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Analyzing the Relationship between Microbial Abundance and Enzyme Activity in Dairy Milk for Quality Assessment

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Abstract

The quality of dairy milk depends heavily on microbial contamination and enzyme active measures. Research analysis examined the yeast and mold counts along with Aerobic Plate count (APC) and enzyme activities, including alkaline phosphatase and phosphatase, using 525 pasteurized, 560 raw, and 645 sterilized milk samples. Research measured pH levels and counts of Aerobic Bacillus (AB) and the concentration of Volatile Fatty Acids (VFA). Results indicated that 10.20% of raw milk and 2.45% of pasteurized milk samples exceeded the threshold for APC count. Yeast and mold counts were significantly higher in raw milk (8.56%) compared to pasteurized (5.65%) and sterilized milk (9.30%). AB abundance was highest in raw milk (6.32%) and lowest in sterilized milk (5.65%). Alkaline phosphatase activity (ALP) levels exceeded the critical threshold in raw milk, pasteurized milk and sterilized milk samples. Raw milk had pH of 6.90, slightly higher than pasteurized milk (6.78) but similar to sterilized milk (6.88). VFA concentrations were higher in raw milk (0.40 mM) than in pasteurized (0.28 mM) and sterilized milk (0.35 mM), indicating greater microbial metabolic activity in raw samples. These findings highlight the microbial and enzymatic variations across different milk processing methods, emphasizing need for effective thermal treatments to ensure milk safety. Research established that Bacillus microbes linked positively to ALP measurements in raw milk samples, while pasteurized milk activity levels were related negatively to microbial populations. The analysis demonstrates that dairy milk assessment depends heavily on microbial counts combined with enzymatic activity as well as quality measure indicators.

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Introduction

The quality attributes milk possesses are controlled by intrinsic and extrinsic factors where microbial abundance and enzyme activity play decisive roles (Quintieri et al., 2024). Milk is colonized by bacteria, yeasts, and molds, which can either enhance or deteriorate its quality (Komorek et al., 2024). The production of dairy products like yogurt, cheese, and kefir relies on beneficial microorganisms for fermentation (Sungur et al., 2021; Sibanda et al., 2024). Harmful microorganisms in dairy products can lead to spoilage, unpleasant flavors, and potential health hazards for consumers (Samyuktha et al., 2022; Piras et al., 2024). Microbial levels in milk are directly influenced by dairy handling, storage, and processing conditions, which are crucial for quality control (joshi et al., 2024). Natural enzymes in milk play a crucial role in modifying its sensory characteristics and functional properties. The milk proteins contain three key enzymes: lipases, proteases, and lactase (Pieta & onyeka, 2024). Enzymes split fatty acids, proteins, and lactose, influencing milk's taste, texture, and shelf life. Enzyme activities enhance several dairy processes, but uncontrolled or excessive enzyme activity results in negative outcomes, such as bitterness and rancidity as well as protein breakdown (Choudhary et al., 2024). The activity of enzymes is regulated by microbial processes through the creation of enzymes by numerous microorganisms that break down milk quality (Wang et al., 2024). A great understanding of the relationship between microbial abundance and enzyme activity is essential to ensure good-quality milk (Smihunova et al., 2024). The relationship between these two could be complex because intrinsic and extrinsic factors like environmental factors and forest microbial species have been reported to either enhance or inhibit enzyme activity in milk (Mezzetti et al., 2024).

