1.00 g
Sodium lauryl sulphate salt	0.25 g
Cefsulodin 10 mg/1 vial	312.5 μ l
Gentamicin sulphate + Amoxycillin (1:1 ratio)	10 μ l (5+5 μ l)
pH adjustment	7.4 (adjusted with 0.1 N HCl)

The objectives of the present study were to develop such a method which will provide results of coliform test in just 12 hrs by using enzyme substrate interactions. Along with this, it was attempted to develop an interpretation chart for quantitative detection of coliforms which would be helpful in decision making when counts laid in a specific range.

Materials and methods

The study was planned to develop a lateral flow enzyme substrate assay strip for qualitative estimation of coliforms. The work was conducted in the Department of Dairy Microbiology, SMC College of Dairy Science, Kamdhenu University, Anand. It was planned to use formulated selective broth to test the performance of coliforms detection strip from spiked samples.

Formulated selective coliforms broth was developed with addition of Sodium lauryl sulphate salt @ 0.2g, Gentamicin sulphate + Amoxycillin (1:1 ratio) @ 10 μ l and Cefsulodin @ 312.5 μ l per 100 ml which exhibited strong inhibition of targeted organism like *Salmonella typhi* ATCC 14028 while promoted the growth of coliforms cocktail and *Escherichia coli* ATCC 25922 (Gawai, 2022b). The composition of the broth is given in Table 1. This formulated selective broth was used to inoculate spike coliforms and later its presence was detected using developed enzyme substrate assay. The cocktail of coliform culture was prepared by mixing equal contents from three positive tubes of MacConkey's broth. This culture was propagated in nutrient broth medium and incubated at 37°C for 24 h and then stored at 5 $\pm$ 2°C. Sub culturing was done at an interval of 7 days during the course of the study.

Protocol standardization for a sample processing

Sample processing protocol was standardized after slight modification in the method described by Prasad et al. (2013) and Makwana et al. (2019). Sample prepared with addition of fixed cells of coliforms as per the spiking protocol in the formulated coliforms broth (Gawai et al. 2017). B-galactosidase and other enzymes present in coliforms are intracellular type, hence to extract these ultra-sonicator was used. Amplitude and time for

ultra sonication were standardized using a statistical program software Response Surface methodology.

Protocol for crude enzyme extraction

Coliforms cells were added by spiking in 9 ml formulated selective broth and incubated at 37 °C up to 10 h. After incubation, test tube was removed and mixed carefully. The cells were harvested at 5000 rpm for 10 min at 4°C in refrigerated centrifuge. The supernatant discarded as it contains extracellular enzymes and the remained cell pellet was mixed with 5 ml of 0.05 M sodium

phosphate buffer (pH 6.8). The cell pellets suspension was mixed thoroughly with vortex for 2-3 min. Further to extract intracellular enzyme, cell disintegration method *i.e.* sonication treatment was used. The cell suspensions were sonicated for 15 minutes (pulse 10 seconds off/15 seconds on and 75 % amplitudes) in ice bath using ultra sonicator (LABMAN, India). After sonication, the extract was centrifuged at 5000 rpm at 4°C for 10 min and obtained the supernatant containing the crude enzymes. This was further used to check the presence of coliforms using ONPG test. The flow chart for crude enzyme extraction for coliform testing using X-gal strip or ONPG assay is given in fig 1.

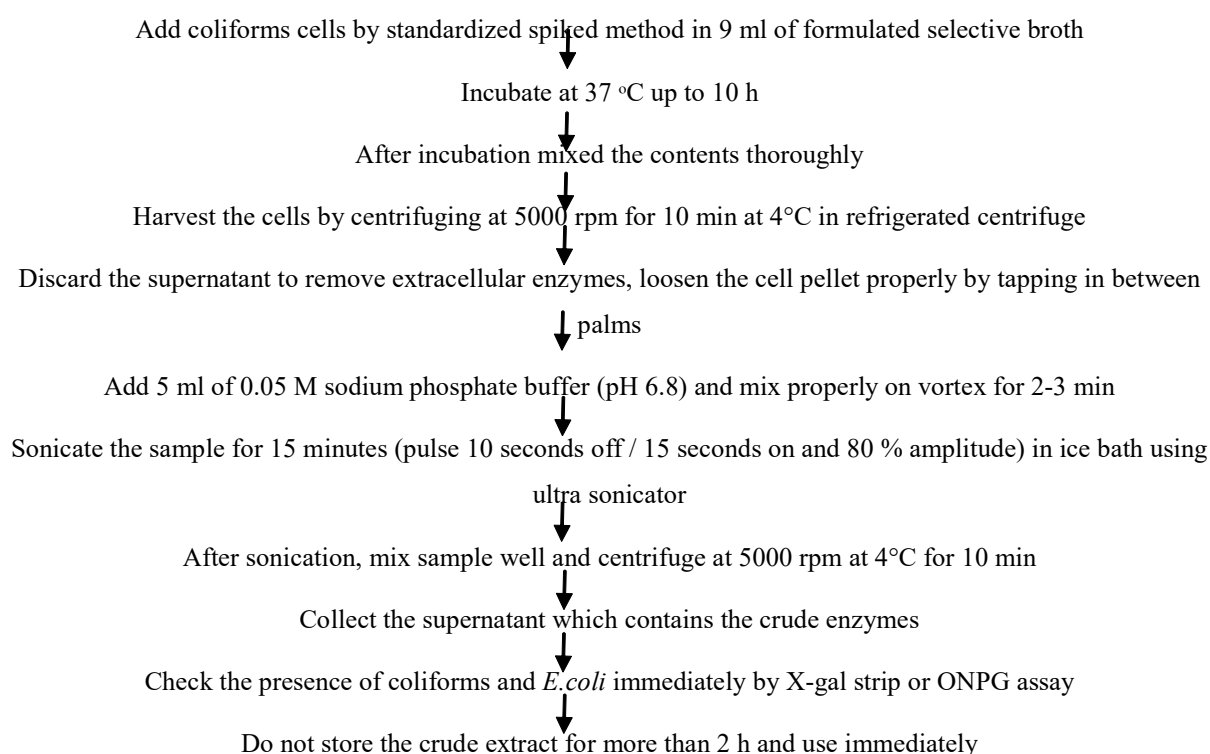


Fig. 1 Flow chart for crude enzyme extraction for coliform testing using X-gal strip or ONPG assay

Table 2: Experimental design matrix (CCRD) for levels of factors: Amplitude and time of sonicator and the results of ONPG test responses

Run	Standard	A: Amplitude of Sonicator	B: Time of Sonication	Response of ONPG test in min
1	7	80.00	07.99	140
2	8	80.00	22.07	55
3	9	80.00	15.00	16
4	4	95.00	20.00	75
5	6	101.21	15.00	110
6	13	80.00	15.00	28
7	10	80.00	15.00	20
8	3	65.00	20.00	60
9	1	65.00	10.00	80
10	2	95.00	10.00	60
11	5	58.78	15.00	40
12	11	80.00	15.00	22
13	12	80.00	15.00	30

Preparation of enzyme substrate assay strip test

A strip was used to make interaction of enzymes extracted from the sample and impregnated dried substrate. For preparation of a strip, an absorbent pad (Axiva Chemicals Limited, New Delhi) was used. It was cut in size of 8 cm x 8 cm. Precautions were taken to avoid any contamination of strip from sweat and other chemicals which may interfere with the results. On the strip, X-gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside) (100 mg/4 ml Dimethyl sulfoxide) solution was added @ 20 μ l using 2.5 ml medical grade syringe and allowed it to dry for 4 h. These strips were stored in cool and dry place till onset of experiment. A properly dried dip strip aseptically added in sterilized empty test tube. To this, 1000 μ l of prepared crude extract was added and incubated the test tube at 37°C for 15 min in an incubator and observed for change in the colour of a strip from white to blue.

Results and Discussion

Optimization protocol for the use of ultra sonicator

Endogenous enzyme extraction was carried out in formulated coliform broth medium using Sonicator (LABMAN, India). Prasad et al. (2013) developed method for extraction and characterization of β -galactosidase produced by *Bifidobacterium animalis* spp. *lactis* Bb12 and *Lactobacillus delbrueckii* spp. *bulgaricus* ATCC 11842 grown in whey using 15 minutes (pulse 10 seconds off/ 15 seconds on and 75 % amplitudes) in ice bath using ultra Sonicator (LABMAN, India). For optimization of best suitable condition for extracting β -galactosidase enzyme from coliforms an advanced statistical software programme named Design Expert 10.0.1 was employed. Here, amplitudes of sonicator were selected from the range of 65 to 95, time from 10 to 20 min. Among the various combination of amplitude and time, it was considered the best wherein ONPG test took the minimum time to indicate a positive result.

Influence of varying levels of amplitude and time of sonicator on early positive ONPG test

Table 3: Partial coefficients of regression equations of suggested models for early positive result of ONPG at different amplitudes and time intervals

Factor	ONPG test Results (Times in min)	
Linear	A	11.75
	B	-15.65
Interactive Quadratic	AB	+8.75
	A ²	21.53 [#]
	B ²	32.77 [*]
R ²		0.7751
Model F value		4.83
Intercept		23.20
APV		5.572
Model		Quadratic

*P < 0.01; [#]P < 0.05; APV= Adequate Precision Value; R²= Coefficient of determination

A:amplitude of sonication and B: to time chose for sonication process

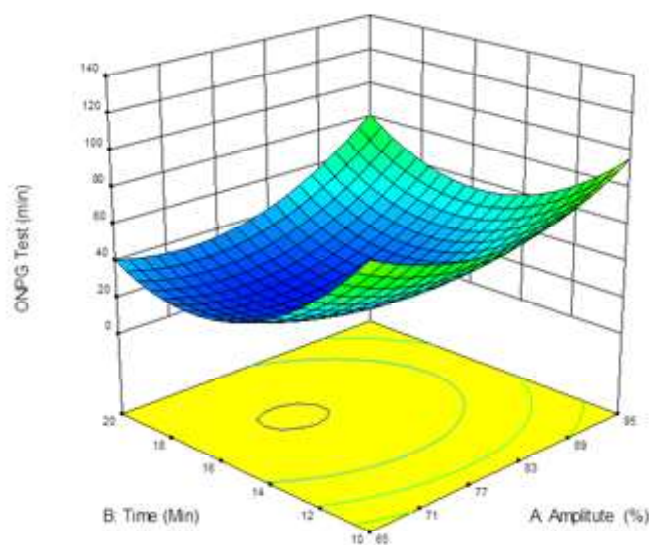


Fig. 2 Response surface for changes in amplitude of sonicator with different time intervals

In all the runs of the experiments suggested by RSM software, spiking of cells of coliforms were kept constant *i.e.* 10 cells per 10 ml of formulated selective broth. The data of the time require to get positive ONPG test is given in Table 2.

Time require to get positive result of ONPG test was observed in the range of 16 to 140 min for all the runs. The coefficient of determination (R²) is the proportion of variability in the data explained or accounted for the model and high value (0.7751) of R² (Table 3) indicated a better fit for the model to the data. Also the adequate precision value (APV) of 5.572 recommended the use of this response to navigate the design.

The values presented revealed that time of sonication (A) and amplitude of sonicator (B) had non-significant effect on the test result of ONPG at linear level. Interaction effect of time of sonication and amplitude of sonicator (AB) also had a non-significant effect on the test result of ONPG.

The square of factor (quadratic) of sonication amplitude (A) had a significant effect ($P < 0.05$) while time of sonication (B) had a highly significant effect ($P < 0.01$) effect on the response of ONPG test. Multiple regression equation generated to predict the result of ONPG test as affected by amplitude and time of sonication is given in fig 2.

Sonication is one of the most widely used methods for disruption of the bacterial cell walls (Engler, 1985). Among the three methods tested; sonication, bead milling and high-pressure homogenizer, sonication was found to be more effective for releasing β -gal (Sakakibara et al. 1994). Berger et al. (1995) compared two physical disruption methods for the extraction of intracellular β -gal enzyme from *Thermus* species and found that the sonication was superior to the glass-bead milling.

Many workers have suggested different protocol for sonication of coliforms depending on different work like extraction of β -galactosidase, certain type of protein *etc.* Jing (2016) demonstrated that 60 min of sonication duration with 80% amplitude released the highest concentration of NS1 protein from the *E. coli* cells.

Cruz-Cansino et al. 2016 investigated the effectiveness of ultrasound for the inactivation of *Escherichia coli* inoculated into cactus pear juices (green and purple). The kinetics of *E. coli* in cactus pear juices treated by ultrasound (60%, 70%, 80% and 90% amplitude levels for 1, 3 and 5 min) were evaluated over 5 days. They reported ultrasound treatment at 90% amplitude for 5min resulted in non-detectable levels of *E. coli* in cactus pear juice for 2 days.

