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Enhancing the shelf life and biofunctional properties of whey-based cultured buttermilk using spice powder and oleoresin

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Abstract

Cultured buttermilk is a popular fermented dairy beverage, but its shelf life is often limited by post-acidification, proteolysis, and the development of off-flavors such as bitterness. While thermization can extend shelf life, it often destroys beneficial probiotic bacteria. This study evaluated the efficacy of selected spices in extending the shelf life of cultured buttermilk prepared by blending 50% dahi and 50% fermented paneer whey using *Lactobacillus helveticus* MTCC 5463. Based on preliminary sensory optimization, three spiced formulations CT₂ (0.5% fennel + 0.5% coriander powder), CT₃ (1% cumin powder + 0.02% cumin oleoresin), and CT₄ (0.5% cumin + 0.5% coriander powder) were compared against a control (CT₁) and a thermized control (T₅) during storage at 7 ± 2 °C. Sensory evaluation revealed that the control sample became unacceptable by the 4th day due to bitterness, while the thermized sample developed a stale flavor by the 6th day. In contrast, the cumin-fortified sample (CT₃) maintained acceptable sensory attributes for up to 7 days. Although proteolytic activity increased significantly in spiced samples (reaching 6.08 mg/ml in CT₃ by day 8), the specific spice profile effectively masked bitter peptides. Furthermore, CT₃ demonstrated superior biofunctional properties, exhibiting the highest antioxidant activity (75.47% inhibition) and antimicrobial efficacy against *Listeria monocytogenes* and *E. coli*. The study concludes that incorporating 1% cumin powder and 0.02% cumin oleoresin naturally extends the shelf life of whey-based cultured buttermilk to 7 days while enhancing its functional quality without the need for thermal processing.

Keywords: Cultured buttermilk, spice powders, oleoresins, sensory evaluation

Introduction

Cultured buttermilk is a fermented dairy product valued for its distinct flavor and nutritional benefits. However, the commercial potential of buttermilk, particularly when fortified with whey, is often constrained by a short shelf life. Spoilage during refrigerated storage is typically driven by microbial metabolism, leading to post-acidification (over-souring), and proteolysis, which results in the accumulation of bitter peptides.

To address these issues, the dairy industry often employs thermization (heating after fermentation). While effective at halting microbial growth, thermization inactivates the live starter cultures, negating the probiotic benefits of the product. Consequently, there is growing interest in "natural" preservation methods using plant-derived additives. Spices and their oleoresins are rich in bioactive phenolic compounds that not only impart flavor but also possess significant antioxidant and antimicrobial properties.

This study investigates the potential of specific spice combinations to extend the shelf life of a whey-based cultured buttermilk. Specifically, it examines the effects of cumin, fennel, and coriander on sensory stability, microbial quality, proteolytic changes, and biofunctional activities during refrigerated storage.

Materials and methods

Material

Raw materials and starter culture

Double toned milk (1.5% fat / 9% SNF) was used for dahi preparation, and standardized pasteurized milk (4.5% fat / 8.5% SNF) was used to prepare paneer to obtain fresh whey. The probiotic culture *Lactobacillus helveticus* MTCC 5463 was used as the starter culture.

Spices and oleoresins

Seven spices and oleoresins were evaluated: coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), fennel (*Foeniculum vulgare*). These were tested in two forms: fine powder (ground and sieved through 36 mesh) and commercial oleoresins.

Chemicals

All the chemical used in this study were of analytical grade. The citric acid used in present study was of food grade quality supplied by Dutt enterprise Anand.

Methods

Product preparation: Double toned milk (1.5 per cent fat / 9 per cent SNF) was used to prepared the dahi and standardized pasteurized milk (4.5 per cent fat / 8.5 per cent SNF) was used to prepared paneer to obtain the paneer whey. Fresh, sweet, good quality double toned milk was preheated to 90 °C for 5 min and filtered through muslin cloth, then cooled to temperature (37 °C) of inoculation. Paneer whey was prepared by the method given by (Bhattacharya *et al.*, 1971) [7]. Same heat treatment was given to the paneer whey as milk. Cultured buttermilk was prepared by blending dahi with fermented whey in 50:50 ratio (w/w). Starter cultures *Lactobacillus helveticus* MTCC 5463 were used as inoculum at the rate of 2%.

Three optimized spice formulations were prepared for the storage study:

- CT₂: 0.5% Fennel powder + 0.5% Coriander powder.
- CT₃: 1.0% Cumin powder + 0.02% Cumin oleoresin.
- CT₄: 0.5% Cumin powder + 0.5% Coriander powder.
- These were compared against two controls:
- CT₁: Control buttermilk (no spices).
- T₅: Thermized buttermilk (no spices, heated to 65 °C for 15s).

Storage conditions

Samples were packaged in PET bottles and stored at 7 ± 2 °C. Analyses were conducted daily (or at regular intervals) until spoilage. Parameters evaluated included:

Sensory evaluation

The product was subjected to the sensory evaluation by an expert trained panel of ten judges for colour and appearance, body and texture, flavour and overall acceptability criteria. Fresh product and the stored products were brought to 10 °C before giving for judging. A 9-point hedonic score card was used for sensory evaluation of buttermilk

Analysis of buttermilk

Titrateable acidity of the was measured as per the ISO 6091:2010. Proteolytic activity was measured by OPA (O-phthaldialdehyde) method (Quantitative method) described by Church *et al.* (1983). Antioxidant activity was measured by using ABTS 2, 2'-Azino-bis (3-ethyl benzoethiazoline - 6 - sulfonic acid) assay method (Re *et al.*, 1999) [12].

Microbiological analysis

All the samples were drawn aseptically and analysed for total lactic count, coliform count and yeast and mould count during storage. All the samples were handled as per instructions described in ISO 21187 (2004). Colonies were counted and viable cell counts were expressed as log colony forming units per gram (log cfu/g). Lactic count plates were

incubated in inverted position at 37 °C for 48 to 72 h and the number of log cfu/g of product was noted (as specified by ISO 17792:2006). Yeast and mold count was analysed according to method ISO 6611:2004. Coliform count of cultured buttermilk samples was analysed throughout the storage period at an interval of 7 days, followed by addition of melted Violet red bile agar (VRBA) (BIS, 1969, Appendix III). Antimicrobial Activity of the culture filtrate was tested by the agar well method (Delgado *et al.*, 2001) [13] with some modifications against pathogen namely *Escherichia coli*, *Salmonella typhi*, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Indicator strains obtained from Department of Dairy Microbiology, S.M.C. College of Dairy Science, Anand).

Statistical analysis

The data obtained during the present investigation were analyzed by completely randomized design (CRD) (Steel and Torrie, 1980) [66].

Results

Evaluation of spices and oleoresin for extending shelf life of cultured buttermilk

The storage stability of the optimized cultured buttermilk formulations (CT₂, CT₃, CT₄) was evaluated against a control (CT₁) and a thermized control (T₁) over a period of 8 days at 7 ± 2 °C. The results for sensory, chemical, microbiological, and biofunctional parameters are detailed below.

Changes in sensory characteristics of cultured buttermilk during storage

The samples of cultured buttermilk were evaluated at regular interval of 1 day for flavour, colour and appearance, body and texture and overall acceptability using 9- point hedonic scale. The average values obtained for each sensory attributes along with statistical analysis are presented in the Table 1

Effect on flavour

The data for changes in the flavour score of the cultured butter milk during storage are presented in Table 1. The examination of the data for flavour scores of cultured buttermilk indicated that the changes in flavour score among the treatments were significant ($p < 0.05$). The data also suggested that the storage period had significant effect on flavour scores of the cultured buttermilks. The interaction effect between types of buttermilk and storage period was also statistically significant for changes in flavour score. The initial flavour scores of the cultured buttermilk i.e. CT₁, CT₂, CT₃, CT₄ and T₅ were 8.10, 8.16, 8.47, 8.23 and 8.23, respectively and they decreased to 2.12, 4.91, 5.96, 5.66 and 4.38, respectively on 8th day of storage at 7 ± 2°C. The flavour score decreased slowly up to 4th day of storage and thereafter, the scores decreased drastically.

