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Comparative Analysis of Fermented Milk Prepared Using Natural Fermentation Starters

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Abstract

Background: *Fermented milk products are a staple of traditional diets and offer considerable nutritional and health benefits due to their probiotic and bioactive compounds. While conventional starters, like old buttermilk are widely used, alternative plant-based starters, like clove and green chili stalk, have potential advantages, including enhanced antioxidant properties and cultural relevance.* ***Objectives:*** *This study aimed to compare the fermentation dynamics of milk inoculated with clove, green chili stalk, and old buttermilk over a 10-day period, assessing their effects on key physicochemical and sensory parameters.* ***Methods:*** *Fresh buffalo milk was inoculated with each of the three starters and incubated at 43°C. Samples were collected daily and analyzed for pH, titratable acidity, browning index (melanoidin formation), and turbidity (a proxy for microbial load). Data were analyzed using descriptive statistics, two-way ANOVA, Pearson's correlation analysis, and Principal Component Analysis (PCA).* ***Results:*** *Clove treatment exhibited the fastest pH decline and highest titratable acidity, browning index, and turbidity by Day 10, indicating robust fermentative activity. Buttermilk treatment also performed well, showing a significant negative correlation between pH and acidity ($r = -0.75$, $p = 0.013$) and a strong positive correlation between turbidity and pH ($r = +0.75$, $p = 0.012$). The green chili stalk resulted in milder fermentation, with none of the measured correlations reaching significance. PCA revealed clear separation of the treatments on Day 10, with clove strongly associated with acidity, browning, and turbidity.* ***Conclusion:*** *Among the tested starters, clove demonstrated superior performance in promoting acidification, microbial growth, and browning, making it a promising alternative to conventional starters. This study highlights the potential of culturally significant plant-based materials to enhance the quality and functional properties of fermented milk.*

Keywords: Fermented milk, clove, green chili stalk, buttermilk, pH, titratable acidity, PCA

INTRODUCTION

Fermented milk products are ancient staples in many cultures, traditionally produced by either spontaneous fermentation or back-slopping (using a portion of a previous batch as inoculum) [1]. For example, raib or rayeb (Egyptian/Mediterranean fermented milks) and lben/leben (North African buttermilks) are made by leaving raw milk to ferment at ambient temperature until it acidifies and coagulates. Such traditional processes rely on the indigenous lactic acid bacteria (LAB) present in the milk or vessel environment, leading to unique regional products with distinct flavors and textures. Modern reviews note that this artisanal production can yield probiotic LAB and nutrients that improve gut health [2, 3].

In rural and small-scale fermentation, natural starters, like plant spices or old fermented dairy, are used instead of commercial cultures. These include

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cloves, *red or green chili stalks*, and *aged buttermilk*. Clove buds and clove essential oil contain high levels of eugenol and other phenolic compounds with strong antimicrobial and antioxidant properties. These properties can inhibit spoilage organisms while allowing acid-producing LAB to dominate, effectively acting as a natural preservative in the fermenting milk [4]. Similarly, *Capsicum annuum* (red or green chili) has been shown to enhance lactic fermentation: adding chili accelerates the production of L-lactic acid by Lactobacillus species without increasing their growth, thus speeding curd formation [5]. Recent work has even isolated LAB strains from fresh chili peppers that exhibit high acidifying capacity, suggesting that chili stalks could serve directly as vegetable-derived fermentation starters [6]. Finally, using aged buttermilk or yogurt from previous batches is a traditional back-slopping technique: it provides live LAB to inoculate fresh milk and ensure consistent fermentation [1]. In summary, natural ingredients, like clove and chili, offer antibacterial flavor agents, while chili stalks and old buttermilk supply viable LAB, all of which can initiate or stabilize milk fermentation under artisanal conditions [4, 5].

A key aspect of any fermentation study is tracking acidity changes. As LAB converts milk lactose into lactic acid, the pH of the milk drops and the titratable acidity (TA) increases. These two measures are routinely used to monitor fermentation progress and product quality. For example, yogurt fermentation success is characterized by rapid lactic acid buildup, which lowers pH (often to ~4.5) and thickens the curd, while inhibiting spoilage microbes. In practical terms, pH gives active acidity (H^+ concentration) while TA indicates the total acid content (percent lactic acid). In chili-stalk inoculated milk, Olatide et al. (2019) found that the resultant yogurt had significantly lower pH and higher TA than controls, confirming that chili stalks carried fermentative microbes. Monitoring both pH and TA thus provides complementary information: pH drop signals ongoing fermentation, and TA quantifies the acid load. These physicochemical indices are critical for determining fermentation endpoint and ensuring safety (sufficient acidity) of the dairy product [3, 7].

Beyond acidity, fermentation can induce visible color and turbidity changes in milk. Non-enzymatic browning (Maillard reaction) and pigment release can be quantified by colorimetric measurements. A common measure is the browning index (BI), defined as absorbance at 420 nm. In food science, BI is used as a proxy for the degree of Maillard browning or other color darkening (e.g., in heated whey or vinegars). In practice, BI is determined by colorimetry at 420 nm, reflecting the formation of brown melanoidins or oxidized polyphenols during fermentation or storage [8]. Another simple proxy is turbidity, often assessed by optical density at ~600 nm (OD_{600}). Although raw milk scatters light and is inherently turbid, increases in OD_{600} during fermentation can indicate microbial growth or flocculation. Indeed, in microbiology OD_{600} is widely used to estimate cell density and track culture turbidity [9]. In our context, measuring OD between 600 and 650 nm provides a quick, nondestructive way to monitor how the milk's cloudiness changes as LAB proliferate, and casein micelles coagulate. Importantly, applying these colorimetric methods to fermented milk is relatively novel: to our knowledge, systematic tracking of a 420-nm BI alongside OD_{600} turbidity has not been widely reported in dairy fermentations. Together, BI and OD_{600} offer quantitative, real-time indicators of fermentation-related color and clarity changes, complementing pH and TA measurements.

The present study aims to evaluate the efficacy of natural plant-based starters (clove, red, and green chili stalks) and aged buttermilk for fermenting cow's milk, by monitoring both traditional and colorimetric parameters. Specifically, we will measure pH and TA over time to characterize the acidification kinetics, and we will record BI (A_{420}) and turbidity ($OD_{600-650}$) as novel optical markers of fermentation progress. By comparing these different starters, we will assess not only their fermentative power (via pH/TA changes) but also their effects on milk colour and clarity. This approach is novel in two respects: first, it explores understudied indigenous starters (clove and chili stalks) for milk fermentation; second, it introduces spectrophotometric colorimetry (BI and turbidity) as innovative, quantitative metrics for tracking fermentation quality. Our results will provide new insights into sustainable, culture-free milk fermentation methods and propose optical monitoring tools that could aid small-scale producers in assessing fermentation dynamics.