Optimization of varying level of amplitude of sonicator and time to get positive ONPG Test

In the process of optimization of levels of amplitude and time, RSM suggested the best solution with 75.03% for amplitude and 16.41 min for time with desirability of 0.975. However, in case of actual trials, it was observed that amplitude of sonication @ 80, time for sonication @ 15 min was found the best for the positive ONPG test response. The process was replicated seven times. The selected factors and the average values of the results were derived. The values of the selected constraints/responses were compared statistically using paired t-test with that of the predicted values as shown in Table 4. The calculated values of all these selected constraints suggest that the calculated values of 't' for all the constraints were less than the table values, thus it was inferred that there was non-significant ($P > 0.05$) difference between the predicted and actual values of responses. Thus it was confirmed that the selected combination of the factors (amplitude of sonication @ 80 %, time for sonication @ 15 min) was the best in terms of the responses delineated at the study.

Testing of samples using enzyme substrate assay based strip for coliforms detection

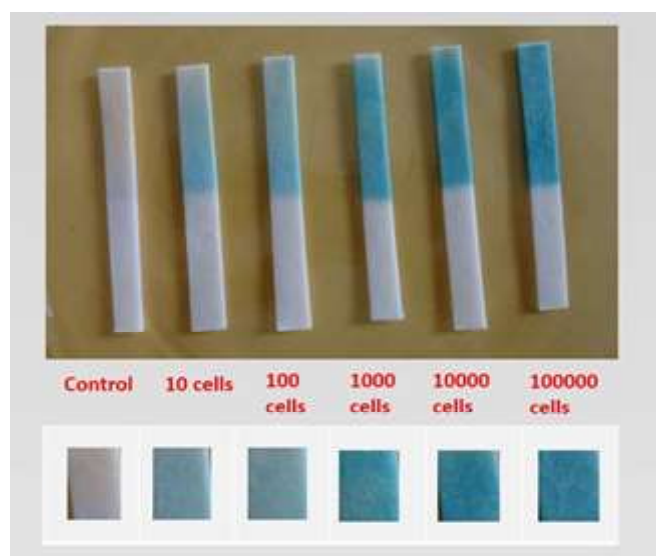


Fig.3 Interpretation chart of the results accessed by X-gal strip method

Spiked samples of coliforms @ 10, 100, 1000, 10000 and 100000 cells were tested with developed strips impregnated and dried with X-gal substrate as per the protocol described earlier. The strip without addition of the cells of coliforms acted as a control and did not develop any change in its colour and remained white. Rest of the strips showed incremental development of blue colour. As numbers of coliforms cells spiked were increased, the intensity of colour also increased. Interpretation chart for use of this developed strip to quantify the population of coliforms in the sample is given in fig 3.

In food hygiene, coliforms acts as index indicator organisms and it is important basis for the assessment of good manufacturing practice. X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside), a soluble colorless compound consisting of galactose linked to a substituted indole reacts with β -galactosidase extracted from coliforms and is the best standard for this interaction (Hahn and Wittrock, 1991). There is high specificity for the galactose part of β -galactose substrates but low specificity for its organic moiety. Thus, an insoluble intensely blue product is produced as hydrolyzed X-gal product, releasing the substituted indole that spontaneously dimerizes. In chromogenic medium containing X-gal, colonies of *E. coli* that have an active β -galactosidase become blue because of this reaction (Sedzro et al. 2018, Pala et al. 2020).

Kilian and Bulow (1976) surveyed the *Enterobacteriaceae* and reported that glucuronidase activity was mostly limited to *E. coli*. The prevalence of this enzyme and its utility in the detection of *E. coli* in water were later reviewed by Hartman (1989). β -D-glucuronidase-positive reactions were observed in 94-96% of the *E. coli* isolates tested (Kilian and Bulow, 1976), while Chang et al. (1989) found a higher proportion of β -D-glucuronidase-negative *E. coli* (a median of 15% from *E. coli* isolated from

Table 4: Comparison of predicted v/s actual values of responses used for process optimization of varying level of amplitude and time of sonicator suggested to get positive ONPG Test

Responses	P-value	Predicted Value	Actual value	Calculated t value	Level of significance
ONPG test	0.18	19.04	23.2	1.60	NS
Predicted values of Design Expert 8.0.3 package					
@Actual values (average of seven trials) of the optimized process protocol					
t-values at 5 % level of significance					
NS = Non Significant					
Tabulated t-value = 2.776					

human fecal samples). In contrast, β -D-glucuronidase activity is less common in other *Enterobacteriaceae* genus, such as *Shigella* (44 to 58%), *Salmonella* (20 to 29%) and *Yersinia* strains and in *Flavobacteria* (Kilian and Bulow, 1976). β -D-galactosidase, catalyzes the breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group within the *Enterobacteriaceae* family.

In the line of the present study Gunda et al. (2017) developed strip test device to detect presence of coliforms from water sample. In their study, the reaction zone was formed below the hydrophobic barrier by depositing the 100 μ l of custom formulated chemical composition (Red-Gal, B-PER and LTB) using pipette and the resulting paper strips were completely dried for one hour under a fume hood before dipping into test sample. They reported that a test with dip time of 2 min, it was able to detect as low as 200 cfu/ml in 180 $\pm$ 20 min and higher concentrations such as 2 $\times$ 10⁵cfu/ml within 75 $\pm$ 12 min. However, for a dip time test of 90 min, the developed DipTest device was able to detect as low as 200 cfu/ml in 54 $\pm$ 8 min and higher concentrations such as 2 $\times$ 10⁵cfu/ml within 28 $\pm$ 5 min.

Gunda et al. (2016) used a novel hydrogel based porous matrix to encapsulate the optimized chemical compounds and incorporated it within a readily available plunger-tube assembly. This overall system allows efficient, field deployable, rapid testing of water samples by simultaneously pre-concentrating and detecting *E. coli* within one integrated unit. They were able to detect *E. coli* concentrations of 4 $\times$ 10⁶cfu/ml to 4 $\times$ 10⁵cfu/ml within 5 min and 4 $\times$ 10⁴cfu/ml to 400 cfu/ml within 60 min using the integrated plunger-tube assembly containing the hydrogel matrix.

Rapid response time and simplicity of use are important for point of use systems. To achieve rapid response times and simple 'dip and use' utility, the enzyme and a colorimetric substrate were not allow contact on strip to prevent mixing before immersion. The close proximity of enzyme and substrate provides a rapid sensing platform. To create the colorimetric response needed for visual detection of bacteria, can adopt a sensing construct that uses β -galactosidase and some specific substrate complimentary to it.

Dasgupta et al. (2016) developed a paper strip to detect the presence of *E. coli* made of a Grade GB003, Whatman absorbing

gel blotting paper with one edge of the strip coated with wax hydrophobic barrier and the opposite edge (attraction zone) coated with D-glucose (dextrose) solution. Under the hydrophobic barrier it contains Red-Gal substrate (6-Chloro-3-indolyl- β -D-galactoside), bacterial enzyme (protein) extracting reagent (B-PER) and nutrient medium Lauryl Tryptose Broth (LTB).

When compared the present work with the work in the same line, this developed method is rapid and could gave results in less time. This could be better alternative to industry people to release a lot of products in approximately 50 % less time in comparison of the results obtained by conventional methods. This method needs lesser capital investment and operating cost wise also affordable.

Conclusion

In the present investigation, the developed formulated broth was used to cultivate coliforms culture and a protocol was standardized for testing presence of coliforms in the spiked broth. It was observed that ultra-sonication technique was the best to extract the intra-cellular enzymes. This method takes approximately 12 hrs to get the results and there is not any need to get further confirmation of results. Use of this technique can provide help to researchers and Indian Dairy Industry in saving time, space and affordable alternative to conventional method. However, for better precision and authenticity, it needs testing of large number of samples. This developed protocol needs evaluations and confirmation with raw and pasteurized milk samples.

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Genome-wide SNP identification and annotation from high coverage whole genome sequenced data of Bhadawari buffalo

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Abstract: Eukaryotic genomes are rich in Single Nucleotide Polymorphisms (SNPs) which have gained immense importance as the genetic marker of choice especially for the production traits in livestock species. The high throughput sequencing technologies, which offer quick and greater coverage genome sequencing data has improved the accuracy of SNP discovery through various bioinformatic pipelines. This is the preliminary study to uncover the SNPs from high coverage (45x) sequenced data of the unique Bhadawari buffalo breed of India. Blood was collected from two pure bred animals from the breeding tract and DNA was sequenced using Illumina Highseq technology. The sequences were aligned to the buffalo reference genome and variant calling was done. The variant count (SNPs, Insertions, and deletions) discoverable at six ascending read depths (2,5,10, 20, 30 and 40) have been analysed. The Transition to Transversion ratio (Ti/Tv ratio) is found to be nearly 2.2. Chromosome wise distribution of SNPs showed that in all read depths maximum number of SNPs were found to be in the 1st chromosome of Bhadawari buffalo genome.

Keywords: Bhadawari buffalo, Whole genome sequencing, SNP, Read depth

Introduction

Buffaloes are hardy animals maintained as a source of income in the households of marginal farmers of India. The technical barriers for scientific upgrading of indigenous buffaloes have been greatly overcome in the last few years (Kumar et al. 2019). Buffalo genome, especially that of the Indian buffaloes other than Murrah buffalo

remain unexplored to a great extent. Considering the decline in the population of pure indigenous breeds due to intense cross breeding, we are at the verge of losing the diversity of the indigenous buffalo genomes shortly. Especially for the breeds with peculiar properties like Bhadawari. Bhadawari buffaloes are reported to be the Indian buffalo breed with highest milk fat percentage. These animals are reported to be economical for maintaining due to their hardy nature and have less calf mortality rate. Their populations are mainly concentrated in the Indian states of Uttar Pradesh and Madhya Pradesh and scattered in northern parts of India (Pundir et al. 1996). The population of this breed is declining and has reached few thousands in recent years. Recent studies have evaluated the mixing of Murrah genome to Bhadawari due to cross breeding (Tyagi et al. 2021).

The upliftment of a particular breed with a declining population like that of Bhadawari should be done in an intensive manner. Even though selective breeding and improvement can be an option, the time and cost required for such programs limits its usage in comparison to the genomic selection (Choi et al. 2013). Among the various technologies for unveiling the valuable genomic areas of the large animal genome, Whole genome sequencing give benefits like uniformity of read coverage and detecting the polymorphisms outside the coding regions (Meynert et al. 2014). Single Nucleotide Polymorphisms are emphasized now a days for analysing the variations in the genome due to their abundance in the genome (Schultz et al. 2020). Detection of structural variants in livestock carries many challenges and bias due to the quality of the reference assembly, false positives in annotation and difficulty in detecting karyotype errors (Bickhart and Liu, 2014). Most research rely on low coverage data due to the high expense of whole genome sequencing. However, this may result in an error rate more than 15%, affecting further downstream analysis such as Runs of homozygosity (ROH) area detection, emphasising the significance of high coverage data (> 30x) to yield more accurate results. (Ceballos et al. 2018). Recent studies suggest that accuracy and number of structural variants per bovine sample increased as the coverage of the short read whole genome sequences data increased from 15x to 47x (Lee et al. 2023).

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This is a preliminary and first study to uncover the Variants (SNPs and InDels) from high coverage whole genome sequenced data from Bhadawari buffalo breed. The results of this study pave light to the comparison of potential variants in this buffalo breed's genome along with the assessment of counts of SNPs at different filtration level which can only be done using high coverage sequenced data.

Materials and methods

DNA isolation and sequencing

Genomic DNA was isolated by Phenol Chloroform method (Sambrook and Russel, 2004) from the blood collected from two healthy Bhadawari Buffaloes from the breeding tract in Etawah district of Uttar Pradesh. Illumina HighSeq 2000 technology was used to sequence the 150bp paired end libraries to obtain high quality sequenced data.

Pre-processing of data and Mapping

The quality of the whole genome sequenced data was evaluated by FastQC v0.12.1 and quality control was performed by Trimmomatic version 0.39. The quality-controlled reads were mapped to reference genome (NDDB_SH_1) of Murrah buffalo using BWA mem (BWA v 0.7.17).

Variant calling and annotation

Genome wide variants were identified using Samtools (samtools v 0.1.19) and BCFtools (bcftools v 0.1.19). Samtools was used for the conversion of sam file to bam format followed by sorting, indexing, and merging the bam files. The conversion to vcf format was done using Bcftools. The variants were filtered using Vcftools (vcftools v 0.1.16) with a PHRED >30 and keeping maximum read depth 500 and minimum as 2,5,10,20,30,40.

Screening of SNPs annotated with genes related to Fat percentage in buffalo

Candidate genes associated with Milk fat percentage was searched from the literature (Vohra et al. 2021, Deng et al. 2016, Ferritas et al. 2016, Dubey et al. 2015, Tanpure et al. 2012) and the SNPs detected from Bhadawari buffalo genome annotated with these genes were enumerated.

