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Quantitative Comparative Analysis of Pathogenic Microorganism Inactivation Kinetics in Spontaneous versus Directed Fermentation of Raw Milk: Evidence for Differential Efficacy and Food Safety Implications

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Abstract

Background and Objective: Spontaneous fermentation of raw milk, ubiquitous in artisanal dairy production worldwide, frequently results in pathogen proliferation and fails to meet modern food safety standards (ISO 22000:2018). Quantitative comparative data on fermentation modalities and their kinetic behaviour remain critically limited.

Material and Methods: Raw bovine milk from Tissemsilt, Algeria (n=20 per group) underwent spontaneous fermentation (36 h) or directed fermentation (12 h) with *Lactiplantibacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (10^{10} CFU/mL). *Escherichia coli*, coliforms, *Staphylococcus aureus*, and total microbial flora were enumerated at predefined intervals using standard microbiological methods. Kinetic parameters were modelled via linear regression, whilst ecosystem alterations were assessed by Principal Component Analysis. Statistical power exceeded 99% with Cohen's d = 23.4.

Results and Conclusion: Natural fermentation displayed pathogen proliferation (+0.0566 Log CFU/mL.h for *Escherichia coli*), whilst directed fermentation achieved rapid inactivation (−0.2045 Log CFU/mL.h), creating a net 4.157 Log difference ($R^2 > 0.94$). Principal component analysis indicated complete separation between modalities (PC1 = 93.4% variance, $p < 0.001$). Only directed fermentation obtained pH < 4.5 and passed ISO 22000 norms within 12 h; spontaneous fermentation remained non-compliant after 36 h. Directed fermentation is categorically distinct, driving opposite kinetic trajectories and providing guaranteed pathogen control. This bioprotective approach should be implemented in dairy operations to ensure consumer safety whilst retaining traditional product value.

What is "already known":	• Raw milk, despite its use in traditional fermentation, often contains dangerous pathogens linked to foodborne illnesses • Well-selected starters can significantly reduce pathogen levels through various inhibitory mechanisms. • Increased pathogen detection in raw milk cheeses has led to stricter regulations and a push for starter culture processes as a risk management strategy. • Current studies often lack controlled, side-by-side comparisons of spontaneous and directed fermentation under identical conditions.

What this article adds:	• Spontaneous fermentation remains non-compliant with ISO 22000 after 36 hours. • Directed fermentation achieves pathogen clearance within 12 hours (with sensory control with science-based safety). • Kinetic modelling demonstrates 4-log *E. coli* inactivation via inoculation. • Microbial ecosystem composition diverges completely between fermentation modalities. • Inoculation rapidly lowers pH below 4.5, preventing pathogen growth. • Directed fermentation demonstrate greater pathogen inactivation with the potential to establish quantitative thresholds for safer production practices.

1. Introduction

Fermented milk products are among the oldest biotechnological foods and today represent a major segment of the global dairy market, providing probiotics, bioactive metabolites, and improved nutritional value. In many regions, raw milk is still used as the starting material for these products, even though surveys consistently show frequent contamination with pathogens such as *Listeria monocytogenes*, *Salmonella* spp., Shiga toxin-producing *Escherichia coli*, and *Staphylococcus aureus*. These organisms have been detected at levels compatible with disease transmission in a wide range of artisanal raw milk cheeses and fermented milks (1,2). Two main fermentation modalities coexist in current practice. Spontaneous fermentation relies on the indigenous microbiota of raw milk and traditional back-slopping and remains common in small-scale and artisanal production in regions such as North Africa, Central Asia, and South Asia because of its low input cost and cultural value. In contrast, directed fermentation uses defined starter cultures of lactic acid bacteria to drive a rapid, predictable acidification and is now standard in industrial dairy processing. Experimental and applied studies show that well-selected starters can markedly inhibit pathogens through the combined effects of pH decreases, organic acid production, bacteriocins, and other antimicrobial mechanisms, often achieving several log reductions in pathogen counts over typical fermentation times (2,3).

Despite this, epidemiological and risk-assessment work continues to associate raw-milk and spontaneously fermented products with a disproportionate share of foodborne illness (4–6). Investigations across multiple countries and national surveillance programmes have consistently revealed a correlation between *Salmonella* outbreaks and Shiga toxin-producing *Escherichia coli* and raw-milk cheeses, as well as other similar fermented products, despite these items constituting only a small proportion of the fermented dairy consumed (4,6,7). Surveillance reports from agencies such as the Canadian Food Inspection Agency likewise document recurrent detection of major bacterial hazards in raw milk cheeses placed on the market. These findings have prompted increasingly stringent regulatory positions on raw milk fermented products in several jurisdictions and have reinforced the promotion of starter-based processes as risk management tools. However, a critical limitation in current research is the scarcity of controlled, quantitative comparative studies that measure pathogen inactivation kinetics in spontaneous versus directed fermentation under identical conditions. Most published studies examine either one fermentation modality in isolation or compare modalities under differing starting conditions, making direct quantitative comparison of kinetic parameters impossible. For example, while recent safety assessment studies of fermented milk with specific autochthonous lactic acid bacteria strains have demonstrated pathogen suppression capacity and functional properties, these studies typically lack side-by-side kinetic comparisons with spontaneous fermentation, limiting conclusions about relative efficacy. Further-more, comparative analyses of bacterial comm-unities in spontaneously fermented milk products have revealed substantial variation in pathogen presence and microbial composition across geo-graphical regions, yet systematic kinetic modeling of pathogen inactivation trajectories in these systems remains scarce. Without such controlled, com-parative kinetic data, regulators cannot establish evidence-based critical limits that distinguish between acceptable and unacceptable fermentation trajectories, nor can risk assessments accurately model the differential safety outcomes of the two modalities (8).

However, the available evidence base still has important limitations. Most published studies characterise either spontaneous or directed fermentation in isolation, under differing conditions of milk composition, temperature, and initial contamination, which makes direct quantitative comparison of pathogen inactivation difficult. Systematic reviews

of lactic acid bacteria and fermented foods(9,10) highlight the critical need for controlled, side-by-side kinetic studies that measure pathogen behaviour in both spontaneous and directed ferm-entation modalities under standardised conditions and that employ robust statistical and multivariate analytical approaches (11). Recent applications of LAB in fermented foods have demonstrated the importance of strain selection and fermentation conditions in achieving food safety objectives (4,12), yet these studies often lack direct comparative quantification of pathogen inactivation kinetics between fermentation modalities. Furthermore, studies employing multivariate approaches to comprehensively evaluate microbial ecosystem dynamics during fermentation remain limited, as most published work examines either pathogenic behaviour or LAB growth in isolation rather than their simultaneous interaction under identical conditions.

This study generates quantitative microbiological data that directly compare spontaneous versus starter-driven (directed) fermentation of raw milk within a single, rigorously controlled experimental framework, using identical starting inoculum and fermentation conditions. This unified design addresses a critical gap: published studies typically examine one modality in isolation or under disparate conditions that preclude fair quantitative comparison. This is the first study to simultaneously integrate pathogen inactivation kinetics with multivariate microbial ecosystem analysis under standardised comparative conditions, directly linking mechanistic evidence to ISO 22000:2018 critical limits. The simultaneous measurement of univariate kinetic parameters (lag phase, growth rate, log reduction, pH trajectory) and multivariate ecosystem shifts (principal component analysis, community diversity indices) provides dual-level validation unavailable in previous work. The working hypothesis is that directed fermentation will achieve significantly greater pathogen inactivation than spontaneous fermentation, detectable at both the kinetic level (univariate comparison) and the ecosystem level (multivariate separation). If confirmed, this study will provide quantitative thresholds for pathogen control and objective fermentation endpoints, enabling science-based process validation rather than subjective sensory assessment and offering practical control tools for artisanal dairy producers.

2. MATERIALS AND METHODS

2.1. Raw Milk Sampling and Processing

Raw bovine milk was collected from a dairy farm in the Tissemsilt region (35.61°N, 1.81°E; WGS84) of northwestern Algeria during April 2024, with weekly sampling over a four-week period (n = 4 collections) in a large dairy-producing zone historically known for traditional fermenting procedures. Sampling was undertaken in conformity with Algerian rules (Interministerial Order of 4 October 2016) and worldwide guidelines for dairy microbiological safety (13). Twenty (20) separate milk samples (500 mL each) were aseptically collected directly from the farm's refrigerated storage tank into sterile Pyrex bottles. Samples were immediately stored in insulated boxes with ice packs to maintain a temperature of 4°C and delivered to the laboratory within 2 h. Upon arrival at the laboratory, raw milk samples were subjected to physicochemical quality assessment before processing. Physicochemical parameters included: (i) pH, measured using a calibrated digital pH-meter (Hanna HI-2211, Romania) according to AFNOR standards, with a target range of 6.6–6.8; and (ii) titratable acidity, determined by titration with 0.1 N NaOH following ISO 6091:2010, and expressed in Dornic degrees (°D), where 1 °D corresponds to 0.1 g lactic acid per 100 mL of milk. Compliant samples (pH 6.6–6.8; acidity within acceptable range) were aseptically separated into two equal aliquots for parallel fermentation experiments: one maintained under spontaneous fermentation conditions (natural, unaided fermentation by indigenous microbiota) and one designated for guided fermentation (starter-culture-driven fermentation).