According to (Ding et al., 2024), commercial sterility does not guarantee that Ultra-High Temperature (UHT) milk will be stable for more than six months. Pseudomonas and Staphylococcus are the dominant bacteria present both in normal and degraded UHT milk. The bacterial counts are increased in degraded milk such that the threshold is inversely related to protease resistance. Srivastava et al., (2025) suggested a global need for safe food products and amenities is growing, and installing a Food Safety system can boost overall performance. Hazard Analysis and Critical Control Points (HACCP) and production processes promote healthy eating. Improved methods of detection can help to avoid adulteration. This research examines recent technology for improving the safety of food, quality, and ecology (Niazi, 2017). Elhalik et al., (2024) analyzed the ammonifying microorganisms (Amm) are critical to the global usage of farming solid residue (Noubar & Salehy, 2017). Investigating the Effect of Social Networks on Supply Chain in Industrial Enterprises (Food Industry) of Kermanshah Province. *International Academic Journal of Accounting and Financial Management*, *4*(2), 50–64. In a research effort, various procedures were utilized to co-compost cow waste with wheat husk. The results revealed that Amm seeding dropped total organic carbon, organic matter in suspension, Carbon Dioxide (CO_2) generation, and releases of Methane (CH_4) by 35%, 16.7%, 1.75%, and 18.0%, respectively. Amm-4 demonstrated the greatest enhancement for composting, which increased vegetable development and carbon absorption. Xu et al., (2024) introduced the research conducted in China discovered that psychrophilic bacteria, the primary spoilage species in raw milk, are accountable for dairy and dairy product degradation. The research found 40 genera and 185 species of psychrophilic microorganisms with Aeruginosa being the most common. Changing seasons altered the variety of these specific bacteria. Metagenomic analysis offers operational details about these organisms to understand their activities and improve raw milk quality.

Metagenomic sequencing, Liquid Chromatography-Mass Spectrometry (LC-MS), and Gas Chromatography-Mass Spectrometry (GC-MS) were used to examine unsweetened milk, pasteurized cow's milk, and ultra-heated samples of milk analyzed by (Zhang et al., 2024). The results revealed several microbial shifts and metabolic alterations that occurred during storage. The top three genera, Microbacterium, Actinomycetia, and microorganisms, were shown to be the most abundant microbes in pasteurized milk. Zhou et al., (2024) analysis underlines the importance of emerging biomarkers in the food sector towards authenticating milk and its byproducts (Khademi et al., 2015). Traditional methods fail to detect complex adulteration tactics, emphasizing the necessity for innovative alternatives. Biomarker discovery methodologies include genomes, and proteomics, for example, metabolomics, along with other -omics approaches. The evaluation assesses possible biomarker sources, addressing issues, such as standardization, reproducibility, and compatibility with existing authentication systems. Incorporate innovative biomarkers increases customer confidence and happiness in the cheese and dairy industry. Zhang et al., (2024) examined the impact of different levels of proteins on ruminal construction, fermenting properties, digesting enzymes, bacteria, and toxins in Tibetan sheep. It was revealed that a crude protein content of 13.03% increased ruminal shape, and production characteristics, thus stimulating stomach activity of enzymes via altering the microbial population and controlling metabolism. Sun et al., (2024) investigation examine how Effective Microorganisms (EM) inoculation affects compost quality and microbial populations in Compost-Bedded Pack Barns (CBP). The results reveal that EM seeding reduces respirable particles, lowers blood total protein and lipid levels, and boosts the amount of bacterial and fungal variety. Co-supplementing with L, B, plus S strengthens the impact. Romero et al., (2024) suggested that Ohmic Heating (OH) is an option for pasteurizing liquid meals. An investigation explored the impacts of OH on pasteurized dairy products using various voltage gradients and a water bath. The results demonstrated that increased OH inactivates bacteria, decreases warming energy and time, and preserves sensory quality. However, larger voltages efficiently inactivate bacteria while maintaining milk quality.

Objective of the research: To evaluate the relationship between microbial count and enzyme activity in dairy milk by assessing microbial counts, enzymatic markers, pH, and volatile fatty acids (VFA) and their influence on milk quality and safety.

Material and Methods

Microbial and biochemical analysis were performed to assess the quality and safety of raw, pasteurized, and sterilized milk samples. In total, 1730 samples were analyzed for microbial contamination and enzymatic activity. Key parameters are yeast and mold count, a phosphatase activity level, pH with Bacillus presence, and VFA concentration, which were analyzed using standard methods for microbiology and biochemistry.

Microbial and Biochemical Indicators of Milk Quality

Microbiological and biochemical indicators are fundamental in milk quality assessment, safety validation, and determination of shelf-life stability. Because microbial load, enzyme activity, metabolic by-products, and biochemical changes are factors that determine spoilage rates and potential health risks, the monitoring of these indicators will provide insight into microbial contamination, thermal processing efficiency, and general dairy product integrity during storage and distribution.