Results and Discussion

Detection of Variants (SNP and InDels)

45X coverage data was remaining after quality control and adapter removal of the 54 X coverage raw sequenced data. Leading and trailing bases with a quality score less than 5 and reads with a length less than 50 bp and PHRED score less than 33 was removed in this process. The quality-controlled reads of the samples on alignment with *Bubalus bubalis* reference genome (NDDB_SH_1) showed 99.86% and 99.88% mapping rate. The number of SNPs annotated at 6 different filtration levels from Bhadawari buffalo genome are shown in Table 1. The total number of SNPs and InDels were in near range upon filtration at read depth 2, 5, 10 and 20. The number of SNPs annotated was 12,104,222; 10,607,561 and 6,724,029 at read depth 20, 30 and 40. The number of InDels annotated were 1,135,489; 982,999 and 614,616 at read depth 20, 30 and 40 read depth.

In a similar study conducted on a large scale with 71 buffaloes, about 28,347,965 SNPs were detected from 12x coverage data with less than 2 read depth (Chen et al. 2023). In all read depths, largest number of SNPs were detected from chromosome number 1 (Table 2) of the genome followed by chromosome number 2. Majority of the SNPs were in the Intronic region just like the results studies in buffalo (Chen et al. 2023) and other large eukaryotes (Hedayat-Evrigh et al. 2020). The percentage of variants in intronic and intergenic regions were 67.4% and 19.145% respectively (Figure 1). Nearly similar percentages of 61.9 % and 17.71% are reported from ddRAD data of sixty-three Murrah buffaloes (Mishra et al. 2020). In other bovine species including cattle, 6 million SNPs were detected from 15x coverage were data of a single animal at read depth less than 5 (Kawahara-Miki et al. 2011). These results clearly suggest the increase in SNP counts at higher coverage. When the number of purebred animals is less the accuracy and efficiency of detection of variants should be improved by a higher coverage data.

The missense (28.4%) to silent (71.3%) ratio was 0.3 ratio 986 (Table 3). The transition to transversion ratio (Ti/Tv ratio) indicating the rate of substitution mutations (Wang et al. 2014) is also considered as a quality parameter for high throughput sequencing studies (Durbin et al. 2010). The transitions (10,904,964) and transversion (4,897,340) ratio was found to be 2.2 in the current study. A ratio of 2.0-2.1 is mentioned for WGS

Table 1: Number of Single Nucleotide Polymorphisms and Insertion- Deletions (SNPs & InDEls) remaining after filtration at read depth (RD) 2,5,10, 20,30 and 40 from the whole genome sequenced data

Species	<i>Bubalus Bubalis</i> (Bhadawari)					
	RD2	RD5	RD10	RD20	RD30	RD40
SNPs	12,361,170	12,351,047	12,323,806	12,104,222	10,607,561	6,724,029
Ins	557,662	557304	555441	540512	468734	296098
Del	611,371	611045	609529	594977	514265	318518
Total variants	13,530,203	13519396	13488776	13239711	11590560	7338645

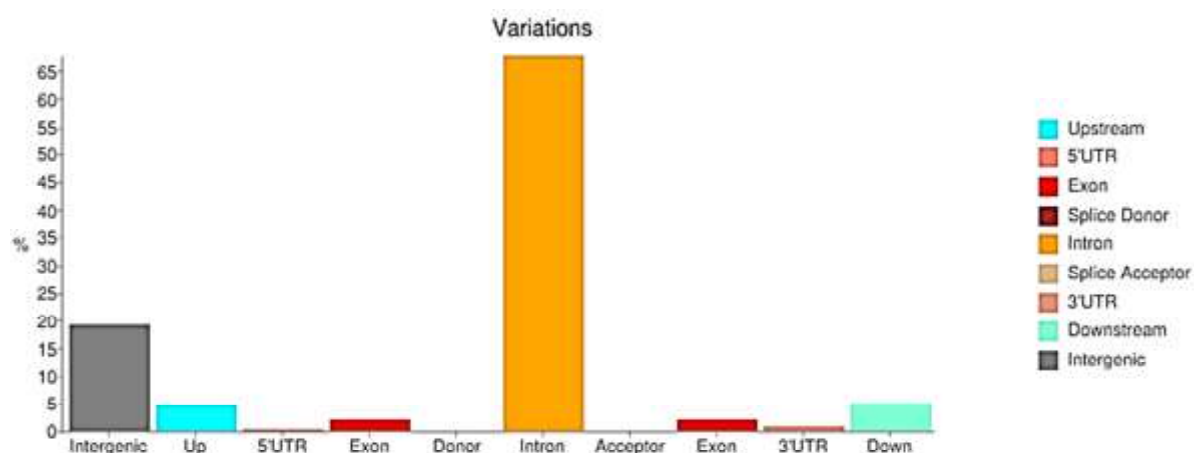


Fig. 1 Percentage of total variants region wise

Table 2: Chromosome wise distribution of SNPs

Chromosome number	Number of SNPs detected at different read depth		
	20	30	40
1	968746	849557	536281
2	858202	753298	479808
3	773600	676048	423107
4	770277	676700	433880
5	616289	540124	342834
6	542018	473920	296750
7	565283	497268	315029
8	555100	487101	307756
9	521672	456142	286810
10	497119	438000	281151
11	473976	415015	263430
12	470116	410797	255327
13	540852	476746	323856
14	374621	326247	201428
15	392421	344207	215738
16	427299	370366	232427
17	353463	309368	194473
18	294834	257556	161822
19	367546	322419	202586
20	324520	282285	175991
21	276071	240628	149460
22	308793	270124	167966
23	257032	225038	140742
24	198184	172223	104650
X	376141	336337	230680
Mitochondrial	47	47	47
Total	12104222	10607561	6724029

data by the International Genome Sample Resource (<http://www.1000genomes.org/>). **Variant annotation**

Table 3: Number of SNPs annotated along with the genes related to milk fat percentage from the Bhadawari buffalo genome

Sl No	Reported gene IDs associated with fat percentage in buffalo	Gene name	The dairy performance pathways and processes associated with the gene	No. of SNPs in Bhadawari genome annotated for the gene
1	CAMTA1	Calmodulin Binding Transcription Activator 1	Fatty acid metabolism (Vohra et al.2021)	3603
2	CCSER1	Coiled-coil serine rich protein 1	Growth and feed efficiency in beef cattle (Abo-Ismael et al. 2018)	7044
3	DGAT1	Type I Diacylglycerol O-acyltransferase	Glycerol 3 phosphate pathway (Khan et al. 2021)	72
4	LEP	Leptin gene	Regulation of bone remodeling (Haruna et al. 2021)	86
5	MC4R	Melanocortin 4 Receptor	Energy metabolism and regulation of feeding behaviour and metabolism (Deng et al.2016)	91
6	SCD	Stearoyl-CoA Desaturase	Saturated fatty acid metabolism (Rincon et al. 2012)	213
7	SREBF1	Sterol regulatory element binding protein 1	SREBF1 pathway, De novo synthesis of saturated fatty acids (Rincon et al. 2012)	60
8	STAT1	Signal transducer and activator of transcription 1	Tri glyceride synthesis (Thaller et al.2003)	188
9	TG	Thyroglobulin	Lipid metabolism (Kaczor et al.2017)	13067
10	ETS2	ETS proto-oncogene 2	Expression of other genes in mammary epithelial cells (Anderson et al. 2007)	97
11	ROR1	Receptor Tyrosine Kinase Like Orphan Receptor1	Mammary gland development (Dillon et al. 2002)	1708
12	CACNG6	Calcium voltage -gated channel auxiliary subunit gamma 6	Calcium channel stabilization (Lee et al. 2010)	119
13	SH3BP5L	SH3 binding domain protein 5	Membrane transport of lactose (Lopdell et al.2017)	59
14	ZNF672	Zinc Finger Protein 672	Energy metabolism (Zhou et al. 2022)	22

SNVs annotated with the genes reported to be related to milk fat percentage were enumerated in Table 3. Among them largest number of SNPs were found to be affecting the gene Thyroglobulin (TG) followed by CCSER1 and CAMTA1. The genomic regions associated with the milk fat related genes ETS2, ROR1, CACNG6, SH3BP5L, ZNF672, CAMTA1, CCSER1 and DGAT1 were found from Genome wide association study of Murrah buffalo (Vohra et al. 2021 & Ferritas et al. 2016). Similarly, the SNPs associated with LEP gene was annotated in earlier studies from Mehsana (Tanpure et al. 2012), MC4R from Chinese

buffalo, SCD from Nileravi buffalo, SERBF1 from Nileravi× Guanxi buffaloes (Deng et al. 2016) and TG from Mehsana and Nileravi buffaloes (Dubey et al. 2015).

Conclusions

This was the first study which explored the Bhadawari buffalo genome to identify the genetic variants through high coverage next generation sequencing technology. The variants were screened at higher read depths of 20, 30 and 40 to get an SNP count of 12, 10.6 and 6.7 million SNPs from 45x coverage data.

Chromosome number 1 harbours most of the SNPs in the Bhadawari genome. Our study suggests to incorporate higher coverage data for variant calling for keeping the accuracy of downstream processing. Further studies with a large number of Bhadawari animals is recommended in future for the genome wide association of the variant positions that are annotated in this study.

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RESEARCH ARTICLE

Genetic blueprinting of novel and performance traits-related SNPs in Indian Gir cattle using latest reference assembly

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Abstract: Among the indicine breeds of cattle, Gir breed thrives in challenging arid habitats, produce more milk with less feed intake and are resistant to tropical diseases. Identification of novel and bias free SNPs are important to include in genetic studies and breeding programs. The current study aimed to explore SNPs in Gir cattle genome followed by annotation of the SNPs to different performance traits. Double-digest restriction site associated DNA sequencing (ddRAD-seq) was done and different bioinformatic tools and software used to mine SNPs and subsequent annotation to different performance traits. A total of 53,243 high quality SNPs were identified using the latest ARS-UCD 1.3 reference genome (GCF_002263795.2). In all, 2,931 SNPs were annotated to 452 candidate genes related to nine different traits of interest such as, milk production, reproduction, adaptation, disease-resistance, growth, carcass, coat thickness, coat colour, and domestication traits. ddRAD-seq offers mining of ascertainment free SNPs in a cost-effective way and hence the SNPs obtained in this studied population can be successfully used for population genetic studies and in breeding programs. The information mined in this study will aid in genetic improvement, designing appropriate breed improvement programmes and leveraging conservation efforts.

Keywords: ddRAD-seq, Gir cattle, Performance traits, SNPs

Introduction

Agriculture is the basic substance of the Indian rural economy with livestock rearing as its lifeline. In India 53 zebu cattle breeds have been registered, including the major milch purpose breeds viz., Sahiwal, Gir, Red Sindhi and Tharparkar cattle breeds (www.nbagr.res.in). The Gir cattle breed is native to Gir forests in Kathiawar region of Gujarat (Gaur et al. 2003). Temperature in their habitat ranges from 7-45°C with an average rainfall of 500-1,500 mm (IMD, 2023). Morphologically, they have leaf-like and the longest ears, sleepy eye appearance, most convex head, largest hump and widest coat colour variation among all Indian cattle breeds (AGRI-IS, 2023). Gir cattle are known for high milk production as compared to other Indian breeds, robustness to stressors as well as tropical diseases and have ~95% A2 allele frequency (Sushil et al. 2018). Gir cattle have acquired cross-country importance shaping Gyr, Girolando and Indubrasil breeds. However, Gir cattle have a lactation yield of about 2,110 kg in India while the one bred overseas, in Brazil has a lactation yield of 3,500 kg (Madalena et al. 2012). Stagnation in productivity and shrinking population size are the main concerns and call for genetic interventions to conserve and bridge the demand-supply gap vis-à-vis growing human population. However, cattle genetics and allied technologies such as SNP arrays and SNP databases developed so far show ascertainment bias (Stothard et al. 2011; Koks et al. 2014; Iqbal et al. 2019) and moreover, exclude discovery of breed-specific or rare variants (Sivalingam et al. 2020; De Donato et al. 2013). Ascertainment bias free markers aid in breed improvement programmes and leveraging conservation efforts. (Jeevan et al. 2019; Kanaka et al. 2023).

Whole Genome Sequencing (WGS) overcomes ascertainment bias but is a costly venture and computationally demanding. Restriction enzyme-based DNA sequencing (RAD-seq) can account for a large number of genetic markers in both referenced and non-referenced organisms and covers up to 40% of the genome (Mishra et al. 2020). Hence, we used double-digest restriction site associated DNA sequencing (ddRAD-seq) (Peterson et al. 2012) which employs rare as well as frequent cutters in this study to omit repetitive and uninformative sequences. Erstwhile, genome-wide identification of SNPs using RAD-seq in seven cattle breeds (Malik et al. 2018), Sahiwal

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(Vineeth et al. 2020), Red Sindhi (Iqbal et al. 2019), Vrindavani (Wara et al. 2019) and Tharparkar (Devadasan et al. 2020) cattle breeds have been exercised in the Indian context. However, there is dearth in literature on genome-wide SNPs annotated to candidate genes for different traits in Gir cattle. This catalyzed our study to discover and thereafter, annotate genome-wide SNPs using ddRAD approach with genes related to performance traits in Gir cattle.