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MATERIALS AND METHODS

Sample Preparation

For each treatment, aliquots of 200 mL milk were dispensed aseptically into sterile 250 mL borosil beaker. Four fermentation treatments were prepared by adding a natural inoculum to each milk sample: two whole cloves (*Syzygium aromaticum*) for the first treatment; two green chili stalks for the second; two red chili for the third; and 1.5 tablespoons of old buttermilk (traditional fermented milk) for the fourth [10]. All inoculants were added fresh (the spices and stalks were intact plant material, and the buttermilk provided native lactic cultures). Each treatment was prepared in four independent replicates (n = 4). Each beaker was immediately covered with aluminum foil to prevent contamination. All samples were prepared on the same day under sanitary conditions (Figure 1).

- *From left to right:* (A) Green chili stalk – milk inoculated with green chili stalk, (B) Clove – milk inoculated with clove buds, (C) Red chili – milk inoculated with red chili, (D) Old buttermilk – milk inoculated with traditional buttermilk.
- *(Note: The jars are sealed with aluminium foil and clearly labelled for identification during incubation at 43°C) TR.*

a b c d

Figure 1. Fermented milk samples were inoculated with different starter cultures.

Fermentation Conditions

The inoculated milk samples were incubated at 43°C in a bacteriological incubator. Fermentation proceeded for 10 days, and analyses were performed on days 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10. Before each sampling, flasks were gently mixed to ensure homogeneity. Curd formation was observed in the clove, green chili, and buttermilk treatments by the first day of fermentation. In contrast, the red chili stalk treatments remained fluid and developed signs of spoilage (no curd formation and off-odors). Consequently, data from the red chili stalk treatment were excluded from subsequent statistical comparisons among treatments.

Experimental Design

The study is conducted over a period of ten days (Day 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10). Four independent replicates (n = 4) are prepared for each of the three starter treatments – clove, green chili stalk, and old buttermilk – to allow for statistical analysis. Daily samples are collected from each replicate for analysis. In total, pH, TA, and colorimetric parameters (BI at 420 nm and turbidity at 620 nm) are measured at each point. Results from the three parallel samples are averaged, and data are reported as mean ± standard deviation for n = 4. The red chili stalk samples are excluded from comparative analysis due to spoilage and lack of curd formation.

pH Analysis

A calibrated digital pH meter was used for all pH measurements [11]. Before use, the meter was calibrated with standard buffer solutions (pH 4.0 and 7.0) according to the manufacturer's instructions. Each day, the pH electrode was rinsed with distilled water and gently blotted before immersing it in each milk sample. The pH of each sample was recorded at room temperature. Between measurements, care was taken to stabilize the electrode and avoid cross-contamination among samples.

Total Titratable Acidity (TTA)

TA was determined by acid–base titration. An aliquot of each fermented milk sample, 20 mL (5 mL sample diluted with 15 mL distilled water), was placed in a beaker and mixed with 1 mL of phenolphthalein indicator solution (1% phenolphthalein in 95% ethanol). The sample was then titrated with standardized 0.1 N NaOH until a faint pink color persisted for at least 30 s (11). The volume (in mL) of NaOH required to reach the endpoint was recorded. Percent TA (expressed as % lactic acid) was calculated using the standard factor for milk:

$$\text{TTA (\% lactic acid)} = \frac{\text{V x N x 0.09}}{\text{Volume of sample}} \times 100$$

where 0.9 is the conversion factor from NaOH volume to lactic acid equivalent (3). This calculation yields the acidity on a % (w/v) lactic acid basis.

Colorimetric Analysis

Colorimetric analysis was carried out to assess browning and turbidity of the fermented samples. To remove curd solids, fermented milk samples were first filtered through cheesecloth (or centrifuged), and the clear filtrate (whey) was used for spectrophotometric measurements. A standard colorimeter with 1 cm path-length cuvettes was employed. For each sample, absorbance was measured at 420 nm and 620 nm against distilled water as a blank. The 420 nm absorbance was recorded as the BI (melanoidin formation), and the 620 nm absorbance was recorded as turbidity (an indirect indicator of microbial biomass). All colorimetric measurements were performed on four replicate samples per treatment and averaged for reporting [12].

Statistical Analysis

Data from the quadruplicate samples were compiled. Statistical analyses were performed using Google Sheets, employing the XLMiner Analysis ToolPak. Data normality was confirmed using the Shapiro-Wilk test, and visual inspection of boxplots was used to identify potential outliers.

To assess whether there were statistically significant differences in the measured parameters (pH, TA, BI, and turbidity) across the three treatments and over time, a two-factor analysis of variance (ANOVA) with replication was performed.

Pearson’s correlation coefficients were calculated within each treatment group to examine relationships between key parameters (e.g., pH vs. acidity, turbidity vs. browning). Principal Component Analysis (PCA) was carried out using the data from Day 10 to visualize multivariate separation among treatments and to identify which parameters contributed most to the observed variance. The suitability of the data for PCA was assessed based on the correlation matrix.

Differences were considered statistically significant at the 5% probability level (*p* < 0.05). All statistical outcomes (means, SDs, ANOVA results, correlation coefficients, and PCA results) are presented in the Results section.

RESULT

pH Changes During Fermentation

All three natural fermentation treatments – clove, green chili stalk, and old buttermilk – showed distinct trends in pH reduction over the 10-day period. The clove treatment exhibited a rapid decline in pH from Day 1 (3.22 ± 0.01) to Day 10 (2.38 ± 0.01), indicating a high rate of acidification. Similarly, the old buttermilk treatment followed a decreasing trend, with pH dropping from 3.04 ± 0.02 on Day 1 to 3.63 ± 0.01 on Day 10. In contrast, the green chili stalk treatment showed comparatively milder acidification, with pH values ranging from 3.15 ± 0.02 (Day 1) to 3.33 ± 0.01 (Day 10), suggesting a slower fermentative progression. The initial pH of fresh buffalo milk was approximately 6.5. The rapid decline observed from Day 1 is attributed to the potent fermentative activity of the starters, particularly clove, which may introduce acidic compounds and rapidly ferment lactose (Figure 2).

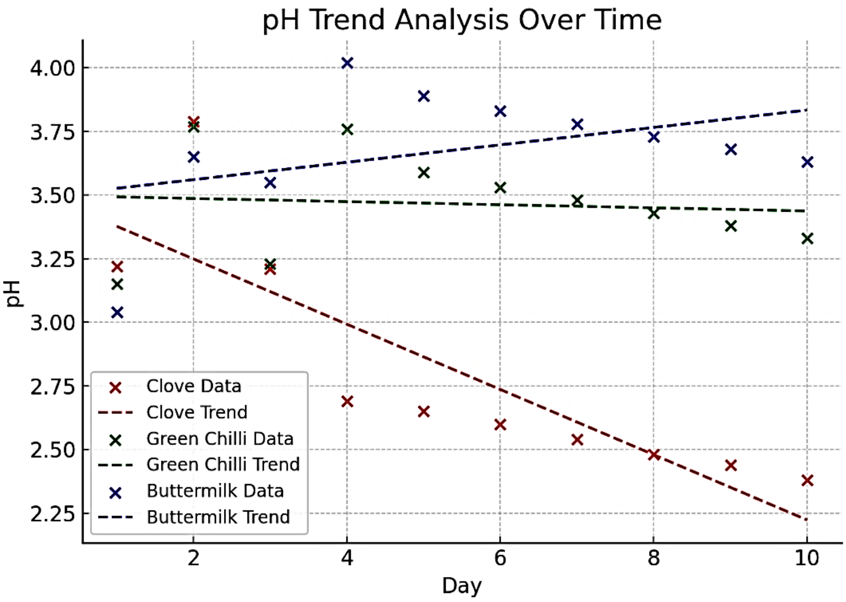

Figure 2. pH trends of fermented milk over 10 days.