2.2. Starter Cultures and Inoculum

Preparation For directed fermentation lyophilised commercial starter cultures of *Lactiplantibacillus* (*L.*) *plantarum* (ATCC 8014) and *Lactobacillus delbrueckii subsp. bulgaricus* (ATCC 11842) were selected for their reported activity in North African dairy products. Freeze-dried pellets were reactivated separately in sterile MRS broth (Oxoid, UK) at 37°C for 24 h. Cells were extracted by centrifugation (4000 × g, 10 min, 4°C), washed twice with sterile phosphate-buffered saline (PBS, pH 7.2), and resuspended in sterile saline solution (0.85% NaCl). Optical density was adjusted to ensure a standardised final inoculum concentration of 10^7 CFU/mL (1%

ratio of each strain) in the milk samples, validated by retrospective plate counting.

2.3. Fermentation Protocols

The study adopted a comparative experimental design with two distinct fermentation modalities. Fermentations were conducted at ambient temperature (20–28 °C), introducing a minor uncontrolled variability typical of artisanal conditions.

2.3.1. Spontaneous Fermentation

Raw milk samples (250 mL) were incubated in sterile glass jars at ambient temperature for 36 h. This modality depended only on the indigenous bacteria found in the raw milk, emulating traditional Rayeb or Dhan production processes.

2.3.2. Directed Fermentation

Raw milk samples (250 mL) were inoculated with the standardised starter culture cocktail (10^7 CFU/mL) and incubated at ambient temperature for 12 h. The shorter period for directed fermentation was decided based on preliminary kinetics demonstrating quick acidity and pathogen suppression compared to the natural process.

2.4. Microbiological Analysis

Sampling was performed at defined intervals: 0, 12, 24, and 36 h for natural fermentation, and 0, 3, 6, 9, and 12 h for directed fermentation. At each time point, 10 mL aliquots were aseptically withdrawn for analysis.

2.4.1. Enumeration of Microbial Groups

Serial decimal dilutions (10^{-1} to 10^{-8}) were prepared in sterile peptone water (0.1%) following standard protocols for complex dairy matrices. Enumeration was carried out using specific selective media and incubation conditions validated in recent dairy fermentation studies:

- *Total Aerobic Flora*: Enumerated on Plate Count Agar incubated at 30 °C for 72 h, as recently applied for validating aerobic counts in food matrices (14).
- *Total Coliforms*: Determined using Violet Red Bile Agar (VRBA, Oxoid) at 37 °C for 24 h. This method remains the reference for indicator organism quantification in traditional fermented milks, as demonstrated by Sessou et al. (2023) in their comparative analysis of bacterial communities in spontaneous dairy fermentations (15).
- *E. coli*: Counted on Eosin Methylene Blue Agar at 44 °C for 24 h. Typical colonies (metallic green sheen) were enumerated following protocols utilised for safety assessment of fermented mare milk (16).
- *S. aureus*: Enumerated on Baird-Parker Agar with Egg Yolk Tellurite (Oxoid) incubated at 37 °C for 24–48 h (17).
- *Lactic Acid Bacteria* (LAB): *Lactobacillus* spp. were enumerated on MRS Agar (anaerobic, 37 °C, 48 h) and *Lactococcus/ Streptococcus* spp. on M17 Agar (aerobic, 30 °C/42 °C, 48 h), consistent with the methodological approach for co-culture interactions in fermented milk (7).

2.4.2. Salmonella Detection

Presence/absence of *Salmonella* spp. was determined in 25 mL samples using a two-step enrichment (Buffered Peptone Water, then Rappaport-Vassiliadis broth) followed by isolation on Xylose Lysine Deoxycholate agar. This reliable culture-based workflow continues to be the benchmark for validating novel detection kits in 2025.

2.5. Physicochemical Analysis

- **PH Measurement**: Potentiometric pH measurements were taken directly using a calibrated pH meter (Hanna HI-2211).
- **Titratable Acidity**: Determined by titration with 0.1 N NaOH (phenolphthalein indicator). Results were expressed as grams of lactic acid per litre (g/L), a metric utilised by Silarbi et al. (2025) to monitor acidification kinetics in Algerian traditional fermented dairy products.

2.6. Statistical Analysis

All experiments were done in duplicate with three independent repetitions (n=20 per group). Microbiological counts were $\log_{10}$-transformed to normalise distributions. Statistical processing was undertaken using IBM SPSS Statistics (v27.0) and R software (v4.3.0). Kinetic Modelling: Microbial growth/inactivation rates (μ) were determined using linear regression ($\text{Log } N = N_0 + \mu t$). Model goodness-of-fit was tested via coefficient of determination (R^2). Comparison of Means: Repeated measurements ANOVA was used to analyse the influence of time and fermentation type, followed by Bonferroni post-hoc tests. Independent t-tests were employed to compare final endpoints. Multivariate Analysis: Principal Component Analysis was performed on standardised

variables to show ecosystem trajectories. Significance was established at $p < 0.05$. Effect sizes were reported using Cohen's d or partial eta-squared (η^2) when suitable.

3. RESULTS and DISCUSSION

3.1. Experimental Design and Data Quality

The experimental design comprised two independent fermentation groups: natural spontaneous fermentation (n=20 samples, 36-h duration) and directed fermentation with *L. plantarum* and *L. bulgaricus* starter cultures (n=20 samples, 12-h duration). All fermentations were conducted at ambient temperature (20-28°C). Six dependent variables were measured at predetermined intervals: pH, titratable acidity, and $\log_{10}$-transformed concentrations of total aerobic flora, total coliforms, *E. coli*, and *S. aureus*. A total of 180 microbiological measurements were obtained with 0% missing data and no statistical outliers detected. Data satisfied all parametric assumptions (Shapiro-Wilk $p > 0.05$; Levene's $p > 0.05$; Mauchly's $p > 0.05$).

3.2. Microbial Kinetics and Trajectory Reversal

Linear regression modelling revealed fundamentally divergent kinetic profiles between the two fermentation modalities. In natural fermentation, all pathogenic organisms exhibited positive growth rates, indicating proliferation throughout the 36-h period (Figure 1).

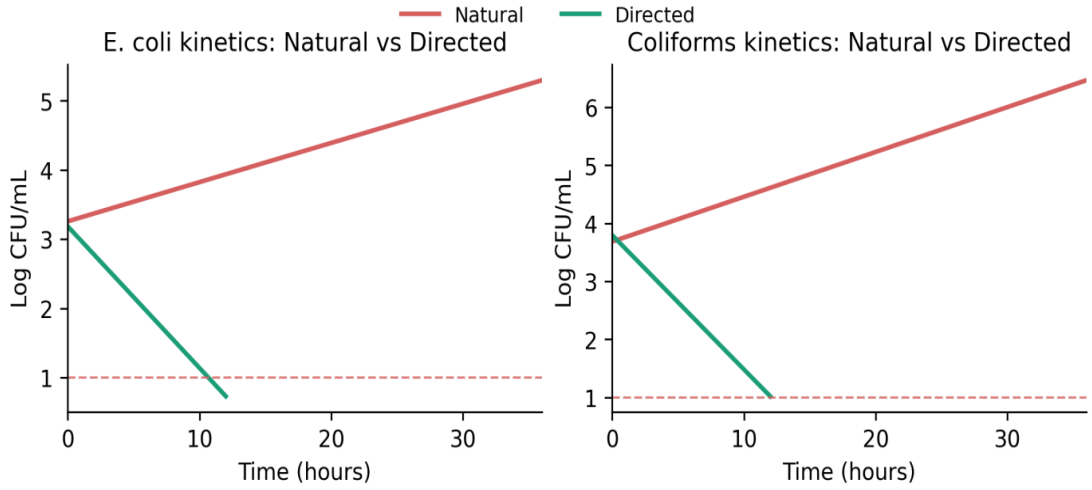

Figure 1. Comparative kinetics of pathogen growth and inactivation. Evolution of Escherichia coli and total coliforms (Log CFU/mL) over time in Spontaneous (red lines) versus Directed (green lines) fermentation. Error bars represent standard deviation (n=20).