Aerobic Plate Count (APC): Quantifies viable microorganisms in milk, serving as a key indicator of microbial quality and hygienic processing. A 25 g milk sample was homogenized in 0.85% saline solution, serially diluted, and plated on plate count agar (PCA). Incubation at 36°C for 48 hours facilitated colony enumeration, expressed as log CFU/mL. The limit of detection (LOD) was 1 $\log_{10}$ CFU/mL. Elevated APC

values indicate inadequate thermal processing or post-pasteurization contamination, highlighting the need for stringent quality control measures to ensure microbial safety and compliance with dairy industry standards.

Aerobic Bacillus (AB) and Thermophilic Bacillus Abundance (TAB): Bacillus species, including thermophilic strains, present spoilage and safety risks in dairy products due to their spore-forming capability. Samples underwent heat treatment (80°C for AB, 100°C for thermophilic Bacillus), followed by incubation on Dextrose Tryptone Agar (DTA) and Milk Plate Count (MPC) agar. Enumeration, reported as log CFU/mL, offered insights into microbial resilience post-thermal processing. Elevated Bacillus levels suggest insufficient pasteurization or improper storage, emphasizing the importance of optimized heat treatment and strict aseptic practices to maintain product integrity and consumer safety.

Yeast and Mold Counts: Fungal contamination in milk compromises shelf life, safety, and sensory quality, necessitating rigorous assessments. Yeast and mold counts were determined by inoculating serially diluted samples onto potato dextrose agar (PDA) incubating at 25°C for five days. Colonies were enumerated as log CFU/mL. Elevated counts suggest post-processing contamination, poor refrigeration, or inadequate sanitation. The presence of mycotoxin-producing molds poses additional risks, underscoring the necessity for early microbial detection, robust cold chain, and meticulous hygiene to prevent fungal proliferation in dairy products.

Alkaline Phosphatase Activity (ALP): It serves as a biomarker for pasteurization efficacy, mirroring the thermal inactivation of pathogens. A 100 µL milk sample was reacted with a chemiluminescent substrate and quantified via luminometry, with an LOD of 1.30 $\log_{10}$ mU/L and an Limit of Quantification (LOQ) of 1.78 $\log_{10}$ mU/L. Persistent ALP activity in pasteurized milk signals suboptimal heat treatment or contamination, necessitating strict monitoring to ensure compliance and safeguard vulnerable groups like infants and immune-compromised individuals.

Phosphatase Activity: Residual phosphatase activity in dairy products is a critical indicator of pasteurization efficacy and microbial safety. Employing a spectrophotometric assay, hydrolysis of p-nitrophenyl phosphate was monitored at 405 nm, allowing assessment of thermal stability and processing integrity. Elevated phosphatase levels in pasteurized milk suggest insufficient heat treatment, correlating with potential pathogen survival. Compliance with regulatory thresholds requires precise thermal treatment, real-time monitoring, and enzyme deactivation to maintain product safety and extend shelf life.

pH Measurement: The milk reveals a great deal about microbial activity and spoilage, along with biochemical stability. A calibrated digital pH meter was used to research samples at room temperature, with any shift from the ideal pH range (6.6 to 6.8) indicating microbial growth or enzymatic activity. Raw milk was more acidic (pH 6.30) because lactic acid bacteria were present, while pasteurized milk remained neutral (pH 7.05), indicative of microbial inactivation. The changes in pH will vary according to bacterial metabolism and proteolysis, suggesting that monitoring should be carried out continuously for spoilage prediction and quality assurance practices throughout dairy production.

Volatile Fatty Acid (VFA) Concentration: It is used as a marker for fermentation and definitely quality deterioration. Solid-Phase Microextraction (SPME) GC-Flame Ionization Detection (GC-FID) analysis identified the important volatile fatty acids, acetic, butyric, and propionic acids. Their significant levels in raw milk (0.96 mM) indicate increased microbial activity, whereas the low levels in sterilized milk (0.28 mM) point toward an effective lethality with heat. Profiles of VFA influence flavor and texture and spoilage, thus determining their assessment in optimizing storage, shelf life, and consumer acceptability in dairy products.