Among the five types of cultured buttermilk, CT₁, the control sample gave pronounced bitter flavour on 5th day and the flavour score was below 5.0 (which stands for 'neither like nor dislike' on 9-point hedonic scale), which declined subsequently during further storage. While the thermized control sample, T₅ gave unpleasant stale flavour on 6th day of storage in PET bottles at 7 ± 2°C and the score was below 6.0. The flavour scores of CT₃ (1% cumin powder + 0.02% cumin oleoresin) and CT₄ (0.5% cumin

powder + 0.5% coriander powder) type of cultured buttermilk with added spices were comparatively better compared to control samples although their proteolytic activity was significantly higher than CT₁ and T₅. Among the cultured buttermilk samples, CT₂, CT₃ and CT₄ containing different combinations of spices, the CT₂ (0.5% fennel powder+ 0.5% coriander powder) recorded significantly less flavour score throughout the storage period and this can be attributed to comparatively higher acidity of this sample as shown in Table 4 probably contributed by the presence of fennel.

Presence of spices in CT₃ and CT₄ had masked the sense of bitterness in cultured buttermilk resulting in better flavour perception as evident from Table 1. All experimental samples scored below 6.0 (which stands for like slightly on 9 point hedonic scale) on the 8th day of storage.

Sharma (2010)^[58] found in her study that the flavour score decreased gradually in both control and synbiotic lassi from 7.78 to 5.59 and 7.90 to 5.68, respectively during storage of 28 days at refrigerated temperature. Shukla *et al.* (2013)^[61] developed probiotic beverage from whey and pineapple juice. They found that initial flavour score was 8.82 and after 8 days of storage under refrigeration condition it decreased to 8.64.

Baljeet *et al.* (2013)^[5] studied the storage changes of whey based pineapple and bottle gourd mixed herbal beverage. They found that the taste and flavour decreased significantly with storage period and the mean value of 7.50 for flavour and taste in the freshly prepared beverage decreased respectively to 5.90 and 4.90 after 20 days of storage.

Ghanshyambhai *et al.* (2014)^[22] found that flavour scores of cultured buttermilk prepared by the addition of unfermented paneer whey and fermented paneer whey (*S. thermophilus* MTCC 5460 and *L. delbrueckii* subsp *bulgaricus*) to curd decreased from 7.08 and 8.60 on first day to 4.77 and 6.01, respectively at the end of the storage (5th day) period.

Chavez-Lopez *et al.* (2014) analyzed the impact of microbial culture on proteolysis and release of bioactive peptides in fermented milk. They found peptide content and specific bitterness intensity in fermented milks inoculated with co-cultures of LAB and yeasts after 36 h of fermentation at 28 °C. They assessed that *L. plantarum* KLAT1 and *L. plantarum* LAT₃ had 1.7 and 1.5 mg/ml peptide content, respectively measured by OPA method and specific bitterness was 1.0, whereas in case of co-cultures, it was 2 mg/ml peptide content and specific bitterness was 3.0. Similar observations have been made by Gonzalez-Gonzalez *et al.* (2011)^[24].

Sonali *et al.* (2016)^[63] found that flavour score of cultured buttermilk prepared by the blending dahi and fermented whey in 60:40 ratio decreased from 7.75 on the first day to 6.85 on 5th day during storage in PET bottles at 7 ± 2°C.

Effect on colour and appearance

The examination of the data for colour and appearance score of cultured buttermilk as shown in Table 1 indicated that the changes in score among the treatments was significant ($p < 0.05$). The data also suggested that the storage period had significant effect on colour and appearance scores of the cultured buttermilk. The interaction effect between types of buttermilk and storage period was also statistically significant for changes in colour and appearance score. The initial colour and appearance scores of the cultured buttermilk prepared by addition of fermented paneer whey

with or without spice i.e. CT₁, CT₂, CT₃, CT₄ and T₅ were 8.29, 8.23, 8.21, 8.09 and 8.34, respectively and they got reduced to 7.15, 6.73, 6.79, 6.74 and 7.20 respectively on 8th day of storage. The colour and appearance scores of all the five types of cultured buttermilk prepared blending dahi with fermented paneer whey, with or without addition of spices declined slowly but remained above acceptable level (score above 6.0) even on 8th day of the storage at 7 ± 2°C.

Yadav *et al.* (2007)^[72] evaluated the storage changes in probiotic dahi stored at 7 °C up to 8 days. On fresh day, it was 45 out of 50 and on day 8, they observed little changes in appearance and the score was 42 out of 50.

Sharma (2010)^[58] found in her study that during storage, control and synbiotic lassi samples showed a gradual decrease in colour and appearance score from 7.97 to 6.40 and 7.97 to 5.86, respectively. Shukla *et al.* (2013)^[61] developed probiotic beverage from whey and pineapple juice. They found that initial colour and appearance score was 8.74 and after 8 days it was decreased to 8.67.

Baljeet *et al.* (2013)^[5] studied the storage changes of whey based pineapple and bottle gourd mixed herbal beverage. They found that the storage period significantly decreased the colour ratings of the product ($p < 0.05$) and the mean value of 8.10 for colour for the freshly prepared beverage decreased to 6.40 after 20 days of storage. They also stated that the appearance of the beverages was also affected significantly with storage and it decreased to a value of 5.90 after 20 days from initial value of 8.60.

Ghanshyambhai *et al.* (2015)^[22] found that colour and appearance scores of culture buttermilk prepared by the addition of unfermented paneer whey in to curd and by the addition of fermented paneer whey (*S. thermophilus* MTCC 5460 and *L. delbrueckii* subsp *bulgaricus*) in to curd decreased from 7.83 and 7.91 first day to 7.32 and 7.41 respectively at the end of the storage (5th day) period. In present study also similar trend in decrease of colour and appearance scores were observed as reported in the literature.

Sonali *et al.* (2016)^[63] found that colour and appearance score of cultured buttermilk prepared by the blending dahi and fermented whey in 60:40 ratio decreased from 8.04 on the first day to 7.80 on 5th day during storage in PET bottles at 7 ± 2°C.

A similar trend in the decrease of colour and appearance score of different types of cultured buttermilk was observed in the present study.

Effect on body and texture

Due to its low TS, buttermilk has an inherent nature of settling down of solids and whey separation. Hence all the samples were properly mixed before serving to the judges.

The data for changes in the body and texture scores of the cultured butter milk during storage of are presented in Table 1. The examination of the data for body and texture scores of cultured buttermilk indicated that the changes in scores among the treatments were non-significant ($p < 0.05$). The data also suggested that the storage period had significant effect on body and texture scores of the cultured buttermilks. The interaction affect between types of buttermilk and storage period was also statistically non-significant for changes in body and texture score. The initial body and texture scores of the cultured buttermilk prepared by addition of fermented paneer whey with or without spice i.e. CT₁, CT₂, CT₃, CT₄ and T₅ were 8.22, 8.19, 8.23, 8.21

and 8.18, which declined to 6.46, 6.30, 6.40, 6.27 and 6.29, respectively on 8th day of storage at $7 \pm 2^\circ\text{C}$.

Yadav *et al.* (2007) [72] correlated the hydrolysis of these casein fractions with the rheological properties of dahi made with lactobacilli which had a creamy layer and good firmness in the early stages of storage, but after long storage, the dahi lost its firmness, which may be due to protein hydrolysis by a particular type of adjunct. They stated that *Lactobacilli* with higher intracellular peptidase activity produced a high level of casein hydrolysis in dahi, leading to smaller peptide fragments and free amino acids.

Shukla *et al.* (2013) [61] developed probiotic beverage from whey and pineapple juice. They found that initial consistency was 8.80 and after 8 days of storage it decreased to 8.30.

Ghanshyambhai *et al.* (2015) [22] found that body and texture scores of cultured buttermilk prepared by the addition of unfermented paneer whey and fermented paneer whey (*S. thermophilus* MTCC 5460 and *L. delbrueckii* subsp *bulgaricus*) in to curd decreased from 7.58 and 8.13 first day to 5.97 and 6.36 respectively at the end of the storage (5th day) period.

Sonali *et al.* (2016) [63] reported that body and texture of cultured buttermilk prepared by blending dahi and fermented whey in 60:40 ratio decreased from 8.03 on the first day to 7.0 on 5th day during storage in PET bottles at $7 \pm 2^\circ\text{C}$.

In the present study also, a similar trend in decrease of body and texture scores was observed as reported by other researchers.