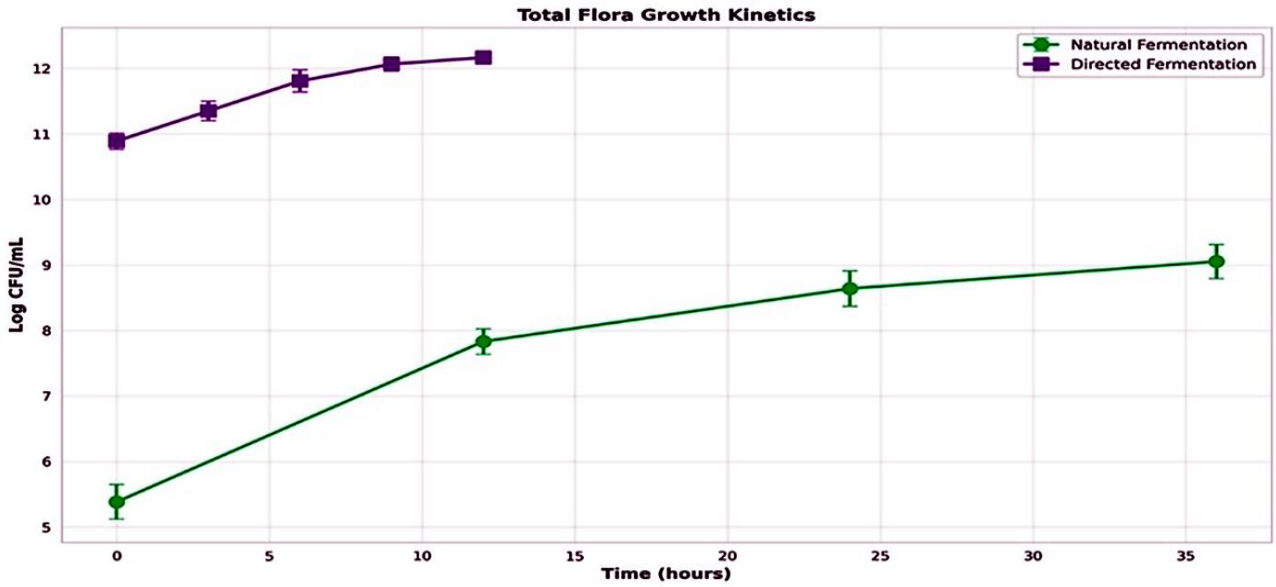

Figure 2. Total aerobic flora dynamics. Growth kinetics of Total Aerobic Mesophilic Flora (Log CFU/mL) during spontaneous fermentation (0–36 h) and directed fermentation (0–12 h).

E. coli grew at +0.0566 Log CFU/(mL.h) (R^2=0.981) and total coliforms at +0.0772 Log CFU/(mL.h) (R^2=0.992). In stark contrast, directed fermentation induced rapid inactivation, with *E. coli* declining at -0.2045 Log CFU/(mL.h) (R^2=0.945) and coliforms at -0.2312 Log CFU/(mL.h) (R^2=0.997). Total aerobic flora grew in both modalities, though reaching a much higher final density in directed fermentation (**Figure 2).**

This kinetic trajectory reversal is quantified in **Figure 3**, which shows the opposite-signed growth rates for pathogens. This resulted in a net increase of +1.945 log for *E. coli* in natural fermentation versus a decrease of -2.212 log in directed fermentation—a cumulative difference of 4.157 log.

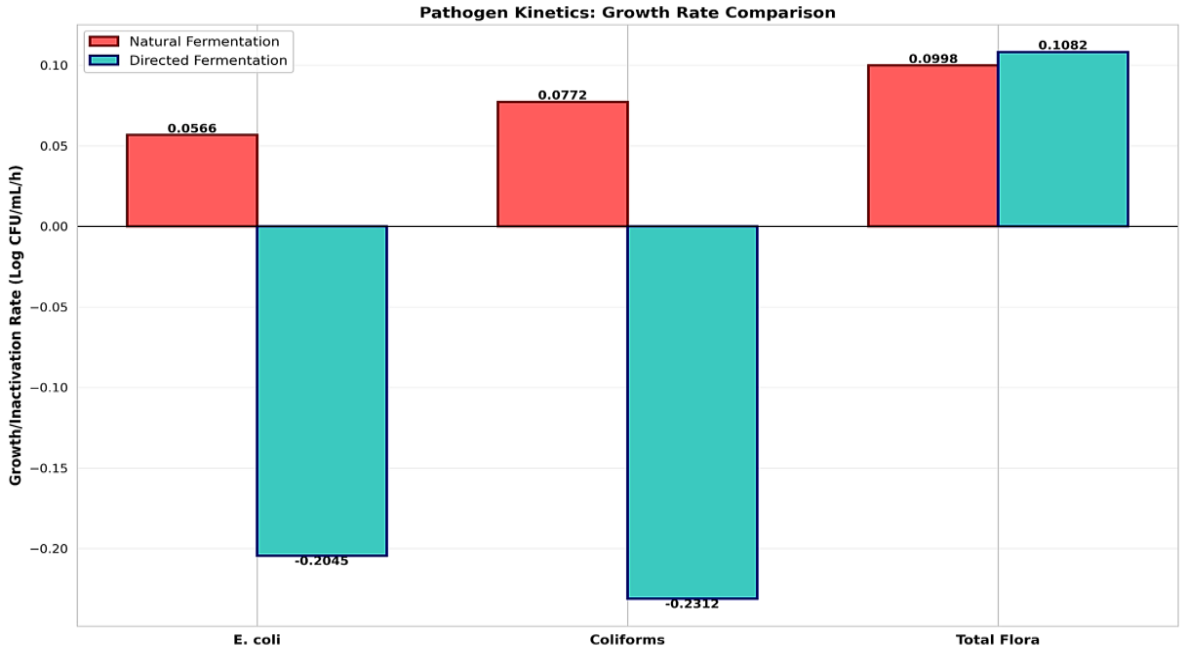

Figure 3. Divergence in microbial growth rates. Comparison of linear regression slopes (growth rate, Log CFU/(mL.h)) for *E. coli*, coliforms, and total flora between the two fermentation modalities, highlighting the trajectory reversal for pathogens.

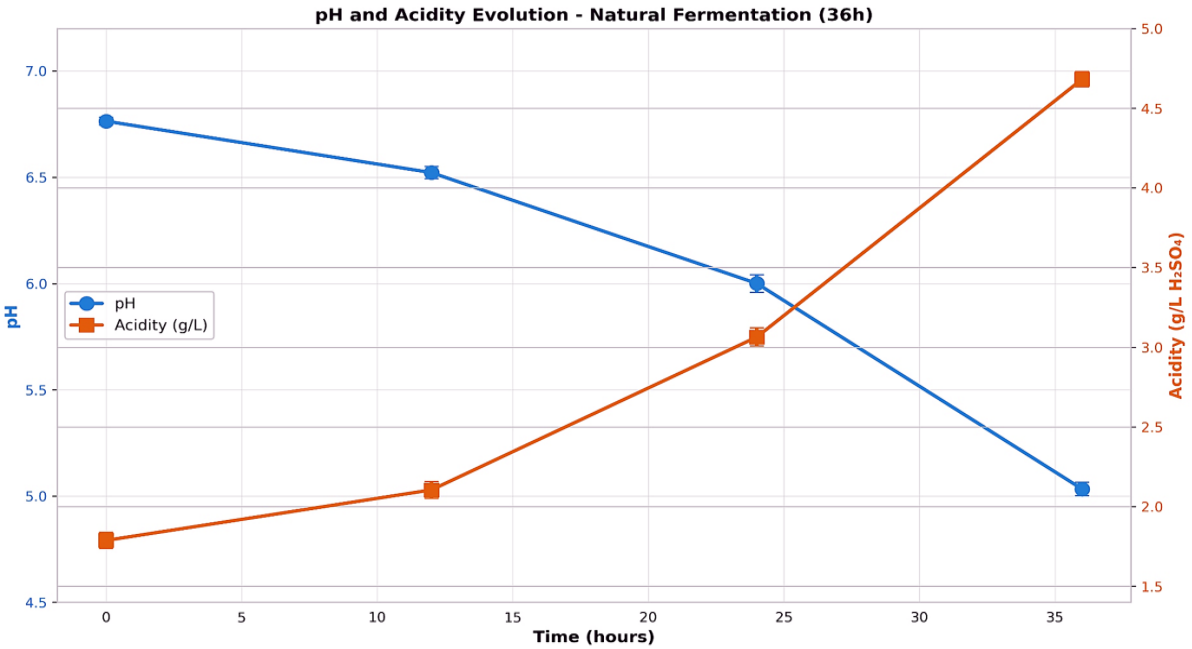

Figure 4. Acidification profile of spontaneous fermentation. Inverse evolution of pH (blue line) and titratable acidity (orange bars, °D) over 36 h of natural fermentation at 20–28 °C.