Data Analysis

The mean, standard deviation, median, minimum values, maximum values, and percentiles for microbial and enzymatic parameters were calculated using IBM SPSS Statistics 27. Multiple regression modeling assessed microbial load, enzyme activity, and biochemical parameters affecting milk quality. Spearman's correlation analysis ($p < 0.05$) identified significant associations, visualized via heat maps. Values below LOD and LOQ were set to zero. Over-limit ratio analysis indicated regulatory non-compliance risks. Results confirmed pasteurization and sterilization effectively reduce microbial and enzymatic activity, ensuring milk safety and quality.

Result

Microbiological and biochemical properties of raw, pasteurized, and sterilized milk, focusing on microbial load, enzyme activity, pH, and VFA, Raw milk exhibited the highest bacterial contamination, while pasteurization and sterilization significantly reduced microbial counts. Enzyme activity, including alkaline phosphatase, confirmed heat treatment efficacy. Yeast and mold levels decreased in processed milk, ensuring improved safety. pH remained stable, with minor variations due to processing. Elevated VFA in raw milk indicated active metabolism. Over-limit ratios suggested potential non-compliance, underscoring the need for stringent quality control to enhance milk safety and regulatory compliance. Figure 1 presents a comparative analysis of microbial contamination, enzymatic activity, and biochemical stability in raw, pasteurized, and sterilized milk. It employs a grouped bar chart to illustrate variations in APC, yeast & mold, Bacillus abundance, alkaline phosphatase, phosphatase activity, pH levels, and VFA concentration across different milk treatments. The substantial microbial load and enzymatic activity in raw milk indicate high contamination and endogenous enzymatic processes. Pasteurization significantly reduces microbial abundance and enzymatic activity, while sterilization nearly eliminates them, demonstrating its superior microbial inactivation efficiency. The pH variation aligns with microbial metabolism, as fermentation processes lower pH in raw milk. The VFA concentration trend further supports microbial activity's role in biochemical deterioration. This figure is directly related to the research as it quantifies the impact of thermal processing on milk safety and quality, reinforcing the necessity of controlled heat treatments.

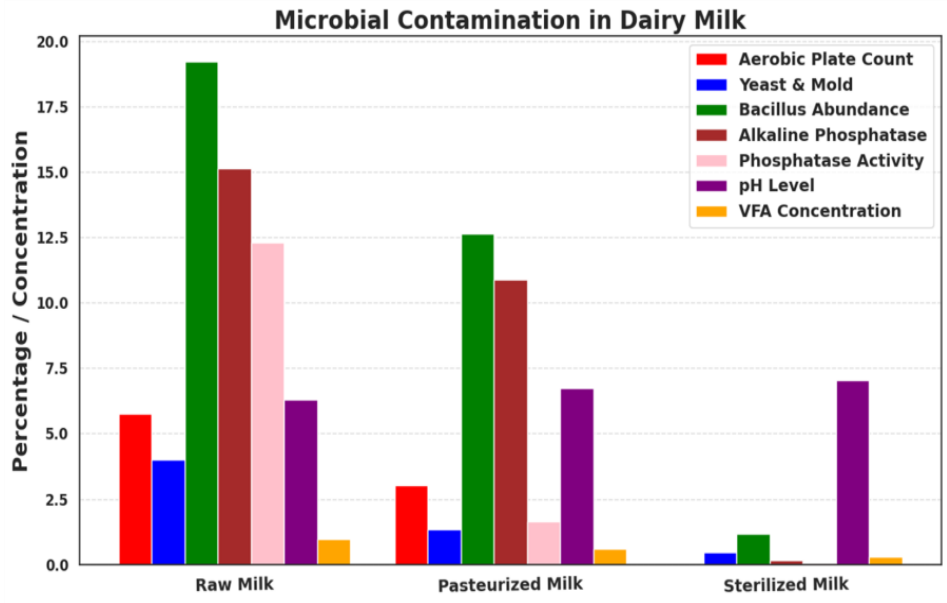

Figure 1. Comparative Analysis of Microbial and Biochemical Changes in Diary Milk