Materials and methods

Ethics Statement

The collection of blood samples from animals in this research project was conducted with the highest regard for animal welfare and ethical principles. The procedures were approved in 43rd Institutional Animal Ethics Committee meeting, in compliance with established guidelines and regulations (43-IAEC-18-9).

Blood collection and DNA isolation

Blood samples, 10 mL each, from seven adult unrelated Gir cows from the Livestock Research Centre at the National Dairy Research Institute in Karnal, Haryana, India (29.704°N Latitude, 76.982°E Longitude) were collected in vacutainer tubes coated with 0.5% EDTA (Ethylene Diamine Tetra Acetic Acid). The genomic DNA was isolated using the phenol-chloroform method as described by Sambrook and Russell (2006).

Library Preparation

The standard RAD protocol (Peterson et al. 2012) was employed for the further sequencing of the DNA after initial evaluation of quality and quantity. The DNA was subjected to double digestion using SphI and MluCI restriction enzymes for constructing the library and thereafter, custom sequencing was done in SciGenom Labs Pvt. Ltd, Cochin, Kerala using Illumina HiSeq 2000 platform.

Bioinformatics analysis

SNPs calling

SNP identification was carried out using the standard procedure (S1). The raw sequencing reads in FastQ format were screened using FastQC v0.11.9 (Andrews et al. 2010), then adapter sequences and barcodes were removed from the sequence reads using PRINSEQ v0.20.4 (Schmieder et al. 2011). STACKS v2.61 (Catchen et al. 2011) was utilized to remove sequences that lacked the restriction enzyme cut site and had a Phred score below 15. The QC-approved reads were then aligned with the *Bos taurus* (ARS-UCD 1.3) reference genome (RefSeq accession: GCF_002263795.2) using *local very sensitive* mode using Bowtie2 v2.5.0 (Langmead and Salzberg, 2012). The alignment resulted in SAM (Sequence Alignment Format) files, which were then converted to BAM (Binary Alignment Format) files, sorted,

indexed, and merged to produce a single BCF (Binary Call Format) file using BCFtools v1.16 (Li, 2011). BCF file was converted to VCF (Variant Call Format) equivalent and genome-wide SNPs and InDels were further detected at read depth (RD) thresholds of ≥ 2 , 5, and 10 with a mapping quality (MQ) ≥ 30 using VCFtools v0.1.16 (Danecek et al. 2011). SNPs were separated from InDels and only those obtained from the aligned reads with ≥ 10 RD were subjected to further quality control to obtain high-quality SNPs, such as Hardy-Weinberg equilibrium (0.001), Minor Allele Frequency (0.01), and Missing Genotypes (1.0), using VCFtools v0.1.16 (Danecek et al. 2011).

Annotation

The high-quality SNPs were then annotated using SnpEff v5.1 (Cingolani et al. 2012). These SNPs located within genes were investigated in the literature to find genes that are linked to performance traits. Graphical maps were generated using MG2C (Chao et al. 2021) to show the placement of SNPs on various chromosomes in relation to different traits. Mutations that were potentially harmful with a SIFT (Sorting Intolerant from Tolerant) score of ≤ 0.05 were recognized and annotated to the relevant protein-coding genes using Variant Effect Predictor, VEP v108.2 (McLaren et al. 2016). Additionally, new SNPs were discovered using VEP v108.2.

Results and Discussion

The objective of this study was genome-wide SNP identification and annotation of SNPs to performance traits in seven Indian Gir cattle using ddRAD-seq. Previous works supporting similar sample size ($n=7$) include Malik *et al.* (2018) and Devadasan *et al.* (2020). On an average, 1.95 million raw reads were obtained per sample. In all, 13.67 million raw reads were sequenced (Table 1). After de-multiplexing, adapter trimming and quality control of raw reads, 98.18% good quality reads were retained (Table 1). The quality reads were aligned with the latest ARS-UCD 1.3 *Bos taurus* reference genome, with an overall alignment rate of 99.85% (Table 1). The number of sequences in two samples were comparatively lower than the other samples (Table 1) due to differences in read depth because of poor DNA quality similar to Malik *et al.* (2018). STACKS was used for trimming of low quality reads as it checks the mean quality score using sliding windows while PRINSEQ trims across mean values (Surya et al. 2019) (Figure 1).

Overall, 254,128 genome-wide variants, inclusive of 211,560 SNPs were mapped to ARS-UCD 1.3, being the latest and representative assembly. Out of them, a total of 53,243 high quality SNPs were sieved with RD ≥ 10 and MQ ≥ 30 for downstream analysis (Figure 2, Table 1, S2). The number of SNPs found are in line with Altmann *et al.* (2012). The number of high quality SNPs is greater than reported 9,638 in Vrindavani crossbred cattle (Wara et al. 2019) and 18,056 in Murrah buffaloes (Jaglan et al. 2023) using

Fig. 1 Schematic representation of steps and tools followed in this annotation

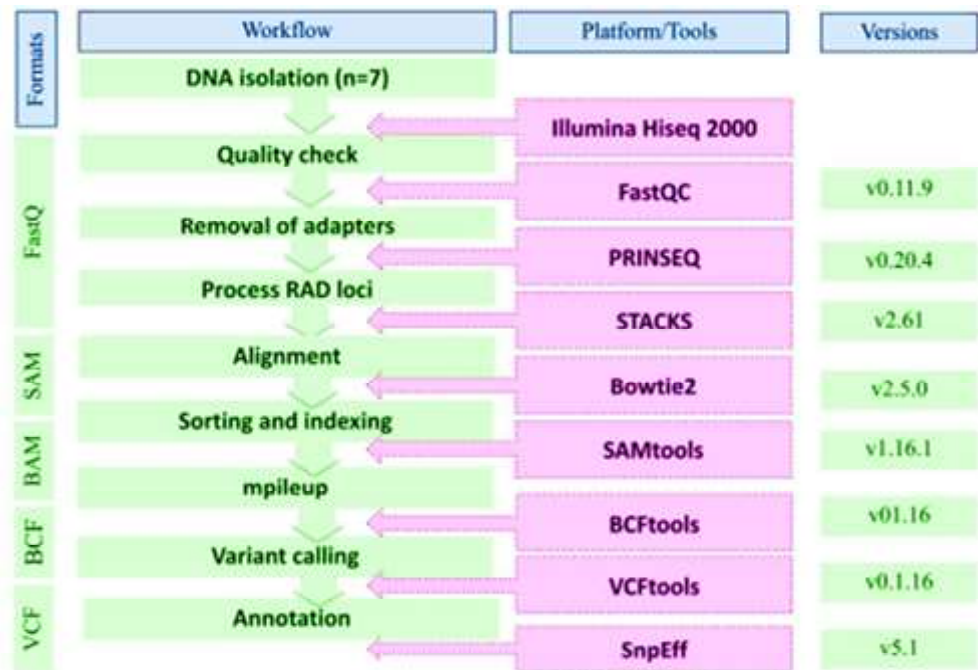


Table 1: Number of raw, processed and aligned reads of Gir Cattle. AR - Alignment Rate, UAR - Unique Alignment Rate. Reads retained = 249,139; Reads removed = 1.82%; Reads retained = 98.18%

No.	Pre-processing of reads			<i>Bos taurus</i> (ARS-UCD 1.3)			
	ID	Raw reads	QC-passed reads	No. of aligned reads	Total aligned reads (in %)	AR (%)	UAR (%)
1	G-1	765,970	754,342	743,780	98.60	99.85	17.42
2	G-2	3,310,806	3,223,269	3,180,160	98.66	99.77	21.55
3	G-3	3,305,652	3,249,045	3,197,532	98.41	99.92	21.82
4	G-4	1,283,522	1,264,553	1,246,962	98.61	99.89	23.03
5	G-5	840,808	829,157	818,436	98.71	99.72	22.45
6	G-6	541,836	533,368	525,862	98.59	99.95	22.42
7	G-7	3,626,672	3,572,393	3,536,918	99.01	99.84	22.24
Average		1,953,609	1,918,018	1,892,807	99	99.85	22

the same QC parameters except missing genotypes > 0.8 and > 0.65. The higher number of SNPs despite stringent filtering implies higher polymorphism in Gir cattle. In concordance with Sivalingam et al. (2020) and Kumar et al. (2020), G/A and C/T substitutions were higher than other base changes. Frequent C > T mutations may be due to the deamination of methyl cytosines in CpG dinucleotides (Jaglan et al. 2023). Evidently, transitions were 2.6' more likely than transversions in line with targeted sequencing approaches (Keller et al. 2007; Patel et al. 2017; Ba et al. 2017; Kraus et al. 2011). Average inter-SNP distance in Gir was 49 kb, which was similar to 45 Kb in Indian cattle breeds identified by genotyping by synthesis method (Malik et al. 2018). Maximum numbers of annotated SNPs were discovered in the transcript region, followed by intron and intergenic regions (Table 2), which was consistent with prior reports on SNPs annotation in riverine buffalo (Surya et al. 2019) and Tharparkar cattle (Devadasan et

al. 2020). Based on effects by functional class, SNPs within the coding regions were classified as missense (347), silent (627) and nonsense (5).

In contrast to the existing variants, 3,824 (7.2%) SNPs were reported as novel (S). A total of 27 missense variants having SIFT scores d" 0.05 were adjudged deleterious. The deleterious SNPs were annotated to 21 protein-coding genes (S2). On an average, Ts/Tv (Transition and Transversion) ratio of the annotated SNPs was 2.7603 per sample (Table 3). The frequency of G/A and C/T transition substitutions were markedly higher than other substitution types (Figure 3). Sizeable differences were seen in between the number of SNPs in different traits, with maximum number of genes (n=176) and SNPs (n=1,191) traced for the traits related to milk quality and quantity, are in line with Mishra et al. (2020). The deleterious SNPs enumerated (n=27 SNPs) are more than those found in Sahiwal cattle, i.e., 18

Table 2: Region-wise distribution of SNPs

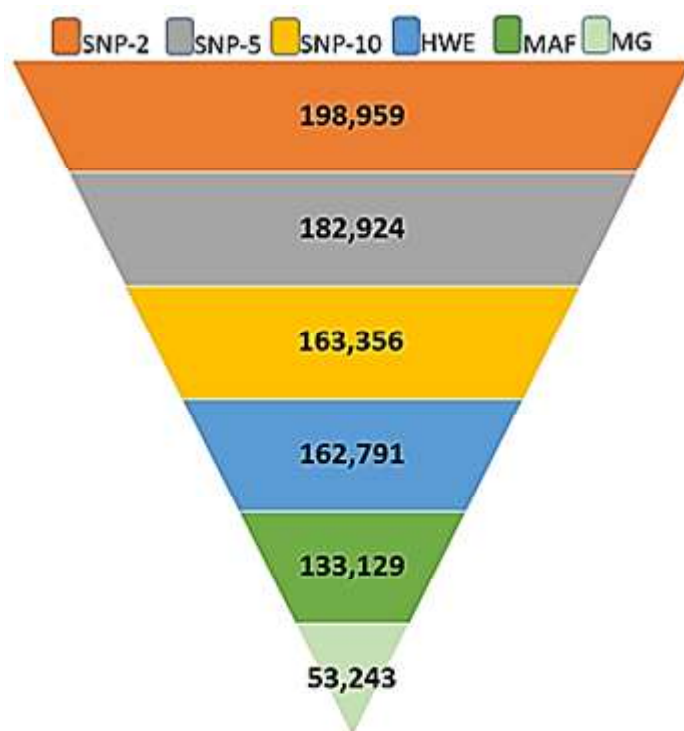
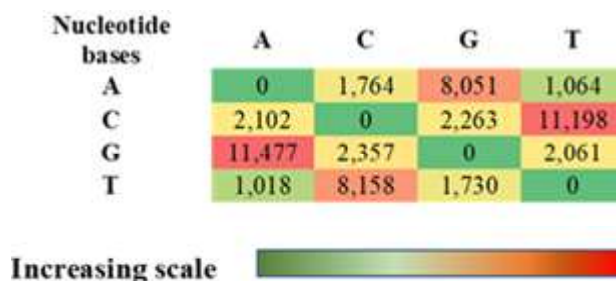
Region	Count	Percent
Downstream	13,962	5.80
Exon	1,361	0.57
Intergenic	29,168	12.12
Intron	90,249	37.51
Splice_site_acceptor	1	0.00
Splice_site_region	123	0.05
Transcript	90,984	37.82
Upstream	13,427	5.58
UTR_3_prime	1,104	0.46
UTR_5_prime	219	0.09

Table 3: Nucleotide base changes in identified SNPs

Nucleotide Change	Count
Transitions	207,345
Transversions	75,116
Ts/Tv ratio	2.7603

deleterious SNPs (Surati et al. 2023), but less than those reported by Kour et al. (2022) in Indian yak (n=166 SNPs). This is suggestive of lesser burden of deleterious variants in Gir cattle due to adaptive selection over many generations. A total of 3,824 novel SNPs were identified, justifying the usage of ddRAD-seq over SNP chips. The novel variants are much lower than 10,519 SNPs reported by Devadasan et al. (2020) may be due to stringent QC applied in this study.