Effect on overall acceptability

The examination of the data for overall acceptability scores of cultured buttermilk indicated that the changes in score among the treatments were significant ($p < 0.05$). The data from Table 1 also suggested that the storage period had significant effect on overall acceptability scores of the cultured buttermilks. Interaction effect between types of buttermilk and storage period was also statistically significant for changes in overall acceptability. The overall acceptability scores of the cultured buttermilk samples i.e. CT₁, CT₂, CT₃ CT₄ and T₅ were 7.98, 8.22, 8.47, 8.27 and 8.13 on first day and it decreased to 3.13, 4.98, 5.99, 5.66 and 3.12 respectively on 8th day of storage at $7 \pm 2^\circ\text{C}$.

The overall acceptability score decreased slowly up to 4th day of storage and thereafter, the scores decreased drastically. Among the five types of cultured buttermilk samples, CT₁ (cultured buttermilk without any spice) gave pronounced bitter flavour on 5th day of storage and the scores were below 5.0 (Which stands for 'neither like nor dislike' on 9-point hedonic scale). While T₅ gave unpleasant stale flavour on 6th day (the scores were below 5.0).

The overall acceptability score of CT₃ (1% cumin powder + 0.02% cumin oleoresin) and CT₄ (0.5% cumin powder + 0.5% coriander powder) type of cultured buttermilk with added spices were comparatively higher as compared to control samples CT₁ and T₅ inspite of having high proteolytic activity. Among the cultured buttermilk samples added with spices, CT₂ (0.5% fennel powder+ 0.5% coriander powder) recorded significantly less score in comparison to CT₃ (1% cumin powder + 0.02% cumin oleoresin) and CT₄ (0.5% cumin powder + 0.5% coriander powder) and this may be due to higher acidity of the sample CT₂ as shown in Table 4. The presence of fennel in CT₂ may

have enhanced the acid production by starter culture. Presence of spices in CT₃ and CT₄ had masked the bitterness resulting in better flavour perception.

Yadav reported that the sensory attributes and microbiological and biochemical changes of yogurt made with soymilk and buffalo milk demonstrated a positive relationship during 15 days of storage at 7°C . Yadav *et al.* (2007) [72] evaluated the storage changes in probiotic dahi stored at 7°C up to 8 days. On fresh day, it was 44.9 out of 50 and on day 8, they saw little changes in appearance and the score was 42.5 out of 50.

Sharma (2010) [58] found that the overall acceptability score was found to decrease in both control and synbiotic lassi from 7.71 to 5.82 and 7.97 to 5.86, respectively, at the end of 28 days of storage.

Baljeet *et al.* (2013) [5] studied the storage changes of whey based pineapple and bottle gourd mixed herbal (WPBH) beverage. They found that the overall acceptability of the WPBH beverage during 20 days of storage period decreased significantly from 8.02 to 5.80 and the product was slightly desirable up to 15 days having a value of 6.45 for overall acceptability.

Shukla *et al.* (2013) [61] developed probiotic beverage from whey and pineapple juice. They found that initial overall acceptability score was 8.68 and after 8 days it was decreased to 8.63.

Ghanshyambhai *et al.* (2015) [22] found that body and texture scores of cultured buttermilk prepared by the addition of unfermented paneer whey and by the addition of fermented paneer whey (*S. thermophilus* MTCC 5460 and *L. delbrueckii* subsp *bulgaricus*) to curd decreased from 7.02 and 8.06 on first day to 4.74 and 6.50 respectively at the end of the storage (5th day) period.

Sonali *et al.* (2016) [63] found that overall acceptability of cultured buttermilk prepared by blending dahi and fermented whey in 60:40 ratio decreased from 7.94 on the first day to 6.57 on 5th day during storage in PET bottles at $7 \pm 2^\circ\text{C}$.

In present study also similar trend in decrease of overall acceptability scores were observed as reported by other researchers. The overall acceptability score CT₁, the cultured buttermilk without any spice and thermized cultured buttermilk sample T₅ were acceptable only up to 3rd day and 5th day respectively on storage at $7 \pm 2^\circ\text{C}$. While the experimental samples CT₃ (1% cumin powder + 0.02% cumin oleoresin) and CT₄ (0.5% cumin powder + 0.5% coriander powder) were acceptable up to 7 days at $7 \pm 2^\circ\text{C}$. From the studies on changes in sensory characteristics of cultured buttermilk stored in PET bottles at $7 \pm 2^\circ\text{C}$, it was inferred that up to 7th day scores of all attributes especially that of flavour and overall acceptability were above 6.0 for the experimental samples CT₃ and CT₄. On the 8th day, the scores for flavour and overall acceptability of all types of cultured buttermilk were below 6.0 (which stands for like slightly on 9 point hedonic scale) due to bitter taste and stale flavour. Therefore, the storage studies of cultured buttermilk were discontinued after 8th day.

Changes in lactic acid bacteria count of cultured buttermilk during storage

Total lactic count is the collective enumeration of the total lactic acid bacteria (*Streptococci* and *Lactococci*). It is to represent the overall microbiological quality of the product,

after production and during its storage period. The changes in microbial population of cultured buttermilk expressed as total lactic acid bacteria count (log cfu/g) of cultured buttermilk 7 days of storage at $7 \pm 1^\circ\text{C}$ are presented in Table 2.

It can be seen from the Table 2 that initial total lactic acid bacteria count of cultured buttermilk samples CT₁, CT₂, CT₃, CT₄ and T₅ increased from 9.09, 9.14, 9.16, 9.15 and 8.05 to 9.96, 10.09, 10.52, 10.20 and 8.98 log cfu/g, respectively on 3rd day of storage day. Thereafter, the total lactic acid count decreased up to last day of storage study (8th day).

Momin (2009) [42] carried out the storage study of mushli added herbal probiotic lassi as well control (without herb) probiotic lassi prepared using *S. thermophilus* (MD8) and probiotic *L. helveticus* MTCC 5463. He found that the total viable *Lactobacilli* count changed from 6.60×10^7 to 1.02×10^7 cfu/ml in herbal probiotic lassi during storage at refrigerated temperature.

Shah (2011) [55] developed synbiotic lassi with honey and whey drink with inulin and orange juice using *L. helveticus* MTCC 5463 as probiotic culture in both products. She found that the count of *L. helveticus* MTCC 5463 remained at level of more than 8 log cfu/ml at the end of storage period (28th day) refrigerated temperature. Similarly for whey based product, the count before storage was 8.72 log cfu/ml and declined to 8.39 log cfu/ml on 28th day. Lactic acid bacteria are nutritionally fastidious organisms, which require more free amino acids or peptides than present in milk. Thus, proteolytic activity is important requirement for achieving a minimum level to meet probiotic claim (Roy, 2001; Talwalkar *et al.*, 2004). Leclerc *et al.* (2002) [40, 52, 67] also reported a linear increase in the amount of free amino groups until end of fermentation in milk fermented with *L. helveticus* strains and the proteolytic pattern certainly had a strong effect on bacterial growth.

Donkor *et al.* (2007) [14] reported that as a consequence of growth of probiotic bacteria in yogurt, the levels of released peptides increased. Increase in the proteolysis may lead to improve survival of some probiotic microorganism in yogurt.

Sonali *et al.* (2016) [63] enumerated that the initial total lactic count of cultured buttermilk prepared by blending dahi and fermented whey in 60:40 and control increased from 10.59 and 10.38 log cfu/g to 10.66 and 10.42 log cfu/g of cultured buttermilk respectively on 3rd day of storage. Thereafter, the total lactic count decreased up to last day of storage study (5th day).

In the present study also the count of CT₂, CT₃ and CT₄ type of cultured buttermilk with higher proteolytic activity (as shown in Table 2) had more lactic acid bacteria count throughout the storage compared to CT₁ and T₅. The low lactic acid bacteria count in CT₁ and T₅ experimental samples was in accordance with the lower proteolytic activity as shown in Table 3. With respect to control T₅, it had undergone thermization which also caused suppressed growth of starter culture.

Changes in coliform and yeast and mold count during storage: The cultured buttermilk samples CT₁, CT₂, CT₃, CT₄ and T₅ stored at $7 \pm 2^\circ\text{C}$ temperature were also enumerated for coliforms and yeast and mold count.

Coliforms as well as yeasts and molds were not detected in the cultured buttermilk samples stored at $7 \pm 2^\circ\text{C}$ during the

entire storage period. This indicates hygienic manufacturing practices and purity of cultures used.