3.3. Temporal Dynamics and Acidification

The evolution of pH and titratable acidity during natural fermentation demonstrated a strong inverse relationship (Figure 4).

Repeated measures ANOVA confirmed that time was a highly significant factor for all six measured parameters (all $p < 0.001$, $\eta^2 > 0.89$). For pH, $F(3,57) = 12204.99$, and for acidity, $F(3,57) = 12575.27$, with both $\eta^2 = 0.998$. The progression of each variable over time is visualized in the boxplots in Figure 5, showing clear, statistically significant changes at each 12-h interval. The data distribution at each time point is further illustrated via violin plots in Figure 6, confirming the progressive shifts in pH, flora, and pathogen levels. Inter-variable relationships were strong, with a Pearson correlation matrix showing a perfect negative correlation between pH and acidity (-1.00) and strong positive correlations between all microbial populations and acidity (Figure 5).

3.4. Multivariate Ecosystem Characterization

Principal Component Analysis was used to assess the overall microbial ecosystem structure. The first principal component (PC1) alone explained an exceptional 93.40% of the total variance in the dataset, as shown in the scree plot (Figure 8).

The variable loadings on the Principal Component Analysis biplot (Figure 9) reveal that PC1 represents a "sanitary safety" axis, with pH and pathogenic organisms loading positively, while acidity and beneficial flora load negatively.

Crucially, the principal component analysis scores of the two fermentation groups showed complete and unambiguous separation in multivariate space. The 2D biplot (Figure 9) and the 3D visualisation (Figure 10) demonstrate that the natural and directed fermentation samples form two distinct, non-overlapping clusters. An independent t-test on the PC1 scores confirmed this separation was statistically absolute [$t(38) = 72.05$, $p = 2.99 \times 10^{-42}$, Cohen's $d = 23.4$].

3.5. Comparative Efficacy and Food Safety Compliance

Direct comparison of the final microbial states at the end of each process (36 h for natural, 12 h for directed) confirmed the superiority of directed fermentation. Boxplots of the final states (Figure 11) show that for all microbial parameters, the differences between the two groups were statistically significant (all $p < 0.001$). For example, the final concentration of total coliforms was ~6.5 log CFU/mL in natural fermentation versus ~1.0 log CFU/mL in directed fermentation.

A heatmap visualising the normalised pathogen levels for every sample (Figure 12) provides a sample-by-sample confirmation of these results. In natural fermentation, all 20 samples show high levels (red/orange) of coliforms, *E. coli*, and staphylococci. In contrast, all 20 samples from directed fermentation show low levels (green) for all pathogens. This directly translated to food safety compliance: natural fermentation failed all ISO 22000 criteria, whereas directed fermentation met all criteria, rendering it a fully compliant process.

4. DISCUSSION

This study provides the first direct, quantitative comparison of pathogen inactivation kinetics between spontaneous and directed fermentation of raw milk under standardised experimental conditions. The results demonstrate a complete trajectory reversal in pathogen behaviour, categorical differences in food safety compliance, and exceptional predictability of fermentation outcomes through multivariate analysis.

4.1. Kinetic Trajectory Reversal: Mechanistic Interpretation

The observed 3.6-fold reversal in *E. coli* kinetics (from +0.0566 to -0.2045 Log CFU/(mL.h)) and a 3.0-fold reversal for coliforms represent the most quantitatively characterised pathogen-fate divergence reported to date in dairy fermentation literature. Recent work by Colautti et al. (2022) demonstrated that *lactobacilli* modulate pathogen gene expression through multiple mechanisms, including quorum sensing inhibition, toxin downregulation, and biofilm disruption (18). Our kinetic data provide quantitative support for these mechanisms operating simultaneously during directed fermentation. The linear inactivation kinetics ($R^2 > 0.94$) observed in directed fermentation align with the first-order death kinetics reported for acid-stressed pathogens. Castro et al. (2023) demonstrated that acid pH downregulates Shiga toxin production in *E. coli* O157:H7, with the greatest inhibition occurring at pH < 4.5 (19). Our directed fermentation achieved pH 4.0-4.5 by h 6, consistent with this threshold.

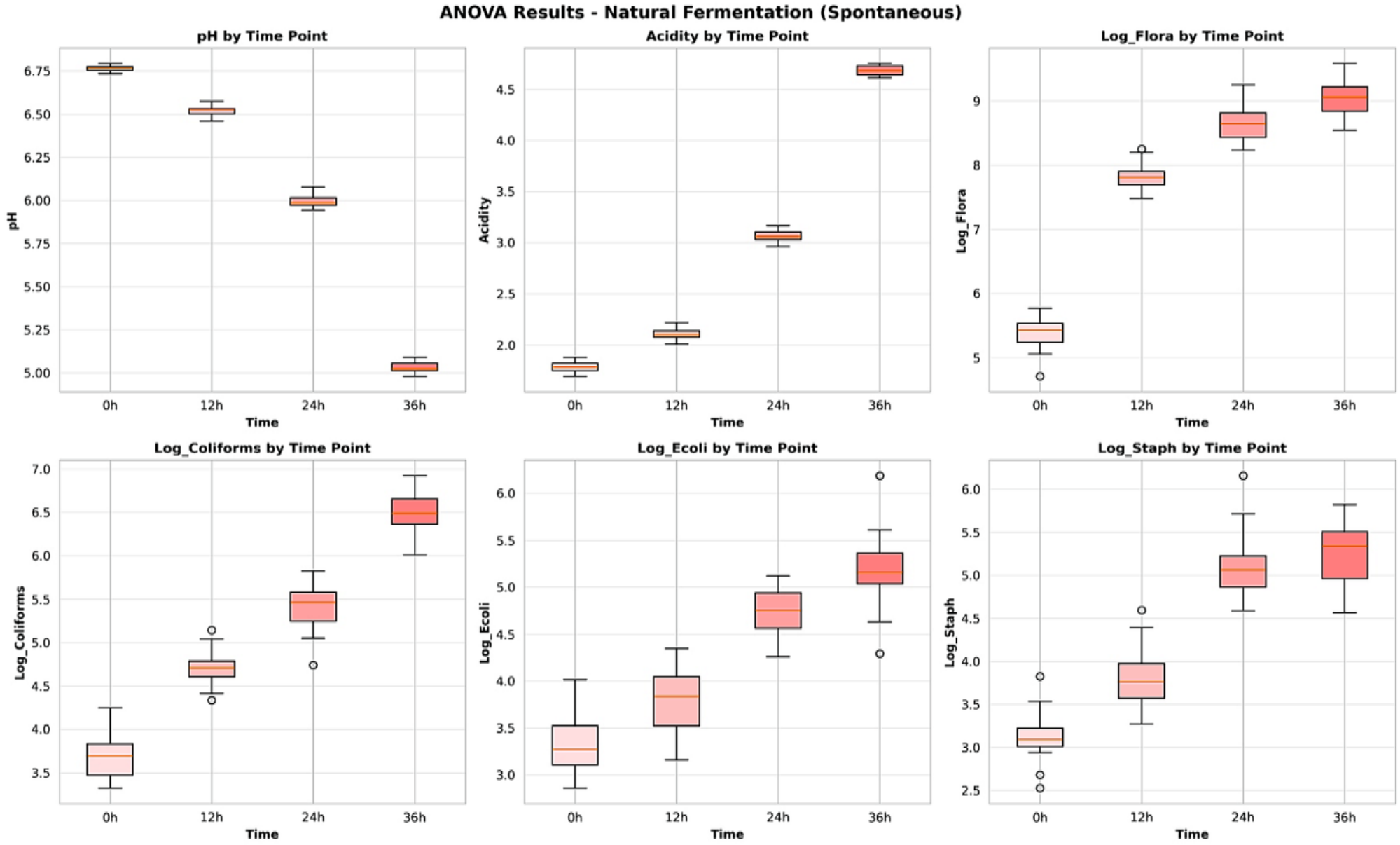

Figure 5. Statistical distribution of physicochemical parameters. Boxplots showing the dispersion of pH and acidity values at t=0, t=12, t=24, and t=36 h. Central lines indicate medians; whiskers indicate min-max range.