A total of 2,931 SNPs were annotated to 452 genes belonging to different traits in the current study (Table 4, Figure 4(i and ii)). Overlapping genes between different traits are given in Figure 5. Devadasan et al. (2020) reported 2,871 SNPs in 383 genes despite greater number of SNPs (n=87,047) derived without quality control. This reflects genomic richness of Gir cattle as compared to Tharparkar cattle in terms of polymorphisms. Some of the important genes annotated are discussed here. Pertinent to milk composition, SNPs were mapped in candidate genes, *COL22A1*

**Fig. 2** SNPs retained after applying quality control. SNP-2: read depth 2, SNP-5: read depth 5, SNP-10: read depth 10, HWE- Hardy-Weinberg equilibrium, MAF- Minor Allele Frequencies, MG- Missing genotype**Fig.3** Nucleotide base changes in identified SNPs**Table 4:** Number of polymorphisms and genes associated with different categories of traits

Traits	SNPs	Genes	Genes with the highest polymorphisms
Production	1,191	176	<i>TG</i> (n=46), <i>RORA</i> (n=38)
Reproduction	729	72	<i>RBFOX1</i> (n=153), <i>RBMS3</i> (n=36)
Disease	615	84	<i>PLXNA4</i> (n=69), <i>PRKG1</i> (n=30)
Adaptation	380	68	<i>CTNNA2</i> (n=41), <i>PRKCB</i> (n=22)
Growth	82	17	<i>PCNX2</i> (n=22), <i>LCORL</i> (n=17)
Carcass	922	101	<i>RBFOX1</i> (n=153), <i>GALNTL6</i> (n=51)
Coat colour	102	25	<i>LCORL</i> (n=17), <i>DOCK8</i> (n=9)
Coat thickness	35	10	<i>TCF7L1</i> (n=9), <i>CUX1</i> (n=9)
Domestication	183	18	<i>ASTN2</i> (n=54), <i>LINGO2</i> (n=30)
Total	4,239	571	
After removing overlaps*	2,931	452	

* Total number of SNPs and genes may not be equal to the arithmetic sum due to the presence of overlaps

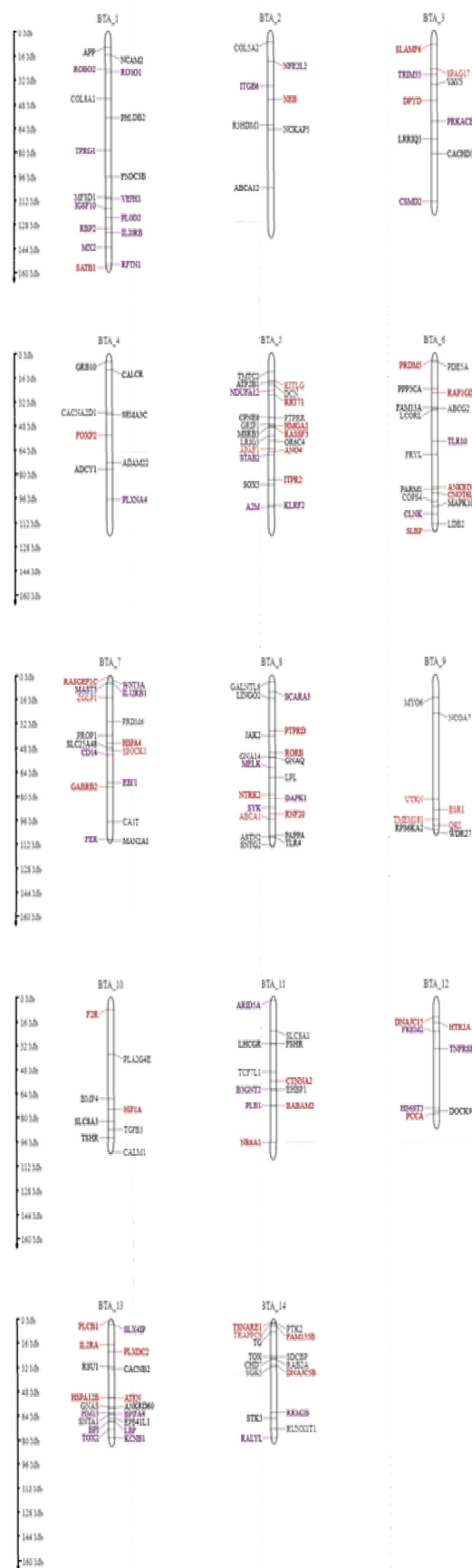
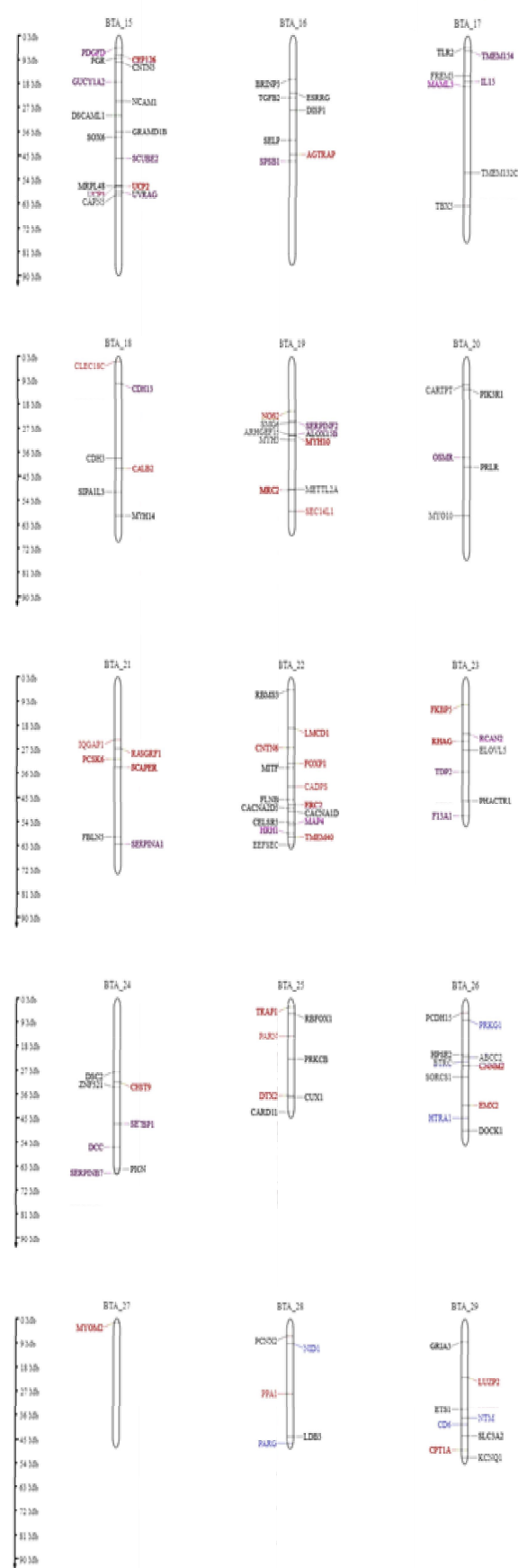


Fig. 4(ii) Chromomap displaying genes and there position on respective chromosomes (BTA 15 to BTA 29). Black-Coat thickness; Blue-Disease; Brown-Growth; Green-Domestication; Orange-Adaptation; Pink-Coat Color; Purple-Carcass; Violet-Reproduction; Red-Production.



and *PTK2* for biological pathways affecting milk fat in dairy cattle (Wang et al. 2013; Buitenhuis et al. 2014). Leptin, a peptide hormone encoded by the *LEP* gene, plays a role in growth, milk production and fertility traits (Clemenson et al. 2011). *IGFBP2* gene influences fertility and milk production in cattle (Klein et al. 2006; Pimentel et al. 2011). Dairy cattle's milk fat and protein percentage are found to be influenced by nucleotide base changes in the *LPIN1* gene (Han et al. 2019). The *EPS8* gene is linked to milk output and milk fat percentage (Raven et al. 2014) as it participates in milk fat biosynthesis in the lactating mammary gland by acting through epidermal growth factor (Chatterjee et al. 2009).

The highest number of SNPs were found in *RBFOX1* gene (n=153), which regulates expression of large genetic networks during early neuronal development (Bill et al. 2013). The *TG* gene (n=46) related to production, encodes thyroglobulin protein, which serves as substrate for the production of thyroxine and triiodothyronine as well as storage form of thyroid hormone and iodine in their inactive forms (Vassart et al. 1985). *PLXNA4* (n=69) is predicted to enable semaphorin receptor activity, TLR signalling and related to sepsis induced cytokine storming (Wen et al. 2010) and is linked to Alzheimer's disease and Parkinson's disease in humans (Schulte et al. 2013; Jun et al. 2014). Another disease related gene, the *PRKG1* gene (n=30) is a crucial mediator of the nitric oxide/cGMP signalling pathway in different cells (Ørstavik et al. 1992). Highest number of polymorphisms in adaptation related genes were found in a tumor suppressor gene *CTNNA2* (n=41) (Fanjul-Fernández et al. 2013) and in a protein kinase known as *PRKCB* (n=22) has been linked to numerous physiological processes, including activation of B cells, induction of apoptosis, proliferation of endothelial cells, and absorption of intestinal sugars (Philippi et al. 2005). *ASTN* (n=54), belonging to astrotactin gene family controls the movement of *ASTNI* during the migration of glial-guided neurons (Wilson et al. 2010) and *LINGO2* (n=30), predicted to act upstream of or within positive regulation of synapse assembly (Su et al. 2010) displayed highest polymorphism related to domestication. The earlier studies support the SNPs found in the Indian Gir cattle with different performance traits.

Conclusions

This study accounts for the richly polymorphic candidate genes related to varied performance traits. The novel variants reported may provide a sizable addition to Gir genetics, which can be further included in existing SNP chips. Overall, this study offers ascertainment bias free markers which may aid in breed improvement programmes and leveraging conservation efforts. The SNPs mined in the current study may need to be validated in larger herds using association studies.

Authorship Contribution Statement

Archana Verma and Jayakumar Sivalingam conceived and designed the study. Anjali Choudhary, M. Joel Devadasan, conducted experiments. Anjali Choudhary, M. Joel Devadasan, D Ravi Kumar, Vineeth M. R., Surya T., Nidhi Sukhija, Kanaka K. K. conducted bioinformatics and data analysis. Nidhi Sukhija, Kanaka K. K. drafted manuscript. Archana Verma, Jayakumar Sivalingam and Niranjana S. K. provided critical inputs during the data analysis and manuscript preparation.

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RESEARCH ARTICLE

Multi-stakeholders' perception in popularization and dissemination of Farmer-led Innovations in Northern India

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Abstract: Agro-ecosystems are subjected to a variety of biotic and abiotic challenges, particularly in developing nations. Farmers' innovations are more quickly adopted by other farmers since they are affordable, accessible, locally relevant, and proven in a real farm setting. The proposed study was conducted in Haryana and Punjab, as these two states hold the greatest number of innovative farmers in India in dairy sector as compiled from different secondary sources (ICAR, NIF, NRDC, TIFAC, NABARD). Each state had two districts chosen at random. A sample size of 70 dairy farmers from each district and 20 Research and Development sector persons (public and private) were selected, thus constituting a sample size of 360 for the study. Exploratory factor analysis (EFA) was carried out using IBM SPSS 20 and the principal component analysis (PCA) using oblique rotation technique. The final regression model explained 74.60% of the variation and was significant ($P > 0.001$). Weighted Mean calculation was done to know how the stakeholders perceive the importance of different attributes of FLIs. Stakeholders were giving prominent importance to the costs of the innovation followed by the proper documentation of the innovations by any sort of internal/external agencies. Our results show that farmer-led innovations generally have a positive effect on welfare, which is consistent with growing arguments that these innovations which have often received less attention than

externally pushed technologies should be encouraged as a supplement to efforts to improve food security and eradicate rural poverty.

Keywords: Perception; Stakeholder; Innovation; Scaling up; Diffusion

Introduction

Research and development (R&D) efforts have led to new ideas, innovations, products, and technology that have significantly shaped agriculture and related industries, with a high social rate of return to investments made (Alston, 2010). However, the results of these initiatives won't be fully realized until the farming community has access to the new information and tools with their ability to connect with the knowledge, institutions and networks, essential to improve their food security, productivity and livelihood opportunities (World Bank, 2011). The links between innovation development to its dissemination, users and support mechanisms must therefore be strengthened in order to facilitate the generation and transfer of innovations. This creates a networking system among all stakeholders that results in a dynamic innovation system.