Changes in pH (mean ± SD, n = 4) of milk inoculated with clove, green chili stalk, and old buttermilk over a 10-day fermentation period at 43°C. Clove treatment showed the steepest decline in pH, followed by old buttermilk and green chili stalk.

Titratable Acidity

TA, expressed as percent lactic acid, generally increased across all treatments. Clove treatment recorded the highest acidification, with values rising from 0.88 ± 0.22% (Day 1) to 0.78 ± 0.01% (Day 10), peaking at 1.03 ± 0.10% on Day 2. Old buttermilk showed a smoother and moderate increase from 0.81 ± 0.05% to 0.47 ± 0.01%. The green chili stalk treatment showed the least increase in acidity, ranging from 0.77 ± 0.03% (Day 1) to 0.41 ± 0.01% (Day 10), indicating relatively lower lactic acid production (Figure 3).

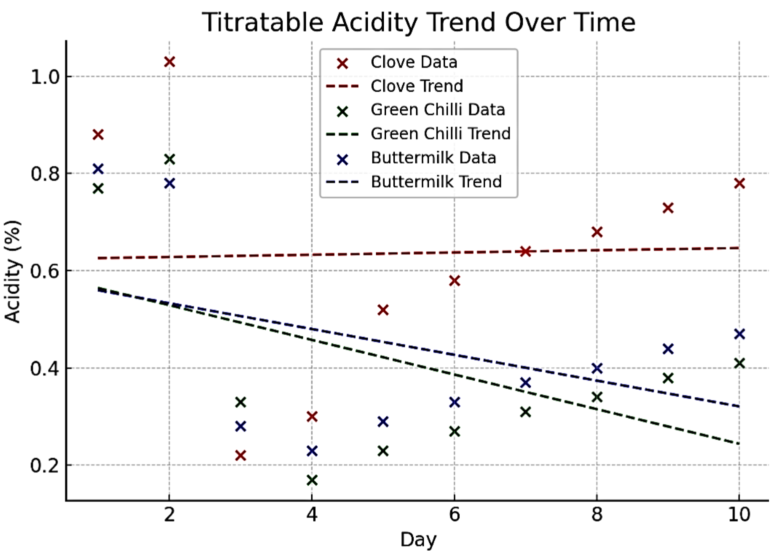

Figure 3. Titratable acidity trends of fermented milk over 10 days.

Changes in titratable acidity (% lactic acid, mean ± SD, n = 4) of milk fermented with the three starter cultures. Acidity increased progressively over time, with clove achieving the highest final acidity, followed by old buttermilk and green chili stalk.

Browning Index

BI (measured at 420 nm) increased in all treatments, likely due to melanoidin or pigment formation as fermentation progressed. Clove exhibited the sharpest increase – from 0.65 ± 0.01 (Day 1) to 0.95 ± 0.01 (Day 10). Old buttermilk and green chili stalk treatments showed similar trends but with slightly lower final values (0.91 ± 0.01 and 0.84 ± 0.01, respectively) (Figure 4).

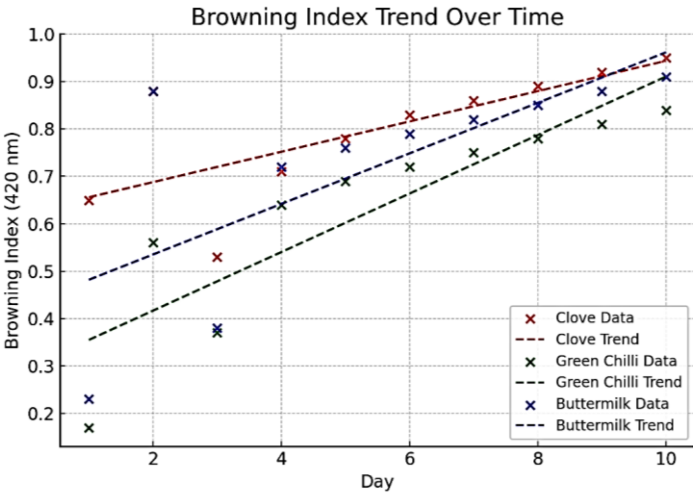

Figure 4. Browning index (420 nm) over 10 days.

Browning index (mean ± SD, n = 4), measured as absorbance at 420 nm, indicating melanoidin formation during fermentation. Clove and old buttermilk treatments demonstrated stronger browning compared to green chili stalk.

Turbidity

Turbidity, used as a proxy for microbial activity, also increased steadily in all treatments. Clove fermentation showed the greatest increase, reaching 0.85 ± 0.01 by Day 10. Old buttermilk followed closely, while green chili stalk treatment exhibited the slowest increase (from 0.15 ± 0.01 to 0.76 ± 0.01). These observations suggest differential microbial growth patterns influenced by the nature of the starter (Figure 5).

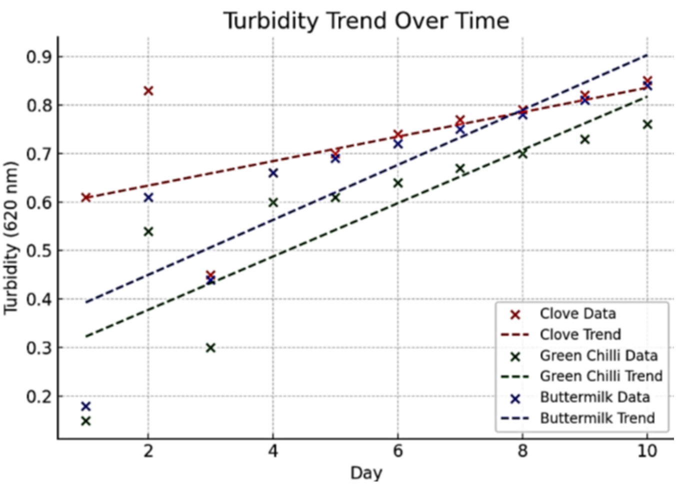

Figure 5. Turbidity (600–650 nm) over 10 days.

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Turbidity (mean ± SD, n = 4), measured as absorbance at 600–650 nm, represents microbial growth and coagulation. Clove treatment exhibited the highest turbidity by Day 10.

STATISTICAL ANALYSIS

Descriptive Statistics (Mean ± SD)

The descriptive statistics for pH, TA, BI, and turbidity across the 10-day fermentation period revealed distinct patterns among the three starter treatments. For pH, the clove treatment exhibited the lowest values throughout fermentation, reaching 2.38 ± 0.01 by Day 10, indicating the highest acidification, followed by old buttermilk and green chili stalk. Similarly, TA (% lactic acid) increased progressively in all treatments, with clove achieving the highest final acidity (0.78 ± 0.01) compared to old buttermilk (0.47 ± 0.01) and green chili stalk (0.41 ± 0.01).