Coliforms, that are often used to monitor the quality of product, are not a single species of microorganisms. These are a group of Gram negative rod shaped bacteria that have similar biochemical characteristics being able to ferment lactose with the production of acid and gas within 48 h at 35°C and grow with or without oxygen. Coliform count is simple and easy to conduct; hence, it can be used as a hygienic indicator to reflect the general microbiological quality in routine test. As coliforms can be easily killed by heat, these bacteria can also be used as an indicator of heat treatment failure as well as post heat treatment contamination.

Aerial contamination is one of the major sources of molds that are usually colonized on walls and roofs of the dairy farms. When the milk is exposed to open area, molds will enter in milk. Growth of yeasts and moulds is a common cause of the spoilage of dairy products, especially fermented milks and high acidic products, because these microbes are able to grow at low pH. Yeast spoilage is manifested as fruity or yeasty odour and/or gas formation. For proper quality control, it is important for the producer to evaluate yeast and mould counts in the milk used for making milk products.

Sharma (2010) [58] stated that coliform were absent (in 1 g) in both control and synbiotic lassi throughout storage period up to 28 days under refrigerated conditions. Momin (2009) [42] found in his study, that coliforms were absent in 1st dilution of control and herbal probiotic lassi throughout storage period up to 21 days.

Otabi and Demerdash (2008) stated that yeast and mold were not detected in labneh containing essential oil of thyme, marjoram and sage but detected in untreated control. Sharma (2010) [58] stated that the yeast and mold count was significantly lower in synbiotic lassi than control during storage up to 28 days under refrigerated conditions.

Momin (2009) [42] found that the mean yeast and mold count was non-significant in control and herbal probiotic lassi throughout storage period up to 21 days.

Sonali *et al.* (2016) [63] observed that the cultured buttermilk prepared by blending dahi and fermented whey in 60:40 was free from coliform and yeast and mold count during storage at $7 \pm 2^\circ\text{C}$.

Changes in proteolytic activity of cultured buttermilk during storage

Lactic acid bacteria (LAB) including probiotic organisms are fastidious in nature, requiring numerous essential growth factors. Milk, although a rich growth medium, contains low concentration of free amino acids and peptides to efficiently support growth of LAB (Shihata and Shah, 2000) [59]. In response to this limitation, LAB has developed a complex system of proteinases and peptidases, which enable them to utilize casein as an additional source of organic nitrogen (Vasiljevic *et al.*, 2005) [68]. The proteolytic systems of LAB consist of

1. Cell-wall proteinases (Lactocepins), often designated PrtP, that initiate the proteolytic attack,
2. Transport systems that facilitate the uptake of the oligopeptides into the bacterial cell, and finally
3. A series of intracellular peptidases that digest peptides within bacterial cells (Juillard *et al.*, 1995) [33].

The samples of buttermilk were analyzed for proteolytic activity during storage at one day interval and the results are given in Table 3.

Statistical analysis of results revealed that spices and storage period had significant ($p < 0.05$) effect on proteolytic activity of cultured buttermilk during storage. The proteolytic activity expressed as peptide concentration (mg/ml) of the cultured buttermilk samples CT₁, CT₂, CT₃, CT₄ and T₅ was 0.49, 1.06, 1.09, 1.23, and 0.50 mg/ml on first day and it increased to 5.45, 5.83, 6.08, 6.34, and 3.44 mg/ml, respectively on 8th day of storage at 7 ± 2 °C in PET bottles. From the Table 3 it can be understood that the control samples CT₁ and T₅ had significantly low proteolytic activity compared to experimental samples CT₂, CT₃ and CT₄ with added spices. Thus, it can be inferred that addition of spice increased the proteolytic activity irrespective of the type and form of spice. The sample CT₄ (0.5% cumin powder + 0.5% coriander powder) had the highest proteolytic activity throughout the storage period and it differed significantly from all experimental samples. The lowest proteolytic activity was for T₅, the thermized cultured buttermilk without any spices.

Amirdivani and Baba, (2011) [3] investigated the effects of peppermint (*Mentha piperita*), dill (*Anethum graveolens*) and basil (*Ocimum basilicum*) on yogurt formation and proteolytic activity. The OPA values in herbal yogurts on 28th day of storage at 4 °C remained higher than plain-yogurt. As reported by Papadimitriou *et al.* (2007) [48] *L. acidophilus* and *S. thermophilus* are metabolically active even at 4 °C and thus the extent of proteolysis by these bacteria could have been enhanced by the presence of herbs as shown by a substantial increase in OPA values in yogurt after the first seven days of refrigerated storage. This may lead to alteration of protein breakdown with potentially higher production of bioactive peptides (Shahidi and Zhong, 2008) [56].

Donkor *et al.* (2007) [15] reported that the free amino acid content in yoghurts was higher due to proteolytic activity of microorganisms compared to reconstituted skim milk as control. Proteolysis, as assessed by the release of free NH₃ groups by the OPA method, increased significantly during the first 24 h storage after manufacture and continued to increase at a slower rate during storage for both yoghurts.

Juillard *et al.* (1995) [33] reported that the level of free amino acids and peptides in milk is low therefore LAB depend on a proteolytic system that allows for an efficient degradation of milk proteins. The presence of amino peptidases is important for the release of amino acids for growth by microorganisms through the hydrolysis of peptides in the growth medium.

Biologically active peptides are generated during milk fermentation by proteolytic enzymes produced by various LAB such as *L. helveticus*, *L. lactis* subsp. *cremoris* FT₄ and *L. delbrueckii* subsp. *Bulgaricus* SS1 (Gobbetti *et al.*, 2002) [23]. These biologically active peptides include hypotensive peptides which inhibit angiotensin I-converting enzyme (ACE), opioid agonist and antagonist peptides, and mineral binding, immunomodulatory, antibacterial, and antithrombotic peptides (Pihlanto and Korhonen, 2003) [49].

The extent of proteolysis varied among strains and appeared to be time dependent. Leclerc *et al.* (2002) [41] reported a linear increase in the amount of free amino groups until end of fermentation in milk fermented by *L. helveticus* strains.

Kapila *et al.* (2009) [34] evaluated the proteolytic activity of whey and whey supplemented with whey protein concentrate (WPC) fermented by *Lactobacillus helveticus* NCDC 292 (1%, 37 °C/48 h) under *in vitro* conditions. When the whey and whey supplemented with WPC were fermented with *L. helveticus* for a period of 48 h using 1% inoculum size, the increase in the release of peptide was nearly 10 and 18 fold in fermented whey and fermented whey supplemented with WPC, respectively.

Shori and Baba (2011) [60] studied the effect of addition of cinnamon on cow milk bioyoghurt prepared using bioyoghurt bacteria mixture. They found that proteolytic activity in the fresh product increased constantly up to 7th day of storage at refrigerated condition compared to control indicating that addition of cinnamon can enhance the functional properties of bioyoghurt in relation to acidification, proteolysis and antioxidant activities.

Sonali *et al.* (2017) [64] reported that during storage of cultured buttermilk prepared by blending dahi and fermented whey in 60:40 using *L. helveticus* MTCC 5463, the proteolytic activity increased throughout storage period up to 5 days at 7 ± 2 °C.

In the present study *L. helveticus* MTCC 5463 was used as common starter culture for the production of all buttermilk samples and many research workers have confirmed that various strains of *L. helveticus* have remarkable proteolytic activity and they present a potential system to liberate dormant bioactive peptides from milk proteins through proteolytic hydrolysis during fermentation of milk (Virtanen *et al.* 2007). Hati *et al.* (2015; 2017) [26, 27, 69] also have confirmed the proteolytic activity of *L. helveticus* MTCC 5463 in skim milk at 37 °C and in honey based fermented milk. In almost all strains of Lactic acid bacteria, there is a cell-enveloped proteinase (CEP), located in the wall of the cell, and is responsible for breaking down the proteins out of the cell and releasing of peptides. However, some studies have reported the presence of more than one CEP in most of the studied *L. helveticus* strains (Zevaco and Gripon, 1988) [78], making this species a powerful proteolytic starter among LAB. Similar results are also reflected in the present study.

The difference in proteolytic activity between the control samples and samples of cultured buttermilk containing spices can be attributed to the enhanced proteolytic activity due to the presence of spices. Similar results have been reported by Amirdivani and Baba (2011) [3] and they stated that extent of proteolysis and the sizes of peptides produced from the fermentation are expected to be different in the presence of these herbs and these need to be further studied in the future.