Innovations in various production methods, markets, and related activities are important drivers of agricultural growth and the advancement of its inclusivity. An idea, behaviour, or other phenomenon that a person or other unit of adoption sees as novel is referred to as an innovation (Rogers, 1963). Innovation has been a lynchpin of Indian agriculture since the dawn of time, and Indian producers are no exception. Farmer-led innovations are those that are created, developed, or tested by a farmer or group of farmers on their own or using ideas from outside sources without the direct assistance of outside agencies or recognized academic institutes (Sule Akkoyunlu, 2013, Wettasinha *et al.* 2008;). Farmers have developed a number of grass-roots improvements over the course of evolution that have increased their profits and turned farming into a viable industry. Farmer-led innovation, in which farmers take the initiative to create new knowledge, technology, and working methods, is increasingly recognized as being crucial to assuring the farming industry's social, economic, and environmental sustainability (Ensor and

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Bruin, 2022). Farm innovators are those who frequently address regional difficulties and generally labour outside of established organizations (European Union, 2011; Olga, 2015; Prolinnova, 2009). The participatory farmer-led techniques in which the farmers acquire and implement the good practices from their peer group, which in turn motivates and empower them to regain control, indicating their effectiveness for generating greater prosperity and welfare. Indian farmers continuously work to make farming more efficient and economical in an effort to raise their standard of living, and these inventions over time served to enhance farming practices and provide better living possibilities. In reality, according to Roling (2009), farmers have been developing new technologies even before official scientific research for development emerged. According to some studies, some scientifically developed technologies were truly based on agricultural innovations made by local farmers. In this context, we have tried to access the multi-stakeholders' perception in the concerned study area.

Materials and methods

Haryana and Punjab states were purposively selected considering their prominence in the number of innovative farmers related to the field of dairying (ICAR, NIF, PPV & FRA, NRDC, TIFAC, NABARD, 2018). From each state randomly two districts were selected. As a whole, a total of four districts were selected. From each district, two blocks were randomly chosen comprising eight blocks as total. From each block 35 dairy farmers were selected comprising a total of 280 dairy farmers as sample. Apart from that 10 government R&D (research and development) persons and 10 private R&D persons related to the field of dairying were selected from the district level. So, a total of 280 dairy farmers and 80 R&D persons (both public and private) were selected as complete sample for the study. 360 samples in total were therefore chosen. A regression analysis was performed to understand the factors impacting innovativeness, with innovativeness as the dependent variable.

$$y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \dots + \varepsilon$$

Table 1: F ratios, standard errors, and regression coefficients of innovativeness on the most crucial factors (Total R = 74.60)

Independent Variables	Co-efficient	Standard Error (SE)	F	Cumulative R
Practical Understanding	0.654	0.231	25.40	38.5
Household expenditure administration	0.004	0.021	7.89	48.5
Managerial Aptitude	0.854	0.679	7.42	52.5
Decision Making Skills	0.472	0.203	7.23	61.3
Co-operative Contact	0.324	0.786	6.87	65.7
Cosmopoliteness	0.657	0.134	5.47	68.7
Maas Media Exposure	0.897	0.067	3.78	70.9
Extension Contact	0.243	0.954	2.98	72.5
Dairy Farming Innovations	0.564	0.654	2.32	74.6

R² = 0.7460; F = 12.89 (for whole model); P > 0.001

Where α is the intercept, β_i 's are the slope between y and the appropriate independent variable x_i , and δ is the error term. Perceptual attributes of the identified Farmer-led Innovations were measured with 37 items covering the aspects of relevancy, profitability, sustainability and adaptability. The weighted mean of the perception scale statements is calculated by multiplying the weight with the quantitative outcome and adding all the products.

Results and Discussion

Innovativeness is defined as "the skill and imagination to develop new things," which highlights its dual nature but only scratches the surface of its significance for business growth and sustainability. Studies have shown that traits like education, income, farm size, cosmopolitanism, membership in farmers' organizations, access to information, and other human factors all favour the adoption of suggested techniques (Rogers and Svenning, 1969; Rogers, 1983). A stepwise multiple regression analysis was done with 27 independent variables with innovativeness score as a dependent variable. The final regression model explained 74.60% of the variation and was significant (P > 0.001). (Table-1)

According to this model, the degree of actual knowledge is a key factor in determining how innovative a farmer is. Having management skills for budgeting, having managerial aptitude, and being more resourceful (in terms of household spending and milk productivity) were all significant predictors of inventiveness. The ability of the farmers to make decisions, their interactions with the local cooperatives, media exposure, contact with extension agents, and cosmopolitanism all had an impact on innovation.

Perception of Stakeholders towards the adoption and popularization of Farmer-led Innovations:

Perceptual attributes of the identified Farmer-led Innovations were measured through 5-point Likert scale. The stakeholders

were asked to read the initial 37 items of the scale under four aspects namely Relevancy, Profitability, Sustainability and Adaptability. KMO and Bartlett's test of sphericity was used to confirm that the data were appropriate for factor analysis. The correlation matrix has elements that need to be discovered, as shown by the KMO value of 0.903 for the perception scale. The results of Bartlett's test of sphericity, which were $2(666) = 9229$, $p 0.0001$, which tells us about their enough co-relation to support the PCA. The results of the two experiments showed that using Principal Component Analysis was adequate. The scree plot generated from PCA recognized 4 components that showed nearly 67.80% of the final scale as compared to the 30.30% of the variation explained in the initial scale. The Monte-Carlo PA software also validated the four components in a parallel analysis. The four components shaped with seven, three, three and two sub-items were also done by Oblique rotation, through a number of iterative process.

The excessive cross-loading of two or more factors resulted in removal of eight items, ten items due to the poor communalities during the extraction process, low factor loading resulted in removal of two items and two items due to the reduction in their cross-relation to other items. Pattern matrix in Table 3 shows the correlation between each item and uncorrelated components that were reinstated by using the iterative process of oblique rotation.

The four aspects of the 15-item scale that were used by PCA were named as relevance, profitability, sustainability, and adaptability in accordance with the fundamental idea expressed by the predominate items. The Cronbach's Alpha accounted for 0.857 for items of the scale measure. The analysis of the inter-item consistency revealed strong internal co-relation; 0.889 for relevancy, 0.750 for profitability, 0.710 for sustainability, and 0.666 for adaptability. On the basis of a meta-analysis, Tornatzky and Klein (1982) identified three innovation characteristics i.e., compatibility, relative advantage, and complexity—as having the strongest, most consistent correlations with innovation adoption. Additionally, Yaacoba and Yusoff (2014) indicated that factors that influence adoption of Farmer-led innovation include compatibility, trialability, outcome demonstrability, image, and visibility.

The content validity of the Perception scale was assured through methodological rigor, which included a review of relevant literature, in-depth interviews with stakeholders, and expert review. By comparing the perception measure with global satisfaction criterion items, concurrent validity was evaluated.

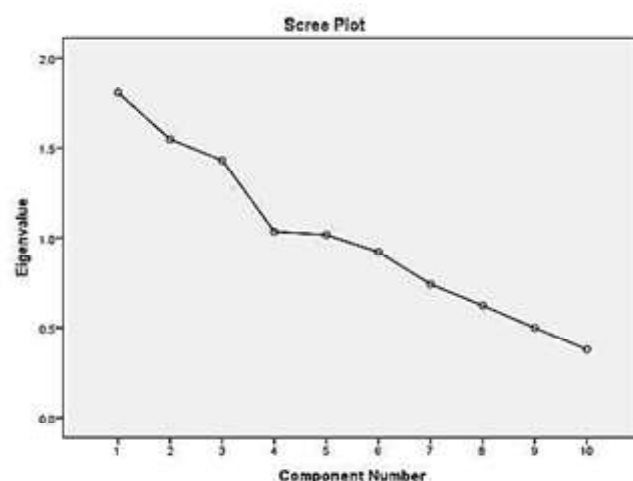


Fig. 1 Scree-plot diagram

The summated perception scale score's Pearson product-moment correlation revealed correlation coefficients of 0.711 ($p 0.001$) and 0.881 ($p 0.001$), respectively. By looking at the component's correlation matrix for the rotated end components, construct-related validity was established. As can be seen in the table below, this suggested that there was little connection among the components. We found low Pearson correlation values between the components. The factor stability of the developmental sample was confirmed using principal component analysis on a random split sample; all four factors were retained with little change in factor loading.

In order to enhance the draft tool's content coverage, the items' applicability to specific local contexts in the specified dimensions, and the scale's face and content validity, a group of experts evaluated it. By showing the stability of the four subscales (relevancy, profitability, sustainability, and adaptability) as well as the scale's high internal consistency, factor analysis supported the scale's construct validity. The observed reliability is within DeVellis's, 2003 acceptable internal consistency range. The four components identified were found to be interrelated with low correlation coefficient which stands out to be good evidence for the scales construct validity. The four factors of relevance, profitability, sustainability, and adaptability are those that stakeholders in the research region believe to be most crucial, which is further strengthened by the scale construct validity computed.

Concurrent validity was established by comparing the summated perception tool to two items from the overall satisfaction tool

Table 2: Oblique rotation statistics for factors with Eigen Values greater than 1

Factors	Eigen Value	% of Variance	Cumulative %
1	8.675	37.6	37.6
2	5.432	12.7	50.3
3	2.324	10.7	61.0
4	1.321	6.8	67.8

that were thought to be closely linked to perception. Concurrent validity was demonstrated by the two measures' highly significant correlations (0.711, $p < 0.001$ and 0.881, $p < 0.001$). Due to the substantial association between the perception scale and measures of overall satisfaction, it is clear that stakeholders who are happy with relevancy and profitability of the Farmer-led innovations have favorable attitude towards its adoption. In response to these shifts, there has been an increase in interest in farmer-led innovation. It is now clear from a growing body of literature (Naouri *et al.* 2020; van Dijk *et al.* 2017; Waters-Bayer *et al.* 2015; Macmillan and Benton, 2014; Lowe *et al.* 2019; Tambo and Wünscher, 2017;) that the governmental agencies and different stakeholders which includes the European Commission, 2016 and the UK's Department for Environment, Food, and Rural Affairs, are coming forward in promoting these Farmer-Led Innovations in a wider scale. (Innovative Farmers, 2020).

Four components were loaded with the 15 items from the final perception measure. To the components that were recovered, the qualities of relevance, profitability, sustainability, and adaptability were given. The scale's criterion-related validity was shown by the final perception scale's high correlation with the global satisfaction ratings. The process of creating the item guaranteed the authenticity of the procedure. Low correlation between the components and high average factor loadings of 0.76 to 0.82 supported the construct validity of the perception measure. To verify the scale's stability, a randomly selected split sample of 120 samples from the validation sample was used. With a score of 0.845, the final 15-item measure demonstrated adequate reliability.

The 15-item perception scale based on relevance, profitability, sustainability, and adaptability was discovered to be a legitimate and dependable indicator of how stakeholders view the

Table 3: Pattern matrix Perception scale

S.N.	Statements	Components				Communality	Component label
		1	2	3	4		
1.	FLI,s helps to enhance farmer's backward and forward linkage with several enterprises.	0.811				0.724	Relevancy
2.	FLI,s helps farmers to empower themselves and their community.	0.792				0.669	
3.	Farmers are not aware of the commercialization process of the FLI,s.	0.789				0.604	
4.	The relative analysis of the costs associated with the innovation was considered.	0.777				0.677	
5.	We must consider refinement and simplification of the innovation for better dissemination.	0.731				0.632	
6.	The potential of the innovation for achieving economies of scale was analysed.	0.724				0.598	
7.	The estimation of the innovations comparative impact and achievement was done.	0.703				0.599	
8.	Ensure proper documentation of the innovation/intervention.		0.826			0.725	Profitability
9.	The innovations discourse a potential need for the stakeholders in the region.		0.820			0.765	
10.	Less number of people were involved in the adoption of an FLI.		0.781			0.725	
11.	The cost effectiveness of the innovations as compared to the existing solutions were properly established.			0.897		0.743	Sustainability
12.	The innovations proportional effect and accomplishment was established.			0.710		0.587	
13.	Demand and supply were chalked out in impacting a large number of beneficiaries.			0.684		0.666	
14.	FLI,s are implementable within existing systems and infrastructure.				0.840	0.760	Adaptability
15.	FLI,s enhances the societal status of any particular individual				0.820	0.718	

Extraction method: Principal component analysis

Rotation method: Oblimin with Kaiser normalization

beginning and growth of farmer-led innovations in dairying. Farmer-led innovations that were developed for practical problem solution or creative application have aided in efficiently handling agricultural activities and maximizing farm profits (Singh N. *et al.* 2018). On the other side, farm innovators might successfully transition into consultants and business owners, opening up options for off-farm income generation. Farmer-led innovations would increase output in developing nations, lowering rural people's levels of poverty (Spielman 2009, Mariam *et al.* 2011). To strengthen the factor structure of the perception scale, additional exploratory and confirmatory factor analysis studies in additional sample areas are required.