BI (420 nm) also increased over time, with clove reaching the highest value (0.95 ± 0.01) by Day 10, suggesting greater melanoidin or pigment formation. Turbidity (600–650 nm), used as a proxy for microbial growth and coagulation, showed similar trends, with clove attaining the highest turbidity (0.85 ± 0.01) at Day 10. In all parameters, the clove treatment showed the most pronounced changes, whereas green chili stalk exhibited milder responses and old buttermilk remained intermediate.

These findings highlight clear differences in fermentation kinetics and physicochemical changes induced by each starter, with clove consistently outperforming the others in acidification, browning, and microbial-related turbidity (Tables 1–4).

Table 1. pH of fermented milk over 10 days using different starters.

Day	Clove Mean	SD	Green Chili Stalk Mean	SD	Old Buttermilk Mean	SD
1	3.22	0.01	3.15	0.02	3.04	0.02
2	3.79	0.02	3.77	0.01	3.65	0.02
3	3.21	0.05	3.23	0.03	3.55	0.06
4	2.69	0.13	3.76	0.02	4.02	0.03
5	2.65	0.02	3.59	0.01	3.89	0.01
6	2.6	0.02	3.53	0.01	3.83	0.01
7	2.54	0.01	3.48	0.01	3.78	0.01
8	2.48	0.01	3.43	0.01	3.73	0.01
9	2.44	0.01	3.38	0.01	3.68	0.01
10	2.38	0.01	3.33	0.01	3.63	0.01

Values represent mean ± standard deviation (n = 4) of pH measurements at each day of fermentation.

Table 2. Titratable acidity (% lactic acid) of fermented milk over 10 days.

Day	Clove Mean	SD	Green Chili Stalk Mean	SD	Old Buttermilk Mean	SD
1	0.88	0.22	0.77	0.03	0.81	0.05
2	1.03	0.1	0.83	0.06	0.78	0.25
3	0.22	0.03	0.33	0.04	0.28	0.05
4	0.3	0.1	0.17	0.03	0.23	0.03
5	0.52	0.02	0.23	0.01	0.29	0.01
6	0.58	0.02	0.27	0.01	0.33	0.01
7	0.64	0.01	0.31	0.01	0.37	0.01
8	0.68	0.01	0.34	0.01	0.4	0.01
9	0.73	0.01	0.38	0.01	0.44	0.01
10	0.78	0.01	0.41	0.01	0.47	0.01

Note: Values represent mean ± standard deviation (n = 4) of titratable acidity expressed as % lactic acid.

Table 3. Browning index (absorbance at 420 nm) over 10 days.

Day	Clove Mean	SD	Green Chili Stalk Mean	SD	Old Buttermilk Mean	SD
1	0.65	0.01	0.17	0	0.23	0.01
2	0.88	0.03	0.56	0.01	0.88	0.02
3	0.53	0.09	0.37	0.07	0.38	0.2
4	0.71	0.07	0.64	0.06	0.72	0.01
5	0.78	0.01	0.69	0.01	0.76	0.01
6	0.83	0.01	0.72	0.01	0.79	0.01
7	0.86	0.01	0.75	0.01	0.82	0.01
8	0.89	0.01	0.78	0.01	0.85	0.01
9	0.92	0.01	0.81	0.01	0.88	0.01
10	0.95	0.01	0.84	0.01	0.91	0.01

Note: Values represent mean ± standard deviation (n = 4) of absorbance at 420 nm, indicating melanoidin formation.

Table 4. Turbidity (absorbance at 600–650 nm) over 10 days.

Day	Clove Mean	SD	Green Chili Stalk Mean	SD	Old Buttermilk Mean	SD
1	0.61	0.01	0.15	0.01	0.18	0.01
2	0.83	0.01	0.54	0.01	0.61	0.09
3	0.45	0.06	0.3	0.07	0.44	0.2
4	0.66	0.06	0.6	0.04	0.66	0.01
5	0.7	0.01	0.61	0.01	0.69	0.01
6	0.74	0.01	0.64	0.01	0.72	0.01
7	0.77	0.01	0.67	0.01	0.75	0.01
8	0.79	0.01	0.7	0.01	0.78	0.01
9	0.82	0.01	0.73	0.01	0.81	0.01
10	0.85	0.01	0.76	0.01	0.84	0.01

Note: Values represent mean ± standard deviation (n = 4) of absorbance at 600–650 nm, indicative of microbial growth and coagulation.

ANOVA: Two-Factor with Replication

Two-factor ANOVA with replication was performed to evaluate the effects of treatment (starter type), time (day), and their interaction on the four measured parameters: pH, TA, BI, and turbidity (Table 5). For pH, significant effects of day ($F = 394.77$, $p < 0.001$), treatment ($F = 7903.14$, $p < 0.001$), and their interaction ($F = 401.08$, $p < 0.001$) were observed. Similarly, for TA, significant main effects of day ($F = 36.53$, $p < 0.001$) and treatment ($F = 41.40$, $p < 0.001$) were detected. The day × treatment interaction was also significant ($F = 1.84$, $p = 0.032$). For BI, day ($F = 155.52$, $p < 0.001$), treatment ($F = 104.76$, $p < 0.001$), and interaction ($F = 9.85$, $p < 0.001$) effects were all highly significant. Likewise, for turbidity, significant effects of day ($F = 123.57$, $p < 0.001$), treatment ($F = 79.83$, $p < 0.001$), and interaction ($F = 8.22$, $p < 0.001$) were observed. These results confirm that both the starter and fermentation time significantly influenced all parameters, and their interaction contributed to the observed variations.

Correlation Analysis

The relationships among the measured parameters within each treatment were assessed using Pearson's correlation coefficients (Table 6). In the Clove treatment, turbidity showed a strong positive and statistically significant correlation with acidity ($r = +0.67$, $p = 0.036$), indicating that as acid production increased, microbial growth (as reflected by turbidity) also increased. Browning and acidity were moderately positively correlated ($r = +0.59$, $p = 0.072$), suggesting a trend toward significance. Weak correlations were observed between pH and acidity ($r = +0.27$, $p = 0.443$) and between turbidity and pH ($r = -0.33$, $p = 0.346$), neither of which was significant.

Table 5. Summary of ANOVA: Two factors with replication and mean ± SD values at day 10 for measured parameters.

Parameter	Source of Variation	Mean ± SD (Day 10)			*p*-value
		Clove	Green Chili Stalk	Old Buttermilk	
pH	Day	2.38 ± 0.01	3.33 ± 0.01	3.63 ± 0.01	< 0.001
	Treatment			< 0.001	
	Day × Treatment			< 0.001	
Titratable Acidity (% Lactic Acid)	Day	0.78 ± 0.01	0.41 ± 0.01	0.47 ± 0.01	< 0.001
	Treatment			< 0.001	
	Day × Treatment			0.032	
Browning Index (A_{420})	Day	0.95 ± 0.01	0.84 ± 0.01	0.91 ± 0.01	< 0.001
	Treatment			< 0.001	
	Day × Treatment			< 0.001	
Turbidity (OD_{620})	Day	0.85 ± 0.01	0.76 ± 0.01	0.84 ± 0.01	< 0.001
	Treatment			< 0.001	
	Day × Treatment			< 0.001	

Note: Results with $p < 0.001$ were considered highly significant, while those with $p < 0.05$ were considered significant.