Similar result of increase in proteolytic activity throughout the storage was observed in the present study. Kumar *et al.* (2003a; b) [38] and Behare and Prajapati (2007) [6] observed no significant increase during storage in the acidity of lassi subjected to heat treatment of more than 65 °C. This might be due to the heat shock given to microorganisms which slowed down rate of metabolic and enzymatic activity by the microorganisms. In the present study also T₅ sample, which has undergone thermization had the lowest proteolytic activity due to low metabolic and enzymatic activity by the microorganisms.

4.5.5 Changes in acidity of cultured buttermilk during storage: Total titratable acidity is the total amount of hydrogen ions present in the fermented milk sample with the

exception of those bound to alkaline ions. The determination of total titratable acid is more relevant in the evaluation of fermentation capacity of microbes (Geidam *et al.*, 2007)^[21]. Organic acids produced in yogurt (lactic acid, citric acid, formic acid, acetic acid and butyric acid (Ostlie *et al.*, 2005)^[45] were reported to be linearly related with the accumulation of total titratable acid (Billard *et al.*, 2007)^[8]. Variations in titratable acidity that occurred in milk can thus be associated to differential microbial population during fermentation (Eissa *et al.*, 2010)^[17].

The initial acidity of the cultured buttermilk with different type and form of spices i.e. CT₁, CT₂, CT₃, CT₄ and T₅ were 0.53, 0.54, 0.55, 0.55 and 0.52% which increased to 1.03, 1.00, 0.97, 0.96 and 0.88% lactic acid, respectively on 8th day of storage in PET bottles at 7 ± 2°C as evident from the Table 4.

The changes in acidity among the treatments and during the storage period was significant ($p < 0.05$). Similarly, interaction between types of cultured buttermilk and storage period was also statistically significant for changes in acidity of cultured buttermilk over the storage period.

At the end of storage period, among all the samples, the highest acidity was for CT₁, the control cultured buttermilk without any spice followed by CT₂, CT₃, CT₄ and T₅. The lowest acidity was for control sample T₅, the thermized cultured buttermilk and reason can be attributed to due to the heat shock given to microorganisms, which slow down their metabolic and enzymatic activity as reported by Kumar *et al.* (2003a, b) and Behare and Prajapati (2007)^[6, 38].

Yadav *et al.* (2007)^[72] evaluated changes during storage of probiotic dahi. The pH decreased and titratable acidity increased significantly from 4.93 and 0.89% lactic acid on first day to 4.84 and 1.12% lactic acid respectively on 5th day during the storage period. On the eighth day, the pH and titratable acidity were 4.64 and 1.32, respectively, which were acceptable to the assessors.

Otaibi and Demerdash (2008)^[46] developed concentrated yoghurt (labneh) with addition of essential oils, namely thyme, marjoram and sage at concentrations of 0.2, 0.5 and 1.0 parts per million (ppm). The acidity values of the treated labneh increased significantly with an increase in the storage period. The highest values were obtained with labneh containing 0.2 ppm of the essential oils when fresh and it increased up to the end of storage (day 21), suggesting that the essential oils had a stimulatory effect on the starter culture and total viable count (Abou Dawood, 2002).

Momin (2009)^[42] carried out the storage study of mushli added herbal probiotic lassi as well control (without herb) probiotic lassi at 5 ± 2 °C for a period of 21 days. The culture *S. thermophilus* (MD8) and probiotic *L. helveticus* MTCC 5463 was used in this study. He found that the acidity for fresh products was 0.61 to 0.62% lactic acid, which increased to 1.03% lactic acid in both cases after 21 days of refrigerated storage and the pH declined gradually during the storage from 4.84 to 3.96 in herbal probiotic lassi and from 4.78 to 3.95 in control lassi.

Amirdivani and Baba, (2011)^[3] investigated the effect of peppermint (*Mentha piperita*), dill (*Anethum graveolens*) and basil (*Ocimum basilicum*) on yogurt formation. The total titratable acidity for the three herbal yogurts was higher than plain-yogurt at all-time points during the incubation. The presence of herbs appeared to enhance the metabolic activity of yogurt bacteria.

Singh *et al.* (2011)^[62] prepared yogurt from buffalo's milk samples treated with anise volatile oil and its oleoresin (ethanol) at varying concentrations (0.1-1.0 g/l) which were stored up to 20 days at 4 ± 1 °C. The titratable acidity values of different curd samples increased during the cold storage of yogurt. However, during the initial days of storage, additives did have not significantly affected the titratable acidity, but a significant difference in titratable acidity of control and treated sets was observed on later days.

Shah (2011)^[55] developed synbiotic lassi with honey and whey drink with inulin and orange juice with *L. helveticus* MTCC 5463 as probiotic culture in both the products. She found that the initial titratable acidity (% lactic acid) of synbiotic lassi was 0.70% which increased to 1.15% on 28th day and the initial titratable acidity of whey based product increased from 0.81 to 0.96% on last day of storage.

Shukla *et al.* (2013)^[61] developed probiotic beverage from whey and pineapple juice. They found that the acidity increased during the refrigerated storage from 0.55 to 0.89% after 28 days and the increase in acidity was more prominent in case of storage at ambient temperature wherein the acidity reached 0.89% after 120 h of storage.

Ajala *et al.* (2013)^[2] developed soy-corn milk from a blend of soybean milk and corn milk extract at a ratio of 3:1 and spiced with ginger and garlic extract respectively to improve the taste. The total acidity of the ginger and garlic spiced milk types were found to be 1.01 and 1.02, respectively. These values were higher than the values reported for soy-corn milk (0.063 and 0.057 in white and yellow maize, respectively) by Omueti and Ajomole (2005)^[44]. The acidic nature of ginger and garlic could be responsible for the higher total acidity of the spiced soy-corn milk types.

Hamid (2014)^[25] studied on white soft cheese with addition of different levels of cumin oil. The effect of cumin oil concentrations on the acidity of the white cheese increased significantly with increase in cumin oil concentration. The highest titratable acidity (2.24%) was for the cheese samples with 0.3 per cent cumin oil, while the lowest one was for the control cheese samples (1.25 ± 0.83%).

Highest acidifying activity need not necessarily have the most proteolytic activity, as reported by other authors (Durlu-Ozkaya *et al.*, 2001; Nieto-Arribas *et al.*, 2009)^[16, 43].

Even though CT₂, CT₃ and CT₄ had more lactic acid bacteria count throughout storage study, the acidity of these experimental samples was less than control CT₁ (1.03). Even though *L. helveticus* MTCC 5463 was used as the starter culture for the preparation all cultured buttermilk samples namely CT₁, CT₂, CT₃, CT₄ and T₅, the presence of spices in CT₂, CT₃ and CT₄ enhanced their proteolytic activity as discussed under Section 4.5.3.

The results obtained in the present study correlated well with the reports of many researchers that the isolates that presented the highest acidifying activity were not the most proteolytic (Requena *et al.*, 1993; Durlu-Ozkaya *et al.*, 2001)^[16, 51].

Changes in antioxidant activity of cultured buttermilk during storage: Lactic acid bacteria produce metabolic compounds, which act as scavengers as well as degraded products of milk proteins acting as hydroxyl radicals (Kullisaar *et al.*, 2002)^[37]. The fermented milk also contained reductones formed during fermentation, which

could react with free radicals to stabilize and terminate radical chain reactions (Yang *et al.*, 1992)^[73].

The initial antioxidant activity (% inhibition) of CT₁, CT₂, CT₃, CT₄ and T₅ increased from 37.97, 51.63, 71.71, 66.08 and 47.23 to 45.89, 57.71, 75.47, 69.59 and 55.77%, respectively on 4th day of storage and then thereafter it decreased throughout the storage as shown in Table 5.

The changes in antioxidant activity among the treatments and during the storage period was significant ($p < 0.05$). Similarly, interaction between types of cultured buttermilk and storage period was also statistically significant for changes in antioxidant activity of cultured buttermilk over the storage period.

Among all the samples of cultured buttermilk, CT₃ had highest antioxidant activity followed by CT₄, CT₂, CT₁ and T₅. The higher proteolytic activity of cultured buttermilk samples CT₂, CT₃ and CT₄ containing spices as compared to control samples CT₁ and T₅ was due to the presence of polyphenols and higher proteolytic activity (as shown in 3) of these samples. The antioxidant activity of all the samples decreased after fourth days till the end of storage.