Table 1 provides a comparative analysis of microbiological and biochemical parameters in raw, pasteurized, and sterilized milk, highlighting the effects of thermal treatments on milk safety and quality. Key indicators include APC, AB, TAB, ALP, yeast & mold counts, pH levels, and VFA concentrations. The data show that raw milk has the highest microbial loads, with elevated APC, AB, and yeast & mold counts, reflecting significant contamination risks. Active enzymatic degradation exists in milk samples because of elevated alkaline phosphatase and phosphatase activity that impairs milk quality. The pasteurization process both decreases pathogenic organisms and breaks down enzymes, yet it leaves behind minimal remaining contamination that meets safety standards. The sterilization process eliminates almost every microorganism for improved safety along with better stability of products. The differences in pH values reveal that microbiological metabolism affects acidity measurements because microorganisms create lactic acids in raw milk samples. The VFA concentration follows a similar trend, further confirming microbial-driven biochemical deterioration. This demonstrates the thermal processing reduces microbial and enzymatic activity. It confirms the role of pasteurization and sterilization in providing milk safety and informs to regarding the microbial and biochemical stability of processed dairy products.

Table 1. Microbiological threshold values for dairy milk

Item	Sample Type	Mean ± SD	Median	Min	Max	40th Perc.	60th Perc.	Over-Limit Ratio
APC (log_{10} CFU/mL)	Raw	4.80 ± 1.55	4.60	<1	8.30	3.35	5.70	10.20% (57/560)
	Pasteurized	3.05 ± 1.30	2.60	<1	7.85	<1	3.20	2.45% (13/525)
	Sterilized	1.60 ± 0.52	1.55	<1	2.60	<1	1.30	8.56% (55/645)
AB (log_{10} CFU/mL)	Raw	2.20 ± 0.88	2.05	<1	5.15	<1	1.90	6.32% (35/560)
	Pasteurized	1.75 ± 0.72	1.60	<1	5.25	<1	1.70	4.85% (26/525)
	Sterilized	1.20 ± 0.22	1.15	<1	1.55	<1	1.28	5.65% (38/645)
TAB (log_{10} CFU/mL)	Raw	1.60 ± 0.50	1.55	<1	2.55	<1	1.70	5.78% (32/560)
	Pasteurized	1.72 ± 0.79	1.65	<1	3.95	<1	1.75	3.92% (21/525)
	Sterilized	<1	<1	<1	<1	<1	<1	7.90% (51/645)
ALP (log_{10} mU/L)	Raw	5.70 ± 0.45	5.72	2.05	6.80	5.58	5.88	9.82% (55/560)
	Pasteurized	2.35 ± 0.38	2.22	<1.80	3.65	<1.80	2.20	9.30% (49/525)
	Sterilized	1.95 ± 0.30	1.90	1.20	2.60	1.70	2.10	9.82% (63/645)
Yeast & Mould (log_{10} CFU/mL)	Raw	1.90 ± 0.50	1.85	<1	3.25	<1	2.00	8.56% (48/560)
	Pasteurized	1.25 ± 0.45	1.10	<1	2.75	<1	1.35	5.65% (30/525)
	Sterilized	1.10 ± 0.30	1.05	<1	1.70	<1	1.20	9.30% (60/645)

pH Level	Raw	6.90 ± 0.15	6.85	6.60	7.25	6.75	6.95	7.90% (44/560)
	Pasteurized	6.78 ± 0.12	6.75	6.60	7.10	6.70	6.82	4.50% (24/525)
	Sterilized	6.88 ± 0.14	6.85	6.65	7.15	6.78	6.90	8.56% (55/645)
VFA (mM)	Raw	0.40 ± 0.15	0.38	0.20	0.70	0.30	0.45	7.12% (40/560)
	Pasteurized	0.28 ± 0.10	0.26	0.15	0.50	0.22	0.32	3.80% (20/525)
	Sterilized	0.35 ± 0.14	0.32	0.18	0.60	0.28	0.40	7.12% (46/645)