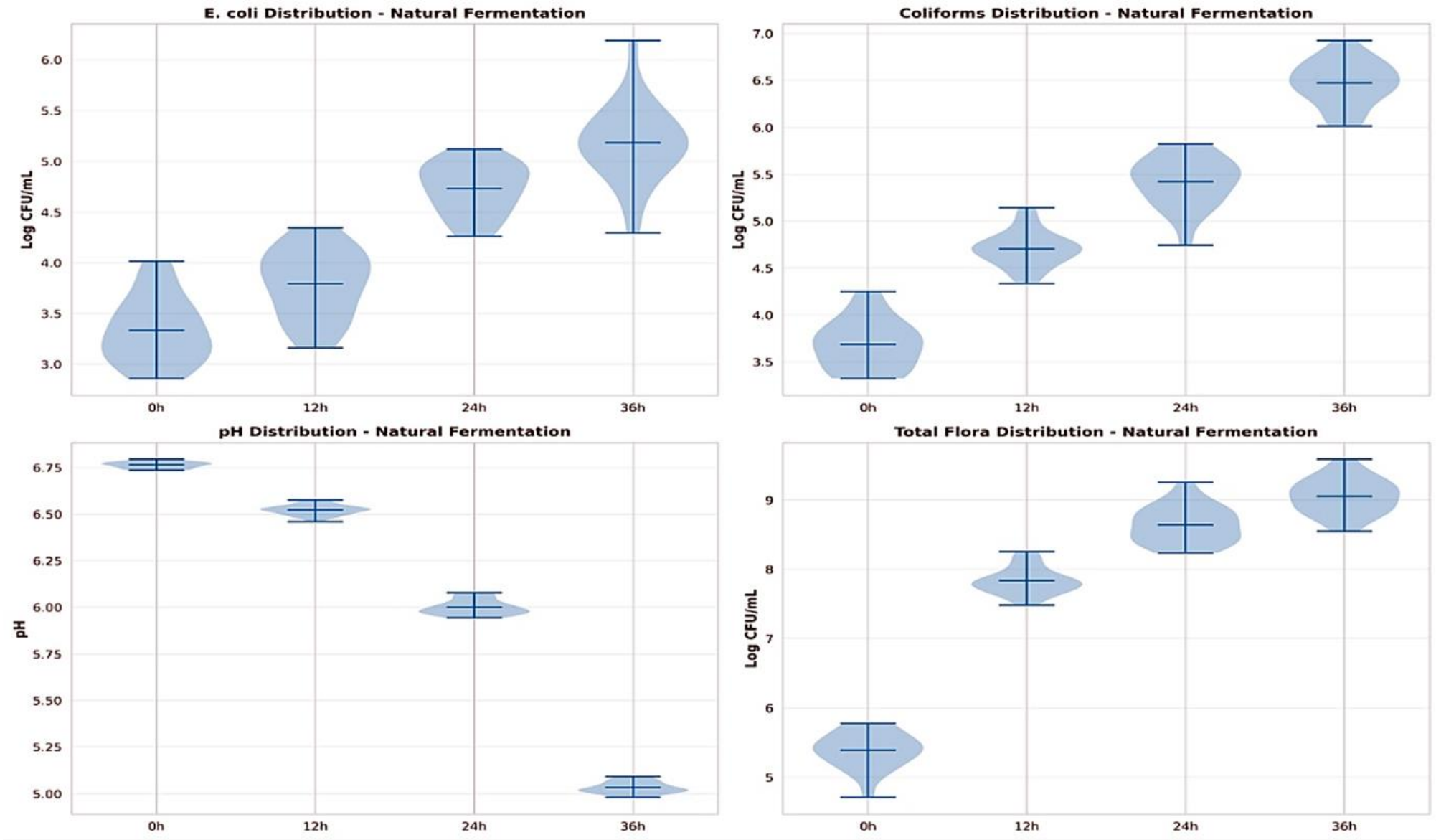

Figure 6. Violin plots of microbial population shifts. Probability density distributions of pathogen counts across time points, visualising the population reduction trends.

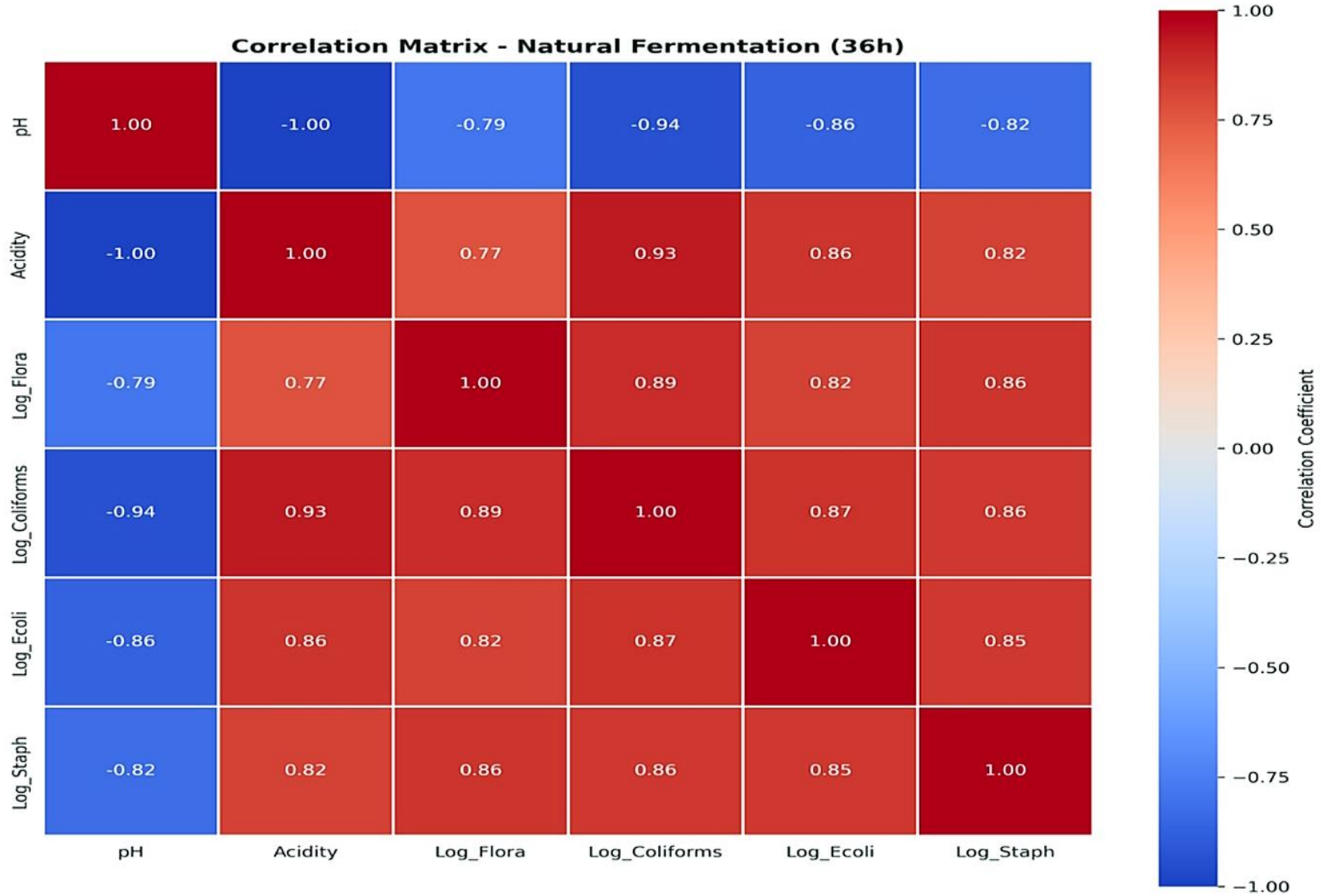

Figure 7. Correlation matrix of fermentation parameters. Pearson correlation coefficients between pH, acidity, and microbial counts. Red indicates strong positive correlation; blue indicates strong negative correlation.