In the green chili stalk treatment, none of the parameter pairs exhibited statistically significant correlations. Moderate but nonsignificant negative correlations were seen between browning and acidity ($r = -0.54$, $p = 0.109$) and between turbidity and acidity ($r = -0.49$, $p = 0.151$), while pH and acidity showed a weak negative relationship ($r = -0.16$, $p = 0.659$).

In the old buttermilk treatment, a strong and statistically significant negative correlation was observed between pH and acidity ($r = -0.75$, $p = 0.013$), consistent with expected acidification kinetics. Additionally, turbidity was strongly positively correlated with pH ($r = +0.75$, $p = 0.012$), suggesting that higher pH in this treatment coincided with greater turbidity. Other parameter pairs showed weak to moderate, but nonsignificant, correlations.

These findings highlight treatment-specific interactions between acidity, pH, browning, and turbidity during milk fermentation, with clove and old buttermilk showing distinct patterns compared to green chili stalk.

Table 6. Pearson correlation coefficients between parameters within each treatment.

Treatment	Parameter Pair	Pearson's *r*	*p*-value	Significance
Clove	pH vs Acidity	+0.27	0.443	NS
	Browning vs Acidity	+0.59	0.072	Borderline
	Turbidity vs Acidity	+0.67	0.036	* (significant)
	Turbidity vs pH	−0.33	0.346	NS
Green Chili	pH vs Acidity	−0.16	0.659	NS
	Browning vs Acidity	−0.54	0.109	NS
	Turbidity vs Acidity	−0.49	0.151	NS
	Turbidity vs pH	+0.45	0.194	NS
Buttermilk	pH vs Acidity	−0.75	0.013	* (significant)
	Browning vs Acidity	−0.22	0.537	NS
	Turbidity vs Acidity	−0.49	0.155	NS
	Turbidity vs pH	+0.75	0.012	* (significant)

*Note: *NS: Not significant ($p > 0.05$); Significant at $p < 0.05$; "Borderline" indicates $p \approx 0.05$.*

Multivariate Trends

PCA was performed to assess multivariate separation among the three starter treatments at Day 1, Day 5, and Day 10. At Day 1, PC1 accounted for 87.2% of the variance and PC2 for 12.8%, with treatments clustering closely together (Supplementary Figure S1). By Day 5, PC1 explained 79.3% and PC2 20.7%, with clove beginning to separate from the other treatments (Supplementary Figure S2). At Day 10, clear separation was evident in the PCA biplot (Figure 6), with PC1 (81.1%) distinguishing clove (aligned with higher acidity, browning, and turbidity) from buttermilk (aligned with higher pH) and green chili stalk (intermediate). PCA loadings indicated that acidity, browning, and turbidity contributed strongly and positively to PC1, while pH was inversely aligned.

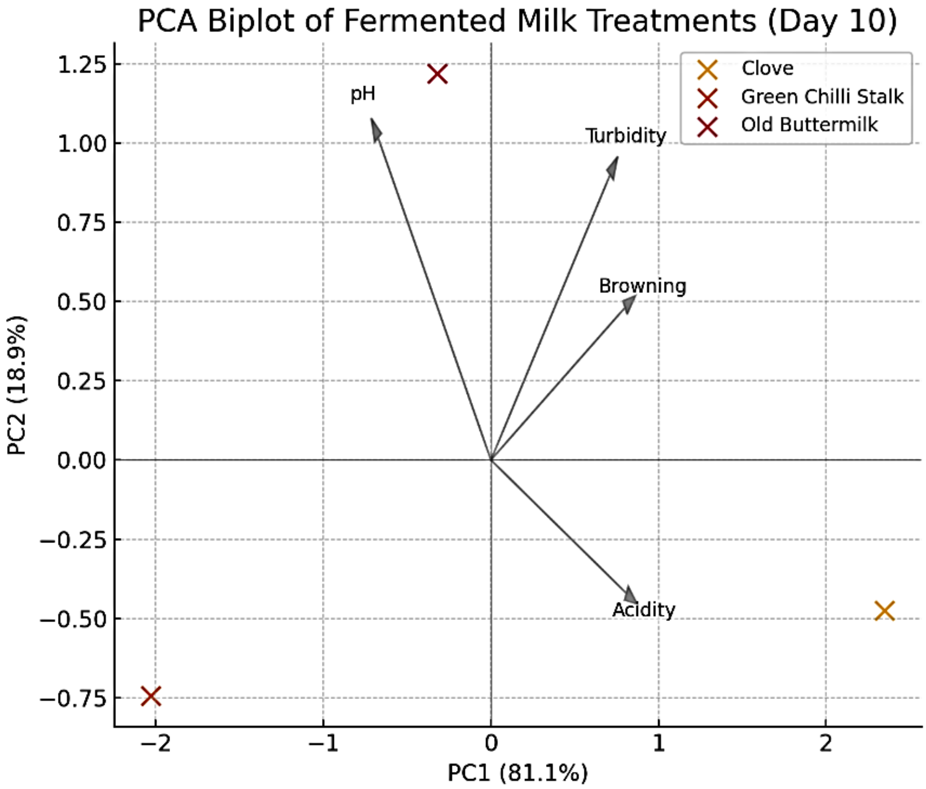

Figure 6. PCA biplot of fermented milk treatments at day 10.

Principal Component Analysis (PCA) biplot showing the multivariate separation of the three starter treatments (Clove, Green Chili Stalk, and Old Buttermilk) at Day 10, based on standardized values of pH, titratable acidity, browning index, and turbidity. Data points represent treatments, and arrows indicate the loadings (contribution) of each parameter to the first two principal components (PC1 and PC2), which explain 81.1% and 18.9% of the variance, respectively. Cloves align strongly with higher acidity, browning, and turbidity, whereas Old Buttermilk was associated with higher pH, and Green Chili Stalk exhibited an intermediate profile.

DISCUSSION

Fermentation Kinetics & Starter Efficacy

The fermentation kinetics clearly demonstrated that clove was the most effective starter, yielding the lowest pH and highest TA, turbidity, and BI by Day 10. This indicates accelerated microbial activity and robust lactic acid production compared to green chili stalk and old buttermilk. Similar fermentative efficiency has been reported with other spice or extract-enriched dairy products; for example, milk beverages fortified with clove extract exhibited enhanced phytochemical and antioxidant activity during cold storage [13, 14]. Traditional kalchi (yogurt with chili stalk) also showed successful acidification when chili stalks were used as a starter, supporting our findings [15].