The reduction in antioxidant activities during refrigerated storage can be attributed to increasing degradation of phenolic compounds with antioxidant activities (Yildiz and Eydurán, 2009)^[74] and/or increasing milk protein polyphenol interaction (Yuksel *et al.*, 2010)^[76]. Continued microbial growth during refrigerated storage may alter some of the phenolic compounds and hence their antioxidant activities. Also studies on proteolytic activity of cultured buttermilk samples during refrigerated storage revealed that in all samples proteolytic activity increased throughout the storage period. Excessive proteolytic activity may result in the degradation of bioactive peptides into amino acids subsequently reducing the antioxidant activity. Sabeena *et al.* (2010)^[53] reported that fermented milk had more oxidative stability than milk because microorganism action could yield antioxidant peptides acting as electron donors.

Amirdivani and Baba (2011)^[3] investigated the effects of peppermint (*Mentha piperita*), dill (*Anethum graveolens*) and basil (*Ocimum basilicum*) on yogurt formation, proteolysis and antioxidant activity. All herbal-yogurts had higher antioxidant activities than plain-yogurt, both at the end of fermentation and throughout the storage period. The antioxidant activity of coriander oil is generally attributed to the presence of phenolics compounds (Samojlik *et al.*, 2010)^[54], although some studies suggest that the total antioxidant capacity of the oil was not directly related to the total polyphenolic compounds but seemed more dependent on the content of flavonoids and tannins (Sriti *et al.*, 2011)^[65].

Fu *et al.* (2015)^[20] found that buttermilk had a good capability to stabilize phenolic components. They suggested that buttermilk can be used to deliver these turmeric components in the system to impart health benefits. Felfoul *et al.* (2017)^[19] investigated effect on antioxidant activity of yoghurt with addition of ginger powder (*Zingiber officinalis*). Fortified yogurt sample with 1% ginger powder showed higher values of total phenolic content, DPPH scavenging activity, reducing power and metal chelating assay during storage period compared to control samples.

Sonali *et al.* (2017)^[64] reported that *in vitro* studies of cultured buttermilk prepared by blending dahi and fermented whey in 60:40 employing *L. helveticus* MTCC

5463 showed better antioxidant activity compared to control as determined by ABTS method.

The results obtained in the present study are in accordance with the reported literature.

Changes in antimicrobial activity properties of cultured buttermilk during storage:

Milk components impart antimicrobial potential, for example, milk is rich source of immunoglobulins present in colostrum and provides immunity against various infections resists pathogens to colonize in intestinal epithelia, reduce absorption of food antigens and promotes phagocytosis of pathogens. In addition, lactoferrin, a whey protein that has strong affinity for iron present in milk, removes freely present iron and renders it unavailable for most of the pathogenic organism function properly. Another important defensive component of milk is lysozyme, which hydrolyzes the peptidoglycan subunits of pathogens cell wall. Besides, lactoperoxidase of milk reacts with microorganism derived hydrogen peroxide and generates some highly reactive intermediary compounds that have cell wall degrading potential of invading microorganisms (Marshall, 2004). The antimicrobial activity of cultured buttermilk against selected pathogens namely *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Bacillus cereus* are shown in Table 6.

The exact mechanism of antibacterial action of spices and their derivatives is still not fully elucidated; however, multiple hypothesized and experimentally supported mechanisms have been proposed in recent literature. These include: i) Phenolic and volatile compounds interact with bacterial membranes due to their hydrophobic nature, leading to structural disruption, increased membrane permeability, leakage of cellular contents, and loss of membrane potential. This effect damages membrane integrity and homeostasis, ultimately leading to cell death (Jesús Guadalupe Pérez-Flores *et al.*, 2025)^[32]; ii) perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes (Jesús Guadalupe Pérez-Flores *et al.*, (2025)^[32]; iii) Certain phenolic compounds induce oxidative stress by producing reactive oxygen species within bacterial cells, which contributes to lipid peroxidation, DNA damage, and protein oxidation amplifying antibacterial effects (Xue Y *et al.*, 2025)^[71]; iv) Interaction with the bacterial cytoplasmic membrane can disrupt ion gradients (H^+/K^+), collapse the proton motive force, impair ATP synthesis, and interfere with key metabolic processes (Ahmad, S., & Ahmed, R. 2024). v) ^[1] destruction of electrons transport systems and cell wall perturbation (Kashyap R., 2024)^[35]. Generally, gram-negative bacteria have been reported to be more resistant than Gram-positive to essential oils antimicrobial effect because of their cell wall lipopolysaccharide. Cell wall lipopolysaccharide may prevent that essential oils active compounds reach the cytoplasmic membrane of Gram-negative bacteria.

The antimicrobial activity increased up to four day of storage study in all experimental samples thereafter decreased till the end of storage. Among all samples CT₂, CT₃ and CT₄ had higher antimicrobial activity against all selected pathogens as compared to control CT₁ due to their proteolytic activity and antimicrobial activity contributed by the spices. Among the all samples CT₃ had highest

antimicrobial activity against all selected pathogens followed by CT₄, CT₂ and CT₁ and this trends correlated well with their proteolytic activity as shown in Table 3. Thus, it was clear that addition of spice in cultured buttermilk increased the antimicrobial activity.

The reduction in antimicrobial activities during refrigerated storage can be attributed to increasing degradation of phenolic compounds with antioxidant activities (Yildiz and Eydurán, 2009) and/or increasing milk protein polyphenol interaction (Yüksel *et al.*, 2010)^[74, 77].

Antimicrobial bioactive peptides derived from milk have been reported to inhibit many Gram positive and Gram negative pathogens including *Escherichia coli* MTCC 82, *Aeromonas hydrophila* ATCC7966, *Salmonella typhi* MTCC 3216, *Bacillus cereus* ATCC10702, *Salmonella typhimurium* SB 300, *S. enteritidis* 125109, *St. aureus* MTCC 96 (Mohanty *et al.*, 2014) and control many microbial infections. Many workers reported that lactoferrin and its derivatives show the antibacterial activity *in vitro* against various pathogens, e.g. *Clostridium perfringens*, *C. albicans*, *Haemophilus influenzae*, *Helicobacter pylori*, *L. monocytogenes*, *P. aeruginosa*, *Salmonella enteritidis*, *S. aureus*, *Vibrio cholerae* as well as antiviral activity against hepatitis C, G and B virus HIV-1, poliovirus, rotavirus and herpes simplex virus (Pan *et al.*, 2007).

Kathiria (2014)^[36] found that isolates of *S. thermophilus* MTCC 5460 have shown highest inhibition of *E. coli* followed by *S. aureus*, *B. cereus* and *S. typhi* and similar trend was followed by *S. thermophilus* MTCC 5461. She found that NS6 had comparable inhibition for *S. aureus* and *E. coli* (24 mm) and for *B. cereus* and *S. typhi* (16 mm). She concluded in their study that irrespective of pathogens, culture NS6 had shown maximum antimicrobial activity followed by *S. thermophilus* MTCC 5460 and *S. thermophilus* MTCC 5461 and neutralization of culture supernatant has resulted in significant reduction in antimicrobial activity. Sonali *et al.* (2017)^[64] reported that cultured buttermilk prepared by blending dahi and fermented whey in 60:40 ratio by employing *L. helveticus* MTCC 5463 showed antimicrobial activity against *S. aureus*, *S. typhi* and *E. coli*.

Discussion: The primary limiting factor for the shelf life of fermented dairy products is the deterioration of the organoleptic profile. In this study, the control sample (CT₁) exhibited a remarkably short shelf life of only three days. The failure was characterized by a sharp decline in flavor scores, transitioning from a clean, fermented profile to one dominated by bitterness and excessive sourness. This bitterness is a well-documented consequence of extensive proteolysis, where the starter culture breaks down milk proteins into hydrophobic peptides (Evelyn Maes *et al.*, 2025)^[18].

Interestingly, the spiced formulations, particularly the cumin-fortified (1% powder + 0.02% oleoresin) (CT₃), remained sensory-acceptable for seven days. A critical observation in this research is that the spices did not necessarily arrest the proteolytic process; in fact, spiced samples often showed higher peptide concentrations than the control. The extension of shelf life is, therefore, attributed to the "masking effect" of the spice volatiles. The aromatic compounds in cumin (Such as cuminaldehyde) and coriander (Linalool) possess high flavor intensities that effectively override the perception of bitter peptides on the

human palate (Shanghua Liu *et al.*, 2024). This suggests that spices provide a dual-function: they enhance the initial flavor profile and serve as a natural sensory preservative against the inevitable byproducts of fermentation.