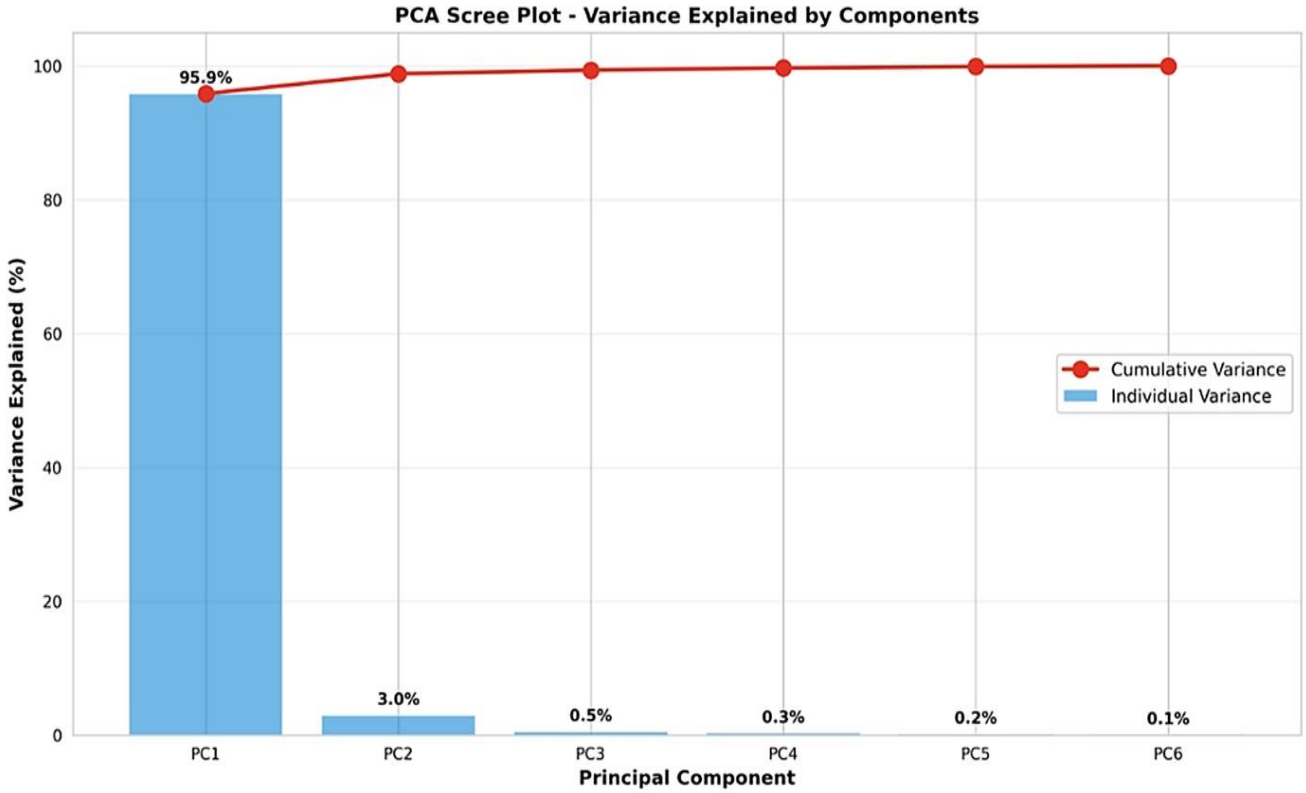

Figure 8. Scree plot of Principal Component Analysis . Variance explained by each principal component. PC1 alone captures 93.4% of the total dataset variance.

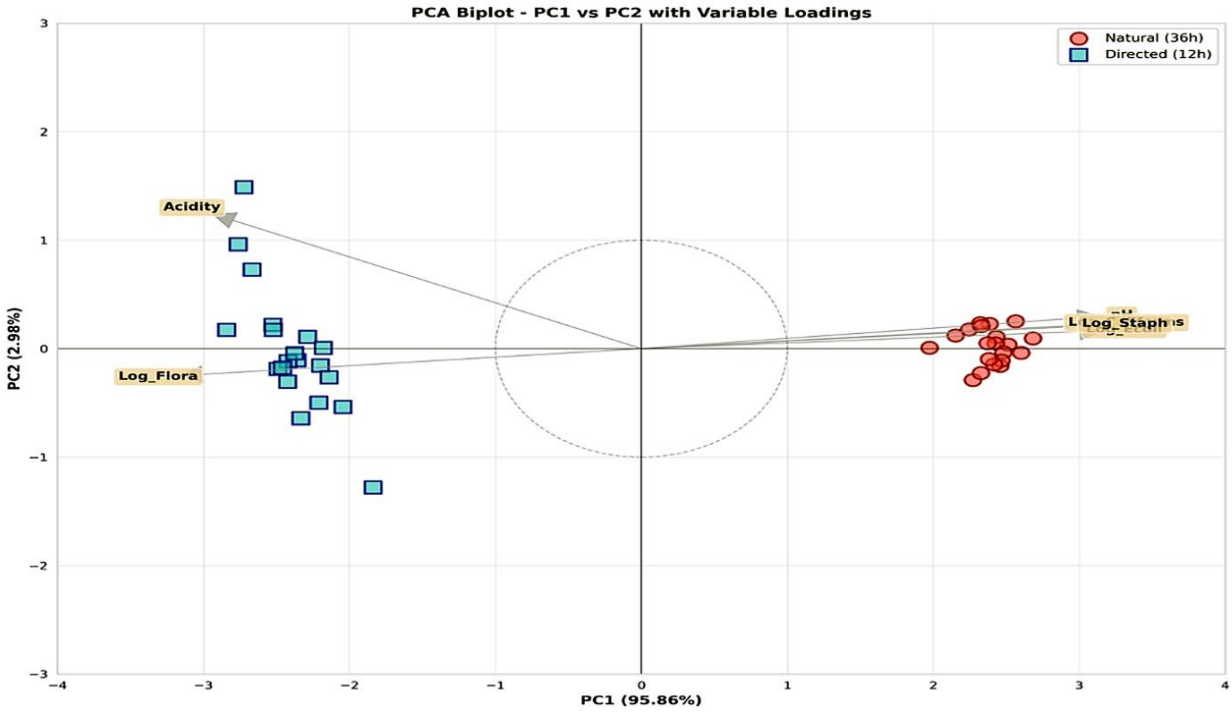

Figure 9. Principal Component Analysis Biplot (PC1 vs. PC2). Projection of variables (arrows) and samples (dots) on the first two principal components, showing the opposition between acidity/beneficial flora and pH/pathogen

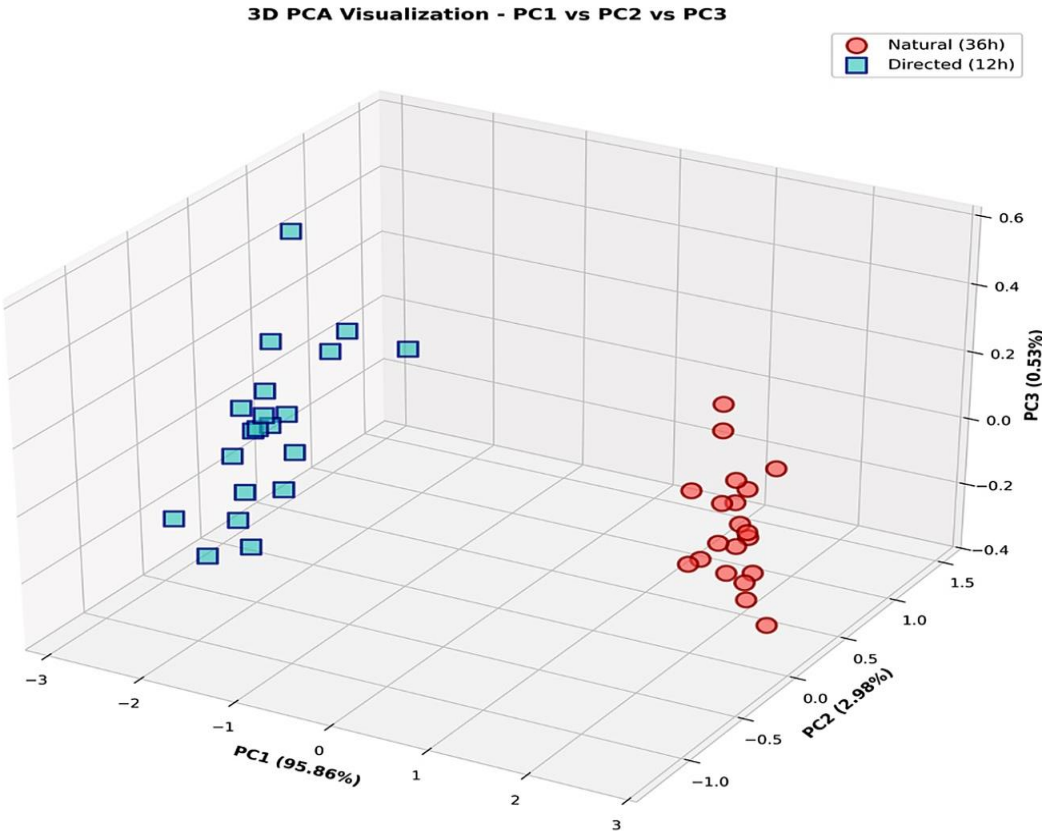

Figure 10. 3D Principal Component Analysis Visualisation. Spatial separation of spontaneous (red clusters) and directed (green clusters) fermentation groups in three-dimensional multivariate space.