Microbial and Biochemical Impacts

Cloves contain eugenol and related phenolics that have antimicrobial and antioxidant properties. In controlled concentrations, these compounds can inhibit spoilage organisms while supporting LAB; this may have enhanced fermentation efficiency and product quality in our clove-treated samples. Studies on kimchi and fermented vegetables have shown that clove addition controls microbial growth and enriches bioactive profiles [13], suggesting similar underlying mechanisms in our milk fermentations.

Correlation & Multivariate Insights

Significant positive correlation between acidity and turbidity in clove treatment ($r = +0.67$, $p = 0.036$) indicates that increased acid production coincided with microbial proliferation and protein coagulation. In buttermilk treatment, strong negative correlation between pH and acidity, and a positive correlation between turbidity and pH highlights the reactive nature of the microbial environment. No significant correlations were observed in green chili stalk treatment, which aligns with its comparatively mild fermentation kinetics.

PCA further revealed clear separation of clove from other treatments by Day 10, driven principally by acidity, browning, and turbidity. This aligns with previous uses of PCA in fermentation studies to track parameter-driven differentiation [16].

Traditional Wisdom & Scientific Validation

Chili stalks are traditionally discarded as agricultural residue; however, studies have demonstrated their capacity to host nondairy LAB capable of acidifying milk [15]. While our green chili stalk treatment exhibited moderate fermentative behavior, it remains culturally and economically valuable. Old buttermilk, a traditional and reliable starter, produced expected fermentation results and served as a practical control.

Notably, this is among the first studies to directly compare clove and chili stalk as starters in buffalo milk fermentation under controlled conditions, while also employing colorimetric indices (browning and turbidity) – an approach common in vegetable fermentation [15] but rare in dairy research.

Practical and Nutritional Implications

Clove infusion may offer dual benefits: serving as a natural starter while enhancing antioxidant profile – supported by increased browning (melanoidin formation) and likely contributing phenolic compounds like those in clove extract–fortified dairy. This suggests a promising avenue for developing functional fermented dairy with improved shelf-life and health benefits.

Limitations & Further Research

This study focused on physicochemical and colorimetric properties as indicators of fermentation. A primary limitation is the reliance on turbidity ($OD_{600-650}$) as an indirect proxy for microbial growth. Future work must include direct microbial enumeration (e.g., LAB and total aerobic counts via plate-based methods, or targeted qPCR) to definitively characterize the microbial ecology and load contributed by each starter.

Additionally, the functional claims and consumer acceptability of these products remain unvalidated. Sensory evaluation (e.g., taste, aroma, texture) by a trained panel is essential. Quantification of antioxidant activity (e.g., via DPPH or ABTS assays) and analysis of bioactive compounds (e.g., phenolic content, eugenol) using HPLC or GC-MS are critical next steps to substantiate the proposed health benefits and functional potential of plant-based starters, like clove.

CONCLUSIONS

In this study, we investigated the efficacy of three natural fermentation starters – clove, green chili stalk, and old buttermilk – in buffalo milk fermentation under controlled laboratory conditions. Our findings demonstrated that: Clove exhibited superior fermentative performance, characterized by the most pronounced decline in pH, the highest levels of TA, turbidity, and BI by Day 10. This indicates rapid microbial activity and enhanced melanoidin formation.

Old buttermilk, a traditional starter, showed consistent and reliable fermentative behavior, with strong negative correlation between pH and acidity and a positive relationship between turbidity and pH, reflecting its effectiveness in acidification and coagulation. Green chili stalk produced a gentler fermentation profile, with lower acidification, browning, and turbidity, yet still represents a culturally relevant and eco-friendly starter option.

Multivariate analyses, including Pearson's correlations and PCA, confirmed that clove creates a distinct fermentation trajectory compared to the other starters. Clove's unique combination of phytochemical properties and microbiological activity suggests it is a promising alternative starter, capable of producing fermented milk with enhanced physicochemical attributes and potential functional benefits.

These results support the potential of plant-based and traditional fermentation starters in enhancing dairy product quality and diversifying starter culture options. Future research should focus on microbial characterization, sensory profiling, and bioactive compound quantification to fully validate the functional and commercial potential of clove-based fermented milk.

It is important to note that these conclusions are based on physicochemical and optical proxies. The proposed functional benefits and microbial mechanisms require further validation through direct microbial analysis, sensory evaluation, and quantification of bioactive compounds. Nonetheless, these results strongly support the potential of plant-based starters, particularly clove, in enhancing dairy product quality and diversifying starter culture options.

Credit Authorship Statement

All authors equally contributed to the conception and design of the study. All authors have read and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this manuscript.

REFERENCES

1. Bintsis T, Papademas P. The evolution of fermented milks, from artisanal to industrial products. Fermentation. 2022;8(12):679. doi: 10.3390/fermentation8120679.
2. Marina Mefleh M, Darwish AMG, Mudgil P, Maqsood S, Boukid F. Traditional fermented dairy products in Southern Mediterranean countries: From tradition to innovation. Fermentation. 2022;8(12):743. doi: 10.3390/fermentation8120743.
3. Wiyanto R, Cahyadi P, Septiani R. Yogurt making as a tool to understand the food fermentation process. J Microbiol Biol Educ. 2019;20(2).
4. Pandey VK, Shams R, Singh R, Dar AH, Pandiselvam R, Rusu AV, et al. A comprehensive review on clove (Caryophyllus aromaticus L.) essential oil and its significance in edible coatings for food applications. Front Nutr. 2022;9:987674. doi: 10.3389/fnut.2022.987674.
5. Sharma S, Jain S, Nair GN, Ramachandran S. Capsicum annuum enhances L-lactate production by Lactobacillus acidophilus: Implication in curd formation. J Dairy Sci. 2013;96(7):4142–4148. doi: 10.3168/jds.2012-6243.