Note: 30^{th} and 60^{th} per denotes the total samples

The contamination levels varied across milk samples. APC exceeded the threshold in 10.20% of raw, 2.45% of pasteurized and 8.56% of sterilized samples. AB was detected in 6.32% (raw), 4.85% (pasteurized), and 5.65% (sterilized), while TAB occurred in 5.78% (raw), 3.92% (pasteurized), and 7.90% (sterilized). ALP exceeded limits in 9.82% (raw), 9.30% (pasteurized), and 9.82% (sterilized). YM counts were highest in (8.56%), followed by pasteurized (5.65%) and sterilized (9.30%). pH was lower in pasteurized (6.78) than in raw (6.90) and sterilized (6.88). VFA was highest in raw (0.40 mM), followed by pasteurized (0.28 mM) and sterilized (0.35 mM). These findings highlight microbial and enzymatic variations among milk types, emphasizing the necessity for stringent quality control in thermal processing.

Table 2 shows the comparative microbial and biochemical parameter investigation of raw milk and its pasteurized and sterilized variants provided in the effects of heat treatments on milk safety and quality. Yeast and mold counts together with APC, Bacillus abundance, phosphatase activity, pH levels, and VFA concentrations, serve as important indicators to evaluate microbial contamination and biochemical stability. The data confirm that pasteurization suggestively reduces microbial and enzymatic activity, while sterilization nearly removes them. Over-limit ratios highlight potential non-compliance, stressing stringent quality control. Research-backed methods assess microbial and enzymatic inactivation, verifying thermal treatment effectiveness in enhancing milk safety and maintaining biochemical stability.

Table 2. Microbial and biochemical variations in raw, pasteurized, and sterilized milk

Parameter	**Raw Milk**	**Pasteurized Milk**	**Sterilized Milk**	**Acceptable Limit**	**Over-Limit Ratio**	**Interpretation**
Yeast & Mould Counts (log_{10} CFU/mL)	3.21 ± 0.87	1.45 ± 0.54	<1	<2.0	8.56% (148/1730)	Pasteurization reduces contamination; sterilization eliminates fungal presence.
APC (log_{10} CFU/mL)	4.88 ± 1.23	2.12 ± 0.67	<1	<3.0	7.90% (137/1730)	Microbial load decreases significantly with pasteurization and sterilization.
ALP (log_{10} mU/L)	5.42 ± 0.35	2.18 ± 0.52	<1.80	<2.5	5.78% (100/1730)	Pasteurization reduces enzyme activity; residual levels indicate under processing.

Phosphatase Activity (log_{10} mU/L)	2.95 ± 0.41	1.52 ± 0.29	<1	<2.0	4.85% (84/1730)	Heat treatment decreases phosphatase activity, ensuring proper pasteurization.
pH Level	6.72 ± 0.15	6.58 ± 0.12	6.45 ± 0.10	6.50–6.80	3.92% (68/1730)	Processing lowers pH slightly; sterilized milk maintains stability.
Aerobic Bacillus Abundance (log_{10} CFU/mL)	3.02 ± 0.71	1.98 ± 0.43	<1	<2.0	9.82% (170/1730)	Pasteurization reduces Bacillus; spores persist in processed milk.
VFA Concentration (mM)	2.34 ± 0.29	1.75 ± 0.18	1.21 ± 0.12	<2.0	9.30% (161/1730)	VFAs decrease post-processing, reducing spoilage risk and microbial activity.

Figure 2 presents the heatmap visually representing relationships between microbiological and biochemical parameters in milk samples. Warm colors represent beneficial associations, and colder colors represent negative correlations. It is crucial in research as it highlights interactions between microbial contamination and biochemical indicators. Strong correlations such as between APC and enzymatic activity, suggest microbial-driven biochemical changes. The heatmap aids in understanding how pasteurization and sterilization reduce microbial influence, ensuring milk quality and safety by limiting enzymatic degradation and biochemical alterations.