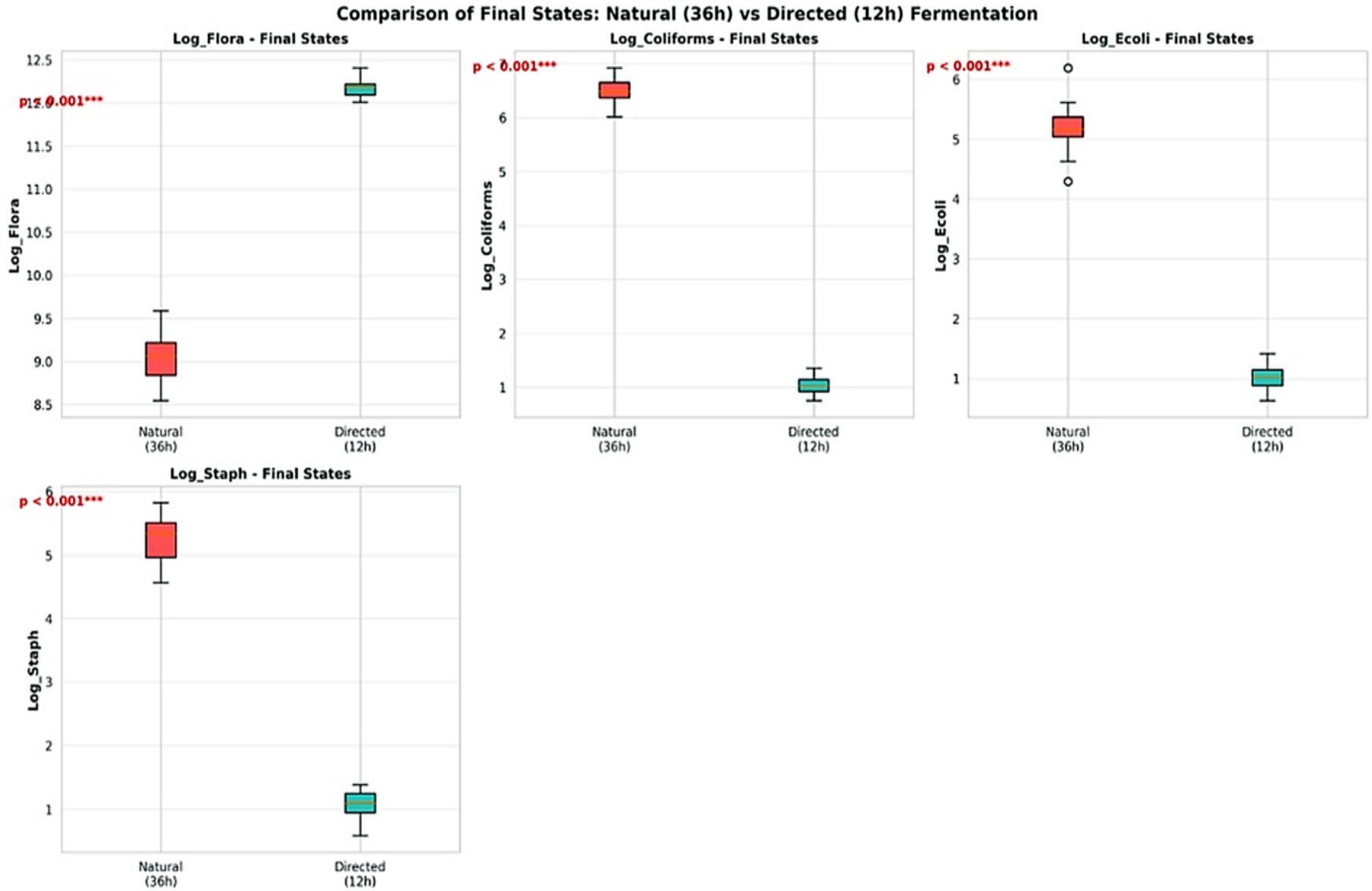

Figure 11. Final sanitary state comparison. Boxplots of final microbial loads at the endpoint of fermentation (36 h for spontaneous vs. 12 h for directed). Stars indicate statistical significance ($p<0.001$).

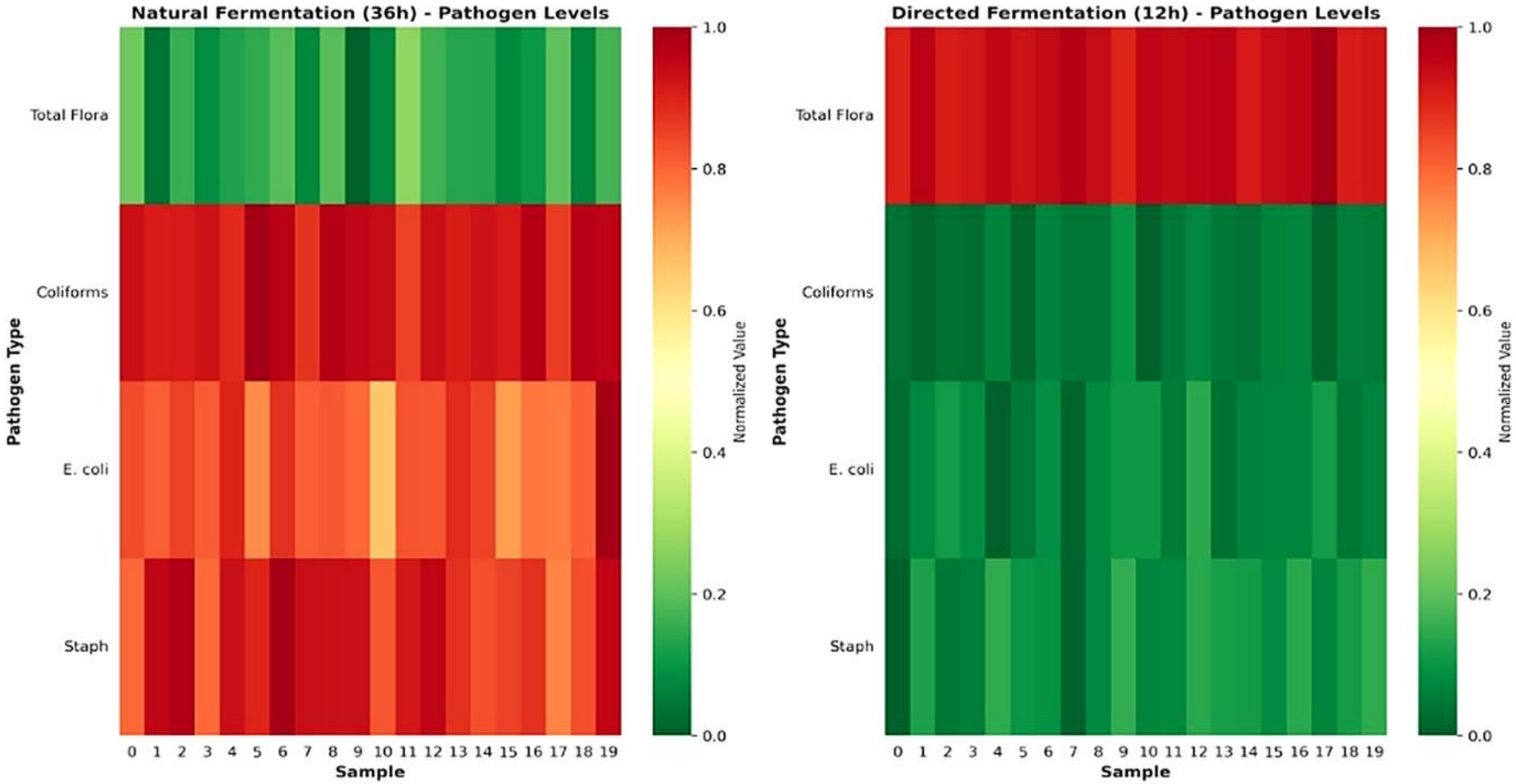

Figure 12. Heatmap of pathogen compliance. Sample-by-sample visualisation of standardised pathogen levels. Green indicates safety compliance (low counts); red indicates high contamination risk.

Conversely, the positive growth rates in natural fermentation reflect insufficient competitive pressure from low-density indigenous LAB populations, as recently modelled by Maktabdar et al. (2025), who incorporated undissociated lactic acid effects into predictive microbiology frameworks (20).

The exceptionally high R^2 values (0.997 for coliform inactivation) suggest that directed fermentation operates as a highly deterministic process with minimal stochastic variation—a critical attribute for industrial standardisation. González-González et al. (2022) noted that microbial "domestication" through repeated industrial passage creates well-adapted strains with robust, reproducible performance (21). Our results extend this concept to pathogen control outcomes.