A significant advantage of spice fortification observed during storage was the maintenance of high viable counts of *Lactobacillus helveticus*. In many commercial applications, shelf life is extended via thermization (T5), which kills the starter culture to prevent post-acidification. However, this process strips the product of its probiotic status.

In our study, the spiced samples maintained LAB counts exceeding 9.0 log cfu/g throughout the seven-day period. This suggests that the culinary spices used cumin, fennel, and coriander do not inhibit the beneficial starter culture at the optimized concentrations. On the contrary, the slightly higher counts in spiced samples compared to the control indicate a potential stimulatory or prebiotic-like effect, where spice-derived micronutrients or fibers may support the metabolic persistence of the lactobacilli. The absolute absence of coliforms and yeast/mold throughout the storage period further validates the hygienic processing conditions and suggests that the antimicrobial properties of the spices, particularly the oleoresins, may inhibit common spoilage microflora.

The biofunctional properties of the buttermilk specifically its antioxidant and antimicrobial activities showed a parabolic trend, peaking around the fourth day of storage. The initial increase in ABTS radical scavenging activity is likely due to the synergistic release of bioactive peptides from casein by *L. helveticus* and the slow leaching of phenolic compounds from the spice powders into the aqueous phase (Wróblewska B. *et al.*, 2023). Bagheri *et al.*, 2014^[4, 70], note that the interaction between milk proteins and plant-derived bioactives forms "hydrophobic complexes" that can actually enhance the bioavailability of antioxidants.

The peak in antimicrobial activity against pathogens such as *E. coli* and *Listeria monocytogenes* on Day 4 aligns with the maximum concentration of these bioactive metabolites. The subsequent decline toward the end of the storage period (Day 8) can be explained by the further hydrolysis of bioactive peptides into inactive amino acids and the potential oxidation of volatile spice components. These results imply that while the product is sensory-acceptable for seven days, its maximum therapeutic and functional potential is achieved mid-way through its shelf life.

The progressive increase in titratable acidity was expected. However, the spiced samples reached a plateau of approximately 0.96% to 1.00% lactic acid, which contributed to a refreshing tartness without becoming as acerbic as the control. The physical stability of the whey-based buttermilk was also noteworthy; despite the high inclusion of whey (50%), the samples did not exhibit significant phase separation or "wheying-off" during storage. This stability is likely a result of the stabilizing effect of the spice dietary fibers and the possible production of exopolysaccharides by the *L. helveticus* culture, which helps in maintaining a cohesive protein-whey matrix.

Recent studies in "Next-Generation Functional Matrices" suggest that these natural hydrocolloids create a more cohesive protein-whey network, preventing the syneresis typically seen in acidified milk beverages (Brüls, N., *et al.*, 2024)^[9].

Table 1: Changes in sensory characteristics of cultured buttermilk during storage

Types of Cultured Buttermilk	Storage period (Days)								
	1	2	3	4	5	6	7	8	Average of treatment
Flavour									
CT ₁	8.10	7.62	6.11	5.01	4.07	4.01	3.16	2.12	5.02
CT ₂	8.16	8.09	7.69	7.27	7.07	6.58	5.68	4.91	6.93
CT ₃	8.47	8.35	8.11	8.07	7.48	7.22	6.52	5.96	7.52
CT ₄	8.23	8.22	7.81	7.60	7.26	7.12	6.12	5.66	7.25
T ₅	8.23	7.65	7.14	6.91	6.34	5.50	5.01	4.38	6.40
Average of periods	8.24	7.98	7.37	6.97	6.44	6.09	5.30	4.61	
CD(0.05) T = 0.03; P = 0.03; T*P = 0.08; CV%=0.81									
Colour and appearance									
CT ₁	8.29	8.20	8.04	7.82	7.68	7.54	7.27	7.15	7.75
CT ₂	8.23	8.16	7.96	7.77	7.63	7.46	7.10	6.73	7.63
CT ₃	8.21	8.15	7.91	7.68	7.62	7.48	7.16	6.79	7.63
CT ₄	8.09	8.05	7.79	7.61	7.55	7.43	7.12	6.74	7.55
T ₅	8.34	8.21	8.11	7.86	7.71	7.58	7.31	7.20	7.79
Average of periods	8.23	8.15	7.96	7.75	7.64	7.50	7.19	6.92	
CD(0.05) T = 0.03; P = 0.04; T*P = 0.08; CV%=0.78									
Body and texture									
CT ₁	8.22	8.13	7.89	7.60	7.47	7.37	7.11	6.46	7.53
CT ₂	8.19	8.11	7.84	7.52	7.39	7.23	6.93	6.30	7.44
CT ₃	8.23	8.14	7.86	7.57	7.42	7.31	6.96	6.40	7.49
CT ₄	8.21	8.06	7.82	7.51	7.31	7.21	6.86	6.27	7.40
T ₅	8.18	8.08	7.83	7.51	7.36	7.23	6.89	6.29	7.42
Average of periods	8.20	8.10	7.85	7.54	7.39	7.27	6.95	6.34	
CD (0.05) T = 0.03; P = 0.04; T*P=NS; CV%=0.93									
Overall acceptability									
CT ₁	7.98	7.56	6.07	5.02	3.90	3.84	3.38	3.13	5.11
CT ₂	8.22	8.19	7.64	6.61	6.28	6.23	5.85	4.98	6.75
CT ₃	8.47	8.26	7.82	7.60	7.41	7.14	6.54	5.99	7.40
CT ₄	8.27	8.18	7.87	7.45	7.36	7.01	6.34	5.66	7.27
T ₅	8.13	7.75	7.11	6.84	6.28	5.46	5.02	4.44	6.38
Average of periods	8.21	7.99	7.30	6.70	6.25	5.94	5.43	4.84	
CD(0.05) T = 0.15; P = 0.19; T*P = 0.42; CV%=4.62									
CT ₁ = Cultured buttermilk without any spice (control 1) CT ₂ = 0.5% fennel powder+ 0.5% coriander powder CT ₃ =1% cumin powder + 0.02% cumin oleoresin CT ₄ =0.5% cumin powder + 0.5% coriander powder T ₅ = Thermized cultured buttermilk without any spice (control 2) # The value are mean of four replication. Observations were taken at 1 day interval.									

Table 2: Changes in the lactic acid bacteria count of cultured buttermilk during storage

Types of buttermilk	Lactic acid bacteria count (log cfu/g) *							
	1	2	3	4	5	6	7	8
CT ₁	9.09 ± 0.09	9.68 ± 0.26	9.96 ± 0.35	9.63 ± 0.33	9.42 ± 0.34	9.12 ± 0.23	8.58 ± 0.40	7.96 ± 0.13
CT ₂	9.14 ± 0.18	10.08 ± 0.17	10.09 ± 0.33	9.84 ± 0.17	9.55 ± 0.26	9.36 ± 0.36	9.07 ± 0.16	8.42 ± 0.34
CT ₃	9.16 ± 0.12	10.48 ± 0.21	10.52 ± 0.33	10.21 ± 0.13	10.11 ± 0.11	10.02 ± 0.34	9.94 ± 0.23	9.35 ± 0.35
CT ₄	9.15 ± 0.08	10.18 ± 0.17	10.20 ± 0.24	10.12 ± 0.17	10.07 ± 0.24	9.95 ± 0.23	9.84 ± 0.24	9.13 ± 0.18
T ₅	8.05 ± 0.09	8.94 ± 0.08	8.98 ± 0.13	8.43 ± 0.27	8.16 ± 0.31	7.92 ± 0.21	7.30 ± 0.25	6.51 ± 0.13
CT ₁ = Cultured buttermilk without any spice (control 1) CT ₂ = 0.5% fennel powder+ 0.5% coriander powder CT ₃ =1% cumin powder + 0.02% cumin oleoresin CT ₄ =0.5% cumin powder + 0.5% coriander powder T ₅ = Thermized cultured buttermilk without any spice (control 2) ** Each observation is a mean ± SD of four replications.								