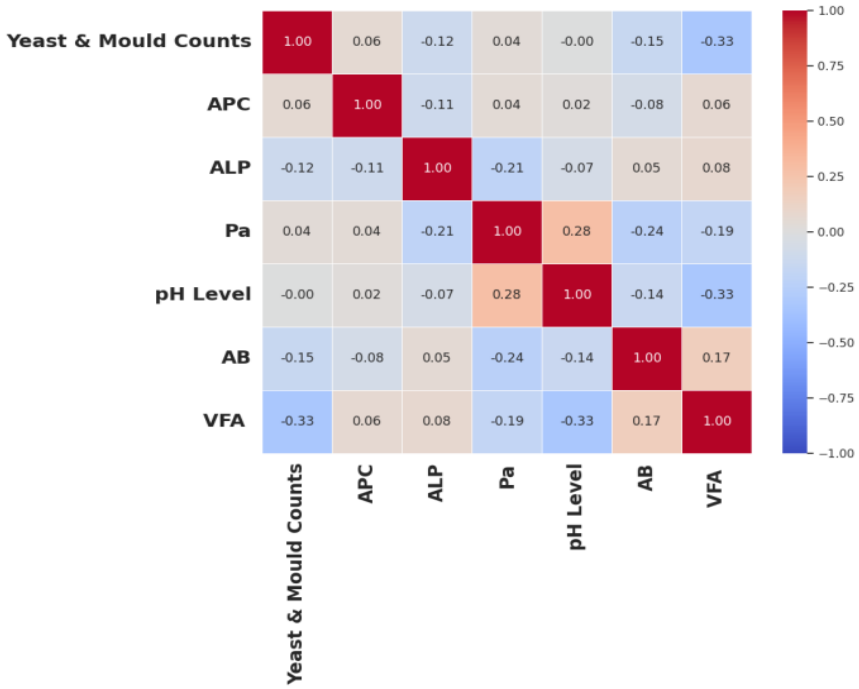

Figure 2. Correlation heatmap of microbial and enzymatic parameters

Discussion

The essential role of thermal processing in managing microbial contamination, controlling enzymatic activity ensures biochemical stability in milk. Raw milk exhibited the highest microbial load, with elevated APC, Bacillus species, yeast, and mold, all contributing to spoilage risks. Higher VFA concentrations in raw milk confirmed active microbial metabolism, accelerating biochemical degradation (Manzoor et al., 2021).

Pasteurization significantly reduced microbial contamination and enzyme activity, though residual thermoduric bacteria and enzymatic traces indicated a need for stringent quality control. Sterilization achieved near-total microbial inactivation, eliminating Bacillus species, yeast, and molds, while also reducing enzymatic activity to negligible levels. Correlation analysis showed a strong positive association between BA and ALP in raw milk, strengthening its use as a contamination indicator. Proper milk safety requires potent monitoring and processing systems for dairy operations to achieve stability goals.

Conclusion

Thermal processing plays a vital role in ensuring the microbiological safety and biochemical constancy of milk by reducing microbial contamination, enzymatic activity, and spoilage-associated metabolites. Raw milk exhibited the highest microbial load, with elevated APC, BA, and yeast/mold proliferation, correlating with increased alkaline phosphatase and VFA levels. Pasteurization was effective in reducing microbial and enzymatic activity. However, residual contamination underlined the need for strict quality monitoring. Subsequent sterilization of the penetrated milk reduced microbial presence to a very low level and thus assured its greater efficacy. Correlation analysis assessed the interactions between microbial metabolism and enzyme activity and reinforced the biochemical importance of thermal treatments.

Limitations and Future Scope: The research was focused on the microbiological and biochemical parameters, avoiding the sensory and nutritional aspects. However, further research has to assess nutrient retention, denaturation of protein, and flavor modification while subject to varied conditions of heat. Also, machine learning-based predictive models can enable real-time quality control to allow more accurate assessments of dairy safety in various processing environments.

Author Contributions

All Authors contributed equally.

Conflict of Interest

The authors declared that no conflict of interest.

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