Weighted importance of Perceptual attributes of Farmer-Led Innovations as reported:

Table 5 makes it abundantly obvious that the stakeholders place a high priority on comparative cost analyses of innovations,

which are then properly documented by all types of internal and external agencies. The stages of scaling up Farmer-led innovations were also highlighted by the World Health Organization (WHO 2010), including scheduling actions, developing capacities, making strategic decisions, and assessing the environment. According to a study by Baliwada *et al.* 2017 scaling up innovations needed commitment and more financial support. They also suggested using a corporate social responsibility fund to involve the private sector in the commercialization of discoveries that are replicable.

The maximum possibilities of achieving economies of scale are also considered for the stakeholders for the effective dissemination of the scale in the different regions. Perceptions of the stakeholders varies from the attributes of cost-effectiveness to the innovations' comparative impact and success. Farmer-led Innovations can also cater a great role in empowering the farming communities and their parallel diffusion

Table 4: Component correlation matrix

Component	Relevancy	Profitability	Sustainability	Adaptability
Relevancy	1.0	0.113	0.356	0.250
Profitability	0.113	1.0	0.019	0.065
Sustainability	0.356	0.019	1.0	“0.009
Adaptability	0.250	0.065	“0.009	1.0

Table 5: Perception Scale statements according to their Weighted Mean Score

Sl. No.	Statements	Weighted Mean Score	Rank
1.	FLI, s help to enhance farmers' backward and forward linkage with several enterprises.	13.79	VIII
2.	FLI, s help farmers to empower themselves and their communities.	14.28	VI
3.	Farmers are not aware of the commercialization process of the FLI, s.	13.76	IX
4.	The relative analysis of the costs associated with the innovation was considered.	16.87	I
5.	We must consider refinement and simplification of the innovation for better dissemination.	12.97	XIII
6.	The potential of the innovation for achieving economies of scale was analyzed.	15.76	III
7.	The estimation of the innovations comparative impact and achievement was done.	13.66	X
8.	Ensure proper documentation of the innovation/ intervention.	15.78	II
9.	The innovations discourse a potential need for the stakeholders in the region.	13.21	XII
10.	FLI requires the involvement of a smaller number of people in the adoption decision.	12.63	XV
11.	The cost effectiveness of the innovations as compared to the existing solutions were properly established.	14.98	IV
12.	The innovations proportional effect and accomplishment was established.	14.65	V
13.	Demand and supply were chalked out in impacting a large number of beneficiaries.	12.89	XIV
14.	FLI, s is implementable within existing systems and infrastructure.	13.97	VII
15.	FLI, s enhances the societal status of any particular individual	13.65	XI

Table 6: Perception level of Stakeholders towards Farmer-led Innovations

S.NO	Level of perception	Frequency	Percentage
1	Low (<0.33)	118	32.78
2	Medium (0.33-0.58)	158	43.89
3	High (>0.58)	84	23.33

amongst the communities. Innovative solutions developed by farmers may be essential for addressing the problem of global food security and eradicating rural poverty (de Janvry and Sadoulet, 2002). Farmers are being urged to use the technological advancements that scientists have been creating and disseminating in field-based innovations more frequently in recent years (Gatzweiler and Von Braun, 2016). Linkages among farmers with the different enterprises can be improved followed by its non-awareness of the commercialization process. Stakeholders also felt the importance of the successful diffusion of Farmer-led innovations into a larger population by giving cognizance to a greater number of people's adoption decisions at the same place. According to several studies (Kummer *et al.* 2012; Tambo and Wünscher, 2017 Reij and Waters-Bayer, 2001;), such farmer-led innovation-generating techniques are critical to tackling food insecurity issues and strengthening community resilience to changing environmental conditions.

Level of perception of Stakeholders towards Farmer-led Innovations:

According to Table 6, 23.33 and 32.78 percent of stakeholders were found to be in the high and low level of perception categories, respectively, while 43.89 percent of the stakeholders fell into the middle level of perception category. This was due to that most of the Farmer-led Innovations were in the incubation stage and around three-five years old. The benefits incurred were not fully utilized in such a short-run period rather they understand it will be beneficial in the long run. The perception-altering effects of these farmer-led innovations show a growing understanding of the significance and value of locally based farming innovations, which support current policies for rural development and poverty reduction while also challenging the traditional technology strategy (Tran *et al.* 2019). The agricultural ecosystem needs innovations to achieve food security and end rural poverty (Leitgeb *et al.* 2011; Brooks and Loevinsohn, 2011). To achieve economies of scale, farmer-led innovations should be sustained for a long time in the field.

Conclusions

Farmer-led innovations must have the most advantageous features possible to draw in prospective users. The innovations' relative edge over the technology it will replace, compatibility with local cultures, and observability are what attract farmers to them most. Simple, practicable, and compatible with the context of farmers and their agricultural circumstances, needs, and experiences are requirements for any innovation or technology.

According to the findings of the current study, each institution working on farmer-led innovations is operating separately, in isolation, with their own resources, and according to a plan of activities that has a very small effect. As a result, institutions focusing on farmer-led innovations should network at the national level. Demonstrating the favourable and statistically significant welfare benefits of farmer-led innovations, the findings support farmers' views as well as the numerous anecdotal accounts of farmer-led innovations' substantial contribution to the livelihoods of rural farm households. A model for scaling up farmer-led innovations in India has been suggested based on the research results of the current study. The consequences for policy-level decisions should be taken into account as this needs to be improved and validated by various experts.

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SHORT COMMUNICATION

Prediction of first lactation milk yield on the basis of test day yield using artificial neural network versus multiple linear regression in Gir cows

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Abstract: The test-day model is a method of choice for the study of milk yield traits and this method is very important in countries like India where herd size is generally smaller and lacking well-established milk recording system. The present study was aimed to predict first lactation milk yield on the basis of test day yield in Gir cows and comparison was made between the relative efficiency of Artificial Neural Network (ANN) and Multiple Linear Regression (MLR) models. First lactation records of 513 Gir cows sired by 75 bulls spread over a period of 34 years (1981 to 2014), maintained at Cattle Breeding Farm, Junagadh were used for the study. The data of monthly test-day milk yield (MTMY) was divided into seven sets. ANN was used with back propagation Bayesian regularization (BR) algorithm and MLR was used with backward elimination method. The accuracy of prediction of first lactation milk yield in MLR was lower than the accuracy of ANN in all the test data sets. The Root Mean Square Errors (RMSE) of prediction were lower in ANN as compared to MLR. The optimum equation had total four variables (test days) viz. TD2 to TD5 for prediction of First Lactation 305-Days Milk Yield (FL305DMY). This equation gave an accuracy of prediction of 76.02% by MLR and 87.69% by ANN model till 125th days of lactation i.e. 5th monthly test day.

Keywords: First lactation 305 day milk yield, MTDMY, ANN, MLR, Gir cow

As per the 20th livestock census India possess over 300 million bovines which includes 192.49 million cattle. A total 50 cattle breeds have been identified and registered by National Bureau of Animal Genetic Resources, Karnal. Gujarat has rich and bio

diverse cattle genetic resources viz. Gir, Kankrej, Dangi and Dagri breeds. Gir cattle are well known milch cattle breed across the whole world. It is known for its integral heat tolerance and disease resistance capacity. The native tract of the breed is Gir hills and forests of Kathiawar including Junagadh, Bhavnagar, Rajkot and Amreli districts of Gujarat (Patbandha et al. 2020).

First lactation 305-days milk yield is considered as an important trait for selection of cows. Test-day milk yield (TDMY) is the measurement of the amount of milk produced by a cow for the period of 24 hours (Schaeffer and Jamrozik, 1996). The test-day model is method of choice for the study of milk yield traits in order to maximize the use of all available information. This method is very important in countries like India where herd size is generally smaller and lacking well-established milk recording. Test day yield model is a substitute of 305-day lactation model because early selection on the basis of test-days could reduce generation interval and using test day yield model, it is possible to economize the genetic evaluation with a better accuracy (Bilal and Khan, 2009).

Various regression models, which are used for future yield predictions are applied in milk yield analyses. However, artificial neural network (ANN) takes an entirely different approach. In practice, ANNs are primarily used in engineering, economic predictions, or in medical diagnoses. There has been relatively little research into the application of ANNs in the field of animal breeding. An artificial neural network, also called a neural network (NN) is a computational model based on biological neural network system. ANNs are used for the evaluation of different parameters (Chaturvedi et al. 2013). Artificial neural networks are based on

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the neural structure of the human brain, which processes information by means of interaction among many neurons. The basic components of an ANN are neurons, weights and learning rules (Stich et al. 2000). The main advantage of neural networks lies in the ability to represent both linear and non-linear relationships directly from the data being modeled. ANN approach needs specified algorithm to be transformed by a computer program (Grzesiak et al. 2003). After applying successful training algorithms, the neural network will be capable to perform classification, estimation, prediction or simulation on new data from the same or similar sources. Therefore, the present study was carried out to predict the first lactation 305-days milk yield by using ANN & Multiple Linear Regression (MLR) model based on monthly test day milk yield records and to find the best early test-day milk yields combination in order to make an early selection of the animals for breeding program. The study also compared the effectiveness of MLR and ANN for prediction of first lactation 305-days milk yield in Gir cows.

First calving decreases the cost of raising the animals to productive life, increases the annual genetic gain and raises the average productive life of the animal. The estimate of additive genetic variability for traits of economic importance gives an idea about the scope of genetic improvement of the trait through selective breeding. The selection and evaluation of breeds to be used as the parental stock is an important step for the success of any animal breeding program.

Data pertaining to of 513 Gir cattle from pedigree cum lactation registers maintained at Cattle Breeding Farm, Junagadh Agricultural University, Junagadh for a period of 34 years 1981 to 2014 were used for the present study. Only those animals whose lactation was normal and has completed at least 100 lactation days were selected in the study. The outliers beyond three-standard deviation on both the tail ends of normal distribution were also excluded from the data. The first test day was considered 5th day (soon after colostrum), the next test day was calculated by adding 30 days to the preceding test day up to 305 days of lactation.

The prediction of First Lactation 305-Day Milk Yield (FL305DMY) was performed utilizing the MLR & ANN.

The MLR was used to develop prediction equations by estimating the regression coefficients for the test-day milk yield records in different combination. Stepwise backward multiple linear regression analysis was used to estimate 305- day milk yield (Singh et al. 2015).

$$\hat{Y}_i = a + \sum b_i X_i$$

Where,

$\hat{Y}_i$ = Estimated first lactation 300 day or less milk yield of the i^{th} animal

X_i = Test day record of i^{th} animal

a = Intercept

b_i = Regression coefficient of first lactation 305 day or less milk yield on text day records

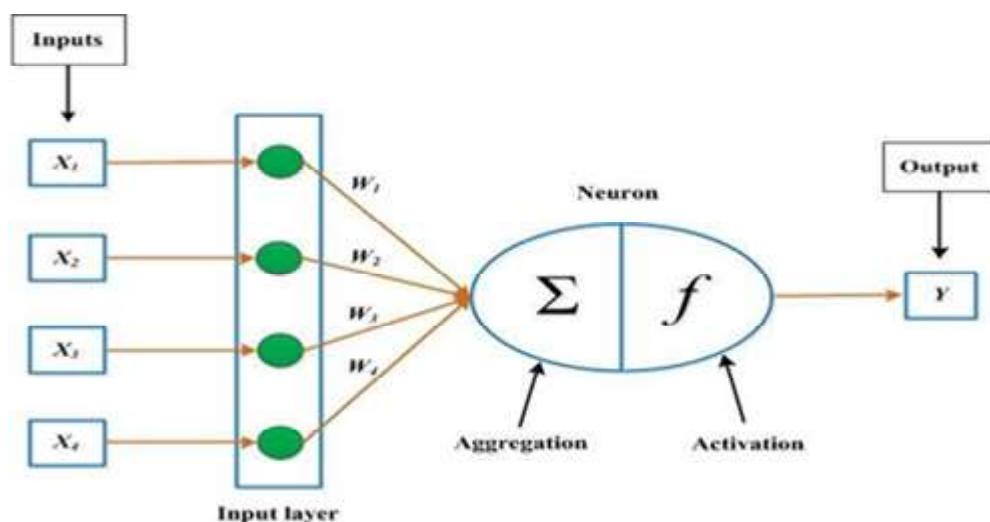
ANN is a multilayer feed forward neural network with back propagation of error learning mechanism was developed using Neural Network Toolbox (NNT) of MATLAB 7.0 to predict the first lactation 305-day or less milk yield (FL305DMY). The network was trained and simulated using back propagation algorithms viz. Bayesian regularization (BR) (Singh et al. 2020) upto 4000 epochs or till the algorithms truly converged. Network parameters such as learning rate, momentum, and error goal were used as the default setting of the algorithms.