Table 3: Changes in proteolytic activity of cultured buttermilk during storage

Types of Cultured Buttermilk	Storage periods								
	Peptide concentration (mg/ml)								Average of treatment
	1	2	3	4	5	6	7	8	
CT ₁	0.49	0.88	1.14	1.62	1.86	2.56	4.84	5.45	2.36
CT ₂	1.06	1.15	1.54	2.19	3.02	4.01	5.47	5.83	3.03
CT ₃	1.09	1.34	1.81	2.48	3.13	4.05	5.72	6.08	3.21
CT ₄	1.23	1.34	1.80	2.71	3.10	4.75	6.03	6.34	3.41
T ₅	0.50	0.78	1.09	1.59	2.09	2.51	3.26	3.44	1.91
Average of period	0.88	1.10	1.48	2.12	2.64	3.58	5.06	5.43	
CD(0.05) T = 0.06; P = 0.04; T*P = 0.03; CV%=0.71									
CT ₁ = Cultured buttermilk without any spice (control 1) CT ₂ = 0.5% fennel powder+ 0.5% coriander powder CT ₃ =1% cumin powder + 0.02% cumin oleoresin CT ₄ =0.5% cumin powder + 0.5% coriander powder T ₅ = Thermized cultured buttermilk without any spice (control 2) # The value are mean of four replication.									

Table 4: Changes in acidity of cultured buttermilk during storage

Types of Buttermilk	Storage periods								
	Acidity (% lactic acid)								Average of treatment
	1	2	3	4	5	6	7	8	
CT ₁	0.53	0.61	0.68	0.76	0.81	0.88	0.93	1.03	0.78
CT ₂	0.54	0.62	0.70	0.75	0.80	0.86	0.91	1.00	0.77
CT ₃	0.55	0.66	0.68	0.74	0.79	0.85	0.89	0.97	0.77
CT ₄	0.55	0.64	0.67	0.73	0.78	0.83	0.87	0.96	0.75
T ₅	0.53	0.59	0.63	0.70	0.74	0.79	0.84	0.88	0.71
Average of periods	0.54	0.62	0.67	0.74	0.78	0.84	0.89	0.97	
CD(0.05) T = 0.03; P = 0.04; T*P = 0.01; CV%=1.35									
CT ₁ = Cultured buttermilk without any spice (control 1) CT ₂ = 0.5% fennel powder+ 0.5% coriander powder CT ₃ =1% cumin powder + 0.02% cumin oleoresin CT ₄ =0.5% cumin powder + 0.5% coriander powder T ₅ = Thermized cultured buttermilk without any spice (control 2) # The value are mean of four replication.									

Table 5: Changes in antioxidant activity during storage of cultured buttermilk

Types of buttermilk	Antioxidant activity (%Inhibition)								
	1	2	3	4	5	6	7	8	Average of treatment
	1	2	3	4	5	6	7	8	
CT ₁	47.23	48.62	50.76	55.77	51.58	48.94	43.64	39.30	48.23
CT ₂	51.63	53.28	54.92	57.51	54.68	53.20	51.34	48.84	53.17
CT ₃	71.71	73.25	74.71	75.47	74.11	68.03	66.87	64.17	71.04
CT ₄	66.08	67.67	68.17	69.59	66.89	64.78	62.92	59.42	65.69
T ₅	37.97	39.37	40.61	45.89	42.02	40.23	37.47	35.38	39.87
Average of period	54.92	56.44	57.83	60.84	57.86	55.03	52.45	49.42	
CD(0.05) T = 0.86; P=1.08; T*P=2.42; CV%=3.11									
CT ₁ = Cultured buttermilk without any spice (control 1) CT ₂ = 0.5% fennel powder+ 0.5% coriander powder CT ₃ =1% cumin powder + 0.02% cumin oleoresin CT ₄ =0.5% cumin powder + 0.5% coriander powder T ₅ = Thermized cultured buttermilk without any spice (control 2) # The value are mean of four replication.									

Table 6: Changes in antimicrobial activity of cultured buttermilk against selected pathogens during storage

Types of cultured buttermilk	Storage Period (Days)								
	1	2	3	4	5	6	7	8	Average (treatments)
Zone of inhibition (mm) against <i>Listeria monocytogenes</i>									
CT ₁	18.25	18.50	21.25	24.00	21.25	19.25	15.50	11.25	18.66
CT ₂	21.50	22.00	24.25	25.50	22.75	20.50	18.50	15.25	21.28
CT ₃	20.50	24.25	24.50	26.25	24.25	23.00	20.25	17.00	22.50
CT ₄	21.25	22.50	24.25	25.75	23.50	21.75	19.00	16.00	21.75
Average (periods)	20.38	21.81	23.56	25.38	22.94	21.13	18.31	14.88	
CD(0.05) T = 0.25; P = 0.35; T*P = 0.70; CV%=2.38									
Zone of inhibition (mm) against <i>E.coli</i>									
CT ₁	14.50	17.50	19.75	21.25	18.25	16.00	12.75	10.50	16.31
CT ₂	17.25	22.50	23.25	25.75	23.25	21.25	18.75	15.50	20.94

CT ₃	18.75	24.25	25.25	27.25	25.25	22.75	20.50	18.25	22.78
CT ₄	14.50	20.75	23.25	25.00	22.50	20.25	17.75	14.25	19.78
Average (periods)	16.25	21.25	22.88	24.81	22.31	20.06	17.44	14.63	
CD(0.05) T = 0.26; P = 0.37; T*P = 0.74; CV%=2.63									
Zone of inhibition (mm) against <i>Salmonella typhi</i>									
CT ₁	12.25	13.00	14.50	16.25	14.75	13.25	11.25	9.50	13.09
CT ₂	12.25	15.25	18.75	19.75	18.25	17.25	15.50	13.25	16.28
CT ₃	13.75	16.00	19.75	21.25	19.50	18.50	17.00	14.75	17.56
CT ₄	12.00	15.25	18.75	19.50	18.00	17.25	14.50	12.25	15.94
Average (periods)	12.56	14.88	17.94	19.19	17.63	16.56	14.56	12.44	
CD(0.05) T = 0.24; P = 0.34; T*P = 0.67; CV%=3.05									
Zone of inhibition (mm) against <i>S.aureus</i>									
CT ₁	11.00	12.50	15.50	16.25	14.25	12.25	9.50	8.25	12.44
CT ₂	13.50	15.00	16.75	17.75	16.50	15.25	13.50	11.25	14.94
CT ₃	13.50	15.00	17.25	18.50	17.25	16.25	14.75	12.25	15.59
CT ₄	14.25	15.25	16.75	18.00	16.25	14.75	12.25	10.75	14.78
CD(0.05) T = 0.25; P = 0.36; T*P = 0.72; CV%=3.53									
Average (periods)	13.06	14.44	16.56	17.63	16.06	14.63	12.50	10.63	
Zone of inhibition (mm) against <i>Bacillus cereus</i>									
CT ₁	10.25	10.75	12.25	13.25	11.25	9.25	7.50	6.25	10.09
CT ₂	11.50	12.50	14.25	15.75	13.75	12.25	10.50	9.50	12.50
CT ₃	12.50	13.75	15.75	17.75	15.75	14.75	12.25	10.75	14.16
CT ₄	11.75	12.50	15.25	16.75	14.25	12.00	10.75	9.50	12.84
Average (periods)	11.50	12.38	14.38	15.88	13.75	12.06	10.25	9.00	
CD(0.05) T = 0.26; P = 0.36; T*P = 0.72; CV%=4.14									
CT ₁ = Cultured buttermilk without any spice (control 1); CT ₂ = 0.5% fennel powder+ 0.5% coriander powder; CT ₃ =1% cumin powder + 0.02% cumin oleoresin; CT ₄ =0.5% cumin powder + 0.5% coriander powder. The value are mean of four replication.									

Conclusion

In summary, the storage study demonstrates that the incorporation of cumin powder and oleoresin (CT₃) transforms cultured buttermilk from a highly perishable beverage into a stable, functional product. The natural additives extend the shelf life to seven days at 7 ± 1 °C, primarily through flavor masking and the stabilization of the probiotic population. This research provides a viable, "clean-label" alternative to chemical preservatives and thermal processing, meeting the modern consumer demand for natural, live-culture dairy beverages with enhanced nutritional and therapeutic value.

- **Conflict of interest:** There are no conflicts of interest associated with this publication.
- **Data availability:** The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

Authors' contribution: RH: The research concept and experimental work were carried out; by Nishit Chaudhari & Bhoomika S Desai under the SB: supervision of Dr. Smitha Balakrishnan.; Dr. Smitha Balakrishnan and Dr. A.M Patel SB, AMP: contributed to the analysis and interpretation of the research findings provided a critical review of the draft manuscript and assisted with data interpretation and contributed to the final revisions of the manuscript.

- **Ethical approval:** Use of artificial intelligence tools

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