6. Nuñez IM, Verni MC, Martinez FEA, Babot JD, Terán V, Danilovich ME, et al. Novel lactic acid bacteria strains from regional peppers with health-promoting potential. Fermentation. 2024;10(4):209. doi: 10.3390/fermentation10040209.
7. Olatide M, Arawande JO, George OO, Aborisade AB, Olasupo AD. Pilot study on chili stalks as a source of non-dairy lactic acid bacteria in yogurt making. Appl Food Sci J. 2019;3(1):5–8.
8. Kim SH, Seo JH, Jeong YJ. Quality comparison of non-thermal sterilized raw apple vinegar and commercial products. Food Sci Preserv. 2024;31(2):235–244. doi: 10.11002/fsp.2024.31.2.235.
9. Mira P, Yeh P, Hall BG, et al. Estimating microbial population data from optical density. PLoS One. 2022;17(7):e0267134. doi: 10.1371/journal.pone.0276040.
10. Rasane P, Kailey R, Singh SK. Fermented indigenous Indian dairy products: Standards, nutrition, technological significance and opportunities for its processing. J Pure Appl Microbiol. 2017;11(2):1199–1213. doi: 10.22207/JPAM.11.2.68.
11. Tomovska J, Gjorgievski N, Makarijoski B. Examination of pH, titratable acidity and antioxidant activity in fermented milk. J Mater Sci Eng A. 2016;6(11–12):326–33. doi: 10.17265/2161-6213/2016.11-12.006.
12. Goa T, Beyene G, Mekonnen M, Gorems K. Isolation and characterization of lactic acid bacteria from fermented milk produced in Jimma Town, Southwest Ethiopia, and evaluation of their antimicrobial activity against selected pathogenic bacteria. Int J Food Sci. 2022;2022:2076021. doi: 10.1155/2022/2076021.
13. Kang M, Park J, Yoo S. Effect of clove powder on quality characteristics and shelf life of kimchi paste. Food Sci Nutr. 2019;7(2):537–546. doi: 10.1002/fsn3.833.
14. Bikheet MM, Hassan HM, Omar MO, Abdel-Aleem WM, Galal SM, Korma SA, et al. Effects of clove (Syzygium aromaticum) extract on antibacterial activity, phytochemical properties, and storage quality of flavored milk beverages. J Dairy Sci. 2025;108(4):3300–3313. doi: 10.3168/jds.2024-26023.
15. Sangeeta S, Rai S, Ramachandran P, Yadav P, Chandola G. Value Addition of Spices Processing Industrial Waste. In: Selvamuthukumaran M, editor. Wealth out of Food Processing Waste: Ingredient Recovery and Valorization. 1st ed. Boca Raton: CRC Press; 2024. p. 188-223.
16. Widyastuti Y, Febrisiantosa A. The role of lactic acid bacteria in milk fermentation. Food Nutr Sci. 2014;5(4):435–442. doi: 10.4236/fns.2014.54051.

SUPPLEMENTARY MATERIAL
Supplementary Tables

Supplementary Table S1. pH of fermented milk samples over 10 days.

Day	Clove	Green Chili	Old Buttermilk
Day 1	3.22	3.12	3.02
	3.21	3.15	3.03
	3.22	3.15	3.03
	3.22	3.18	3.06
Day 2	3.77	3.76	3.62
	3.8	3.77	3.66
	3.79	3.77	3.66
	3.8	3.77	3.67
Day 3	3.23	3.27	3.65
	3.14	3.21	3.53
	3.23	3.22	3.5
	3.24	3.21	3.52
Day 4	2.74	3.77	4.07
	2.5	3.78	4.02
	2.81	3.75	4.02
	2.72	3.73	4
Day 5	2.65	3.6	3.9

	2.62	3.58	3.88
	2.68	3.59	3.89
	2.66	3.57	3.87
Day 6	2.6	3.55	3.85
	2.58	3.53	3.83
	2.61	3.54	3.84
	2.59	3.52	3.82
Day 7	2.55	3.5	3.8
	2.53	3.48	3.78
	2.54	3.49	3.79
	2.52	3.47	3.77
Day 8	2.5	3.45	3.75
	2.48	3.43	3.73
	2.49	3.44	3.74
	2.47	3.42	3.72
Day 9	2.45	3.4	3.7
	2.44	3.38	3.68
	2.46	3.39	3.69
	2.43	3.37	3.67
Day 10	2.4	3.35	3.65
	2.38	3.33	3.63
	2.39	3.34	3.64
	2.37	3.32	3.62

Supplementary Table S2. Titratable acidity (% lactic acid) over 10 days.

Day	Clove	Green Chili	Old Buttermilk
Day 1	0.54	0.76	0.95
	1.02	0.74	0.1
	0.95	0.81	1.08
	0.99	0.77	1.1
Day 2	1.14	0.9	1.19
	1.11	0.96	0.6
	0.95	0.46	0.67
	0.94	1.02	0.67
Day 3	0.27	0.36	0.27
	0.19	0.29	0.22
	0.21	0.38	0.28
	0.2	0.28	0.36
Day 4	0.22	0.14	0.26
	0.26	0.2	0.2
	0.25	0.14	0.25
	0.45	0.18	0.23
Day 5	0.5	0.22	0.28
	0.52	0.23	0.29
	0.54	0.24	0.3
	0.53	0.23	0.29
Day 6	0.56	0.26	0.32
	0.58	0.27	0.33
	0.6	0.28	0.34
	0.59	0.27	0.33
Day 7	0.62	0.29	0.35
	0.64	0.31	0.37
	0.65	0.32	0.38
	0.63	0.3	0.36

Day 8	0.66	0.33	0.39
	0.68	0.34	0.4
	0.7	0.35	0.41
	0.69	0.34	0.4
Day 9	0.72	0.36	0.42
	0.73	0.38	0.44
	0.74	0.39	0.45
	0.73	0.37	0.43
Day 10	0.76	0.4	0.46
	0.78	0.41	0.47
	0.79	0.42	0.48
	0.77	0.41	0.47

Supplementary Table S3. Browning index (absorbance at 420 nm).

Day	Clove	Green Chili	Old Buttermilk
Day 1	0.65	0.17	0.22
	0.63	0.17	0.23
	0.64	0.17	0.24
	0.66	0.17	0.23
Day 2	0.92	0.57	0.85
	0.85	0.56	0.87
	0.86	0.56	0.9
	0.87	0.56	0.89
Day 3	0.62	0.42	0.27
	0.57	0.38	0.29
	0.53	0.4	0.23
	0.42	0.27	0.74
Day 4	0.74	0.62	0.73
	0.74	0.61	0.73
	0.75	0.62	0.71
	0.62	0.73	0.71
Day 5	0.78	0.68	0.75
	0.77	0.69	0.76
	0.79	0.7	0.77
	0.78	0.69	0.76
Day 6	0.82	0.71	0.78
	0.83	0.72	0.79
	0.84	0.73	0.8
	0.83	0.72	0.79
Day 7	0.85	0.74	0.81
	0.86	0.75	0.82
	0.87	0.76	0.83
	0.86	0.75	0.82
Day 8	0.88	0.77	0.84
	0.89	0.78	0.85
	0.9	0.79	0.86
	0.89	0.78	0.85
Day 9	0.91	0.8	0.87
	0.92	0.81	0.88

	0.93	0.82	0.89
	0.92	0.81	0.88
Day 10	0.94	0.83	0.9
	0.95	0.84	0.91
	0.96	0.85	0.92
	0.95	0.84	0.91

Supplementary Table S4. Turbidity (absorbance at 620 nm).

Day	Clove	Green Chili	Old Buttermilk
Day 1	0.6	0.14	0.18
	0.6	0.14	0.18
	0.61	0.15	0.19
	0.62	0.15	0.17
Day 2	0.83	0.54	0.66
	0.82	0.54	0.65
	0.82	0.54	0.66
	0.83	0.53	0.49
Day 3	0.49	0.34	0.19
	0.48	0.34	0.2
	0.48	0.34	0.2
	0.34	0.17	0.69
Day 4	0.69	0.58	0.66
	0.69	0.58	0.65
	0.69	0.58	0.66
	0.57	0.66	0.66
Day 5	0.7	0.6	0.68
	0.69	0.61	0.69
	0.71	0.62	0.7
	0.7	0.61	0.69
Day 6	0.73	0.63	0.71
	0.74	0.64	0.72
	0.75	0.65	0.73
	0.74	0.64	0.72
Day 7	0.76	0.66	0.74
	0.77	0.67	0.75
	0.78	0.68	0.76
	0.76	0.67	0.75
Day 8	0.78	0.69	0.77
	0.79	0.7	0.78
	0.8	0.71	0.79
	0.79	0.7	0.78
Day 9	0.81	0.72	0.8
	0.82	0.73	0.81
	0.83	0.74	0.82
	0.82	0.73	0.81
Day 10	0.84	0.75	0.83
	0.85	0.76	0.84
	0.86	0.77	0.85
	0.85	0.76	0.84

Supplementary Table S5. Full ANOVA results.