The entire data was divided as training (60%), testing (20%) and validation (20%). The network architecture was composed of monthly test day milk yield as the input layer, two hidden layers with five neurons each while the predicted first lactation milk yield was the output layer. To prevent the network from falling into local minima, the momentum coefficient was used of $\alpha = 0.5$. A general schematic diagram of multilayer feed forward network with input layer, hidden layers and output layer is shown in Figure 1.

The statistical analysis was carried out with help of Microsoft Office Excel-2016 and software MATLAB 7 was used for ANN. Effectiveness was compared in both ANN and MLR methods using percent coefficient of determination (R^2 - Value) and root mean square errors (RMSE).

The results showed that the least square mean of first lactation test day milk yield was consistently increasing up to peak yield 6.19kg (MTDMY3) and after that gradually decreasing in phase which showed the typical pattern of lactation curve in Gir cow (Table-1). Singh (1983) reported 8.38 kg peak yield in Gir cows. The overall mean of FL305DMY or less milk yield in the present study was 1448.19 ± 26.61 kg in Gir cow. Savaliya et al. (2016) reported 1554.3 kg and Gadariya et al. (2017) reported 1427.49 kg FL305DMY in Gir cow.

The Multiple linear regression (MLR) analysis revealed that when all monthly test days (TD1 to TD10) were included in equation to predict the first lactation 305 Days milk yield (FL305DMY) the accuracy (R^2 value) was 93.20%. In consonance to the present study, Dongre et al. (2012) observed R^2 value of 92.6% in Sahiwal cows using MLR model. However, when nine variables were included in the equation (TD1 to TD9), 92.58% accuracy was observed. Further, when backward elimination method was

Fig. 1 Schematic diagram of ANN model**Table 1:** First Lactation Monthly Test-Day Milk Yield (FLMTMY) of Gir Cows

Traits	Mean (kg)	Standard error	Standard deviation
MTDMY1	5.33	0.09	2.23
MTDMY2	6.12	0.09	2.25
MTDMY3	6.19	0.10	2.37
MTDMY4	5.84	0.10	2.34
MTDMY5	5.47	0.10	2.35
MTDMY6	5.17	0.09	2.25
MTDMY7	4.85	0.10	2.34
MTDMY8	4.68	0.10	2.26
MTDMY9	4.62	0.10	2.12
MTDMY10	4.37	0.10	2.04
FL305DMY	1448.19	26.61	602.71

Table 2: Test days involved in each input sets and R^2 -value from MLR and ANN

Input sets	Test days included	R^2 (%)	
		ANN	MLR
Set-1	MTDMY1 to MTDMY10	97.00	93.20
Set-2	MTDMY1 to MTDMY9	96.59	92.58
Set-3	MTDMY1 to MTDMY8	95.32	90.83
Set-4	MTDMY1 to MTDMY7	94.36	88.03
Set-5	MTDMY1 to MTDMY6	91.81	82.25
Set-6	MTDMY1 to MTDMY5	88.03	76.42
Set-7	MTDMY2 to MTDMY5	87.69	76.02

applied to predict the first lactation 305 Day milk yield as early as possible and the degree of accuracy was observed decrease (Table 2). Subsequently, formulation of different prediction equations was done by step-wise backward elimination and total 7 sets were analysed. Table-2 revealed that set-7 included minimum no of TDMY (TD2 to TD5) and yielded >75% accuracy. In set-7 the 1st TDMY was not included because it is difficult to record under field condition and mostly missed. Hence, in this study 4 test day milk yields (TD2, TD3, TD4 and TD5) were found to be suitable for prediction of FL305DMY as early as 125th days and considered as a best formula (Table 3). Olori et al. (1999) stressed that $R^2 \geq 0.70$ indicated a very good fit of a model,

while if $R^2 < 0.40$, such model should not be used for prediction. In this study (Table 2), the R^2 - values for all input sets were ≥ 0.76 in MLR model and ≥ 0.87 in ANN model. This indicates all models were very good fitted for Gir cows.

The optimum equation to predict FL305DMY as early as possible suggests that the accuracy obtained by ANN was 87.69% and was higher than the accuracy value obtained for MLR. Further, RMSE estimate was found lower in ANN model as compared to MLR model (Table 3). Similar findings were reported by Singh et al. (2022) in Murrah buffalo. Comparatively, higher estimate was observed in this study than the previous studies conducted

Fig. 2 Prediction of FL305DMY using ANN and MLR model

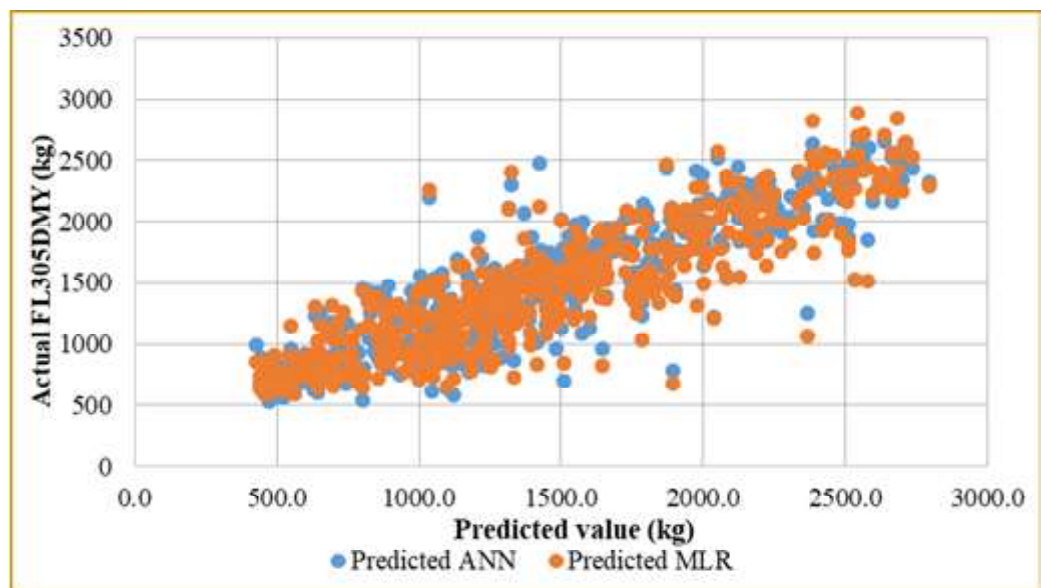
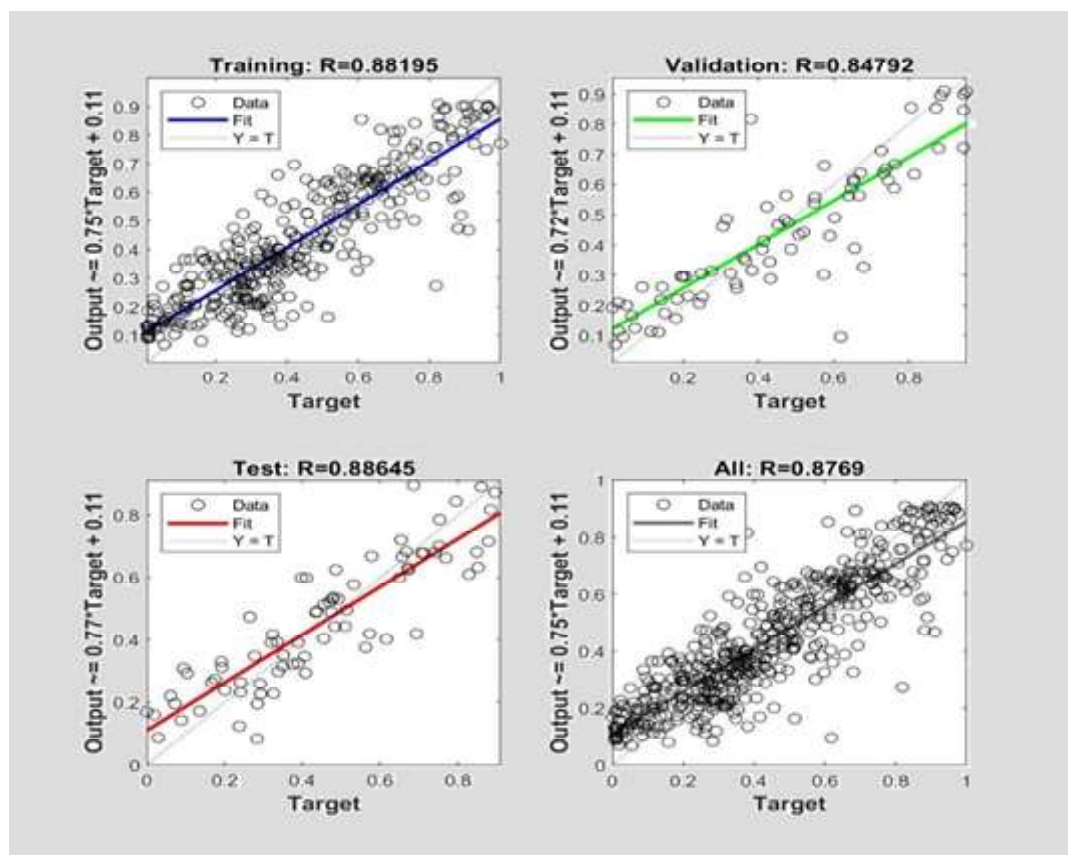


Fig. 3 ANN model for optimum equation



in other breeds of dairy cattle. Lower R^2 -value (72.34%) in MLR model was reported by Debbarma (2010) in Sahiwal cattle. However, Kokate (2009) predicted 61% (MTDY-6) accuracy in Karan-Fries cattle. Saini et al. (2005) reported an accuracy of 78.42% (1st, 2nd and 7th MTDY) in Rathi cattle by MLR and Ramani (2016) reported 77.71% accuracy for prediction of FL305DMY from monthly test day milk yield using MLR analysis

in Gir cattle. While, Dongre et al. (2012) predicted more than 80% accuracy in both methods ANN and MLR for prediction of lactation milk yield by using fortnightly test day yields in Sahiwal cattle, the value which was slightly higher than present estimate value. The higher accuracy obtained must be due to more data allotted for the training.

Table 3: The optimum equation along with their R²-values developed using MLR and ANN

Input Set-7	Optimum Equation by MLR	R ² -value (%)		RMSE(kg)	
		MLR	ANN	MLR	ANN
MTMY2 to MTMY5	$\hat{Y} = 139.19 + (33.36)TD2 + (44.12)TD3 + (64.47)TD4 + (116.61)TD5$	76.02	87.69	13.02	12.41

The difference between best ANN model and MLR model for prediction of FL305DMY are graphically presented in Figure 2. The R² value of optimum equation in ANN model is represented in different category like training, validation, test and based on all test days record (Figure 3). In the present investigation, difference was found between MLR and ANN model to predict FL305DMY in Gir cows. The best ANN network algorithm achieved 87.69% accuracy, whereas the MLR model achieved 76.02% of accuracy for prediction of FL305DMY in Gir cows. Similarly, Dongre et al. (2012) reported 86.08% prediction accuracy of FL305DMY in Sahiwal cattle using best ANN model. However, the accuracy was comparatively higher than the present study in best MLR model (85.16% vs. 76.02%). The variation of results might be attributed to the data sets used by different studies; in this study monthly test day milk yield records used whereas by Dongre et al. (2012) fortnightly test day milk yield records were used. Further, in crossbred cows like Karan Fries and exotic Holstein Friesian cows similar results were observed (Sharma et al. 2006; Njubi et al. 2010). On the other hand, Mundhe et al. (2015) reported higher accuracy for prediction of FL305DMY in Sahiwal cattle using MLR (88.80%) and ANN (89.29%) model. Moreover, the accuracy value shows minor difference between the two models. However, Rana et al. (2012) reported higher accuracy in MLR equation for an early prediction of FL305DMY than the ANN model in Murrah Buffaloes.

Conclusions

It was concluded that the optimum equation for prediction of first lactation milk yield in Gir cattle using Multiple linear regression was $Y = 139.19 + (33.36)TD2 + (44.12)TD3 + (64.47)TD4 + (116.61)TD5$ in this study. It provided an accuracy of 76.02% as early as 125th days of lactation. Artificial neural network proved a better approach with higher degree of accuracy (R²-value 87.69%) for prediction of first lactation milk yield in Gir cattle. It was inferred that FL305DMY could be predicted as early as 125th days of lactation even without having 1st TDMY with high degree of accuracy. Hence, evaluation of Gir cattle at an early stage will help to make selection decisions and could lead to genetic improvement of the animals.

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