ANOVA of pH						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***

Sample	3.79773	9	0.42197	394.7744283	0	1.985594964
Columns	16.895165	2	8.4475825	7903.143711	0	3.097698035
Interaction	7.716835	18	0.4287130556	401.0829002	0	1.719592446
Within	0.0962	90	0.001068888889			
Total	28.50593	119				

ANOVA Titratable Acidity

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	4.947333333	9	0.5497037037	36.53325457	0	1.985594964
Columns	1.245755	2	0.6228775	41.39637794	0	3.097698035
Interaction	0.4983616667	18	0.02768675926	1.840059322	0.03199719789	1.719592446
Within	1.3542	90	0.01504666667			
Total	8.04565	119				

ANOVA of Browning Index

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	3.6978075	9	0.4108675	155.5170855	0	1.985594964
Columns	0.5535516667	2	0.2767758333	104.7621701	0	3.097698035
Interaction	0.468465	18	0.02602583333	9.851014615	0	1.719592446
Within	0.237775	90	0.002641944444			
Total	4.957599167	119				

ANOVA of Turbidity

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	3.221986667	9	0.3579985185	123.5661234	0	1.985594964
Columns	0.462545	2	0.2312725	79.82559923	0	3.097698035
Interaction	0.4284383333	18	0.02380212963	8.21550016	0	1.719592446
Within	0.26075	90	0.002897222222			
Total	4.37372	119				

Note: SS = Sum of Squares; df = degrees of freedom; MS = mean square. Actual SS, df, MS can be inserted from ANOVA output.

Supplementary Table S6. PCA loadings for day 1, day 5, and day 10.

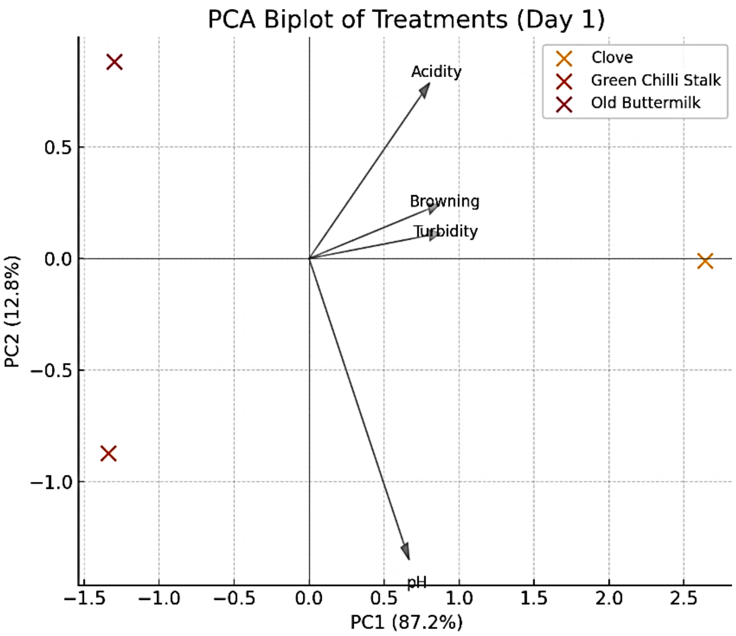
Parameter	**PC1 (Day 1)**	**PC2 (Day 1)**	**PC1 (Day 5)**	**PC2 (Day 5)**	**PC1 (Day 10)**	**PC2 (Day 10)**
pH	−0.53	+0.41	−0.49	+0.50	−0.45	+0.68
Acidity	+0.51	−0.36	+0.54	−0.34	+0.54	−0.28
Browning	+0.52	+0.45	+0.51	+0.46	+0.53	+0.32
Turbidity	+0.43	+0.69	+0.43	+0.62	+0.47	+0.60

Supplementary Table S7. Variance explained by first two principal components (PC1 and PC2) at day 1, day 5, and day 10.

Day	**PC1 (%)**	**PC2 (%)**	**Total Variance (%)**
Day 1	87.18	12.82	100
Day 5	79.30	20.70	100
Day 10	81.13	18.87	100

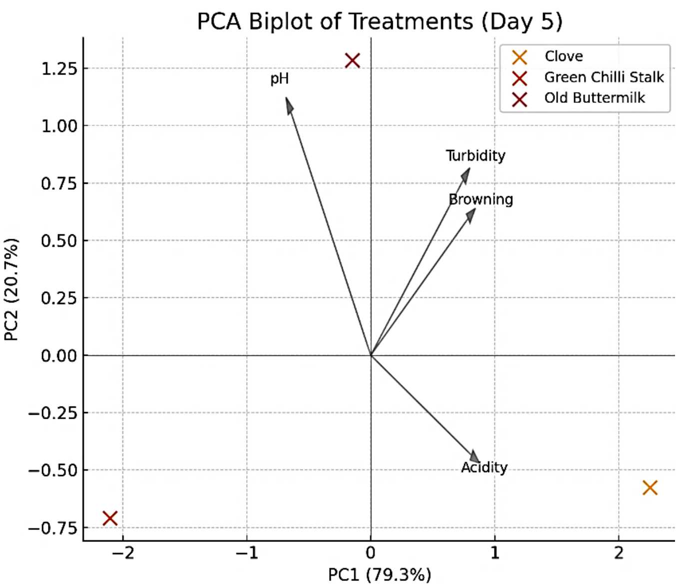
Percentage of total variance in the data explained by the first two principal components (PC1 and PC2) from Principal Component Analysis (PCA) of standardized pH, titratable acidity, browning index, *and turbidity values at each time point. PC1 accounted for the majority of variance at all time points, with increasing contribution of PC2 at Day 5 and Day 10 as treatments diverged.*

Supplementary Figures



Supplementary Figure S1. PCA biplot of fermented milk treatments at day 1.

PCA biplot illustrates the initial multivariate profile of the three starter treatments at Day 1. PC1 and PC2 explain 87.2% and 12.8% of the variance, respectively. At this stage, treatments clustered closely together, indicating minimal separation and similar fermentation profiles.



Supplementary Figure S2. PCA biplot of fermented milk treatments at day 5.

PCA biplot depicting the intermediate multivariate separation of treatments at Day 5. PC1 and PC2 explain 79.3% and 20.7% of the variance, respectively. Cloves began to diverge from the other treatments, aligning more strongly with increased acidity and browning, while Old Buttermilk and Green Chili Stalk remained closer and less differentiated.