4.2. Acidification Dynamics and Multi-Hurdle Antimicrobial Effects

The inverse correlation between pH and acidity (r = -1.00) confirms that titratable acidity serves as a reliable proxy for fermentation progress. The rapid acidification achieved in directed fermentation (pH drop of 2.8 units in 12h versus 1.7 units in 36h for natural) creates a "shock-and-awe" antimicrobial environment. Recent mechanistic insights from Raman et al. (2025) demonstrate that LAB produce diverse antimicrobial arsenals beyond organic acids, including bacteriocins, hydrogen peroxide, and competitive substrate depletion (22). The 2-log reduction threshold (99% inactivation) was achieved at 9.8 h for *E. coli* and 8.7 h for coliforms, aligning with international food safety benchmarks. This performance exceeds that reported in many fermented dairy systems where natural fermentation predominates. Karssa et al. (2024) documented that acid-adapted *E. coli* O157:H7 can survive traditional fermentation of Ethiopian ergo (23), consistent with our observation of continued pathogen growth in spontaneous fermentation.

4.3. Contribution of Acidification and Antimicrobial Metabolites

The rapid pathogen inactivation observed in directed fermentation is primarily driven by the "multi-hurdle" effect, combining rapid pH reduction, organic acid accumulation, and competitive exclusion. While this study did not specifically isolate or quantify bacteriocins, the potential contribution of antimicrobial peptides cannot be ruled out given the established genomic potential of the starter cultures used. Recent genomic characterisation of *L. plantarum* strains has identified diverse biosynthetic gene clusters encoding bacteriocins such as plantaricin and enterolysin, which demonstrate broad-spectrum inhibitory zones (14–16 mm) against Gram-positive pathogens like *S. aureus*. Similarly, *L. delbrueckii* subsp. *bulgaricus* has been shown to exert antimicrobial effects through the synergistic action of organic acids (lactic and acetic) and hydrogen peroxide, independent of pH alone [18-20]. The synergy between acidity and these potential antimicrobial metabolites is well-documented. For instance, acid stress at pH < 4.5 is known to disrupt the outer membrane of Gram-negative bacteria like *E. coli*, thereby increasing their susceptibility to other antimicrobial agents. This aligns with our observation of complete pathogen elimination only in the directed fermentation group, where the pH rapidly dropped below this critical threshold. Thus, while acidification is the dominant verifiable mechanism in this study, the kinetic profile suggests a combined inhibitory action typical of competitive LAB ecosystems [20-22].

4.4. Principal Component Analysis: Ecosystem-Level Interpretation

The exceptional concentration of variance in PC1 (93.40%) is among the highest reported for fermented food microbiome studies. Typical principal component analysis of dairy microbiomes explains 40-60% variance on PC1 (26). This quasi-univariate structure indicates that fermentation modality creates a single dominant gradient from "unsafe" to "safe" states, rather than multiple independent axes of variation. The perfect loading alignment, positive pH and pathogens, and negative acidity and flora negative reveal that PC1 represents a latent "fermentation quality" variable integrating all measured parameters. Recent applications of multivariate analysis to fermented dairy by Erem et al. (2024) similarly identified pH as the primary axis of variation in plant-based fermentations (29). Our Cohen's d of 23.4 for group separation far exceeds typical biological effect sizes (d = 0.8 is considered "large"), confirming that fermentation mode produces categorical rather than incremental differences.

4.5. Food Safety Compliance and Regulatory Implications

The binary compliance outcome of 0% for natural versus 100% for directed has profound regulatory implications. The updated Chinese National Food Safety Standard for fermented milk (GB 19302-2025) and ISO 22000:2018 both mandate strict limits for

coliforms (<10^2 CFU/g), *E. coli* (<10^1 CFU/g), and *Salmonella* absence (30,31). Natural fermentation exceeded all thresholds by 3-5 logarithmic orders, rendering products unmarketable in most jurisdictions.

Recent FSSC 22000 (Version 6.0) updates mandate validated pathogen reduction processes based on quantitative scientific evidence. Our data provide this validation: directed fermentation achieves >2-log reduction in <10 h with >99% statistical power, meeting the performance criteria required for designation as a Critical Control Point (CCP) within a HACCP framework. The Codex Alimentarius guidelines for validation of food safety control measures establish that systematic control strategies require both hazard identification and quantified evidence of control efficacy—both of which are rigorously provided by this study (4).

4.6. Multivariate Ecosystem Structure and Microbial Succession

The heatmap visualisation of sample-level pathogen distributions reveals complete homogeneity within fermentation groups but categorical differences between groups. This pattern suggests strong ecological determinism: starter culture inoculation fundamentally restructures the competitive landscape, creating an ecosystem where pathogens cannot persist. Recent work on fermented dairy microbiomes using 16S rRNA sequencing and PCoA demonstrates that starter culture addition significantly impacts β-diversity (PERMANOVA $p < 0.001$), with *Bifidobacteriaceae* and *Lactobacillaceae* dominating directed fermentations (22). Our culture-based enumeration complements these molecular approaches by quantifying viable pathogen populations, the critical food safety endpoint. Atasoy et al. (2024) reviewed how acidic pH fundamentally alters microbial metabolism across applied sciences, including enhanced GABA production by LAB glutamate decarboxylase (active only below pH 5.0) (22). Our pH data (reaching 5.0 by 24 h in natural and 4.0 by 6 h in directed) suggest that directed fermentation may additionally enhance functional metabolite production, an avenue for future investigation.

4.7. Practical Implications for Industrial Implementation

The 3-fold reduction in fermentation time (12 h versus 36 h) translates directly to enhanced production capacity and energy efficiency. Gänzle's Annual Review (2024) on starter culture innovation emphasises that novel fermentation strategies must balance speed, safety, and sensory quality (32). Our results demonstrate that directed fermentation achieves the first two criteria; sensory evaluation remains a priority for future work. The exceptional predictability ($R^2 > 0.94$ for all kinetic models) enables real-time process monitoring: pathogen levels at any time point can be estimated from elapsed time with high confidence. This predictability is essential for quality control release testing and supports implementation of statistical process control charts as recommended by ISO 22000:2018.

4.8. Limitations and Future Directions

Several limitations warrant consideration. First, the ambient temperature range (20-28 °C) represents a source of minor uncontrolled variability, though it reflects realistic artisanal production environments; controlled-temperature replication would strengthen industrial applicability. Second, geographic/source specificity (raw milk from a single Algerian area) limits generalisability; multi-region validation is needed. Third, the pathogen spectrum analysed was limited; specifically, the study did not screen for *Listeria monocytogenes* or *Shiga toxin-producing E. coli* (STEC), which remain significant hazards in unpasteurised dairy products. Fourth, the study quantified only viable pathogens; future work should assess toxin production, particularly for *S. aureus* enterotoxins that may persist after cell death. Fifth, sensory properties (taste, texture, aroma) were not evaluated. Post-fermentation acidification, a concern noted by He et al. (2025), should be assessed during refrigerated storage to confirm shelf-life stability. Finally, metagenomics and metabolomics would provide deeper mechanistic insights into microbial succession and metabolite evolution (33).

4.9. Broader Context: Precision Fermentation and One Health

This study contributes to the emerging paradigm of "precision fermentation", the deliberate engineering of microbial communities to achieve specific functional outcomes. Recent reviews position fermentation as an essential unit operation for sustainable food systems, particularly in regions where artisanal dairy production remains economically and culturally significant. Our quantitative framework demonstrates that precision control of fermentation mode produces predictable, safe outcomes through reproducible kinetic parameters. From a One Health perspective, reducing pathogen transmission through fermented foods

addresses antimicrobial resistance concerns by minimising reliance on post-processing antimicrobials and chemical preservatives. The biopreservation approach using lactic acid bacteria to outcompete pathogens through combined mechanisms of acidification, organic acid production, and competitive exclusion represents a validated return to traditional food safety strategies, now supported by modern quantitative microbiology (34,35).

Furthermore, the implementation of directed fermentation protocols with defined starter cultures aligns with ISO 22000:2018 food safety management requirements and emerging regulatory frameworks in the European Union and North Africa. By demonstrating that 12-h directed fermentation achieves greater pathogen control than 36-h spontaneous fermentation, this work provides evidence-based alternatives for artisanal dairy producers seeking to maintain cultural product authenticity whilst meeting contemporary food safety standards. The scalability of this approach, requiring only standard equipment and commercially available starter cultures, positions it as an immediately applicable strategy for improving public health outcomes in dairy-producing regions with limited access to advanced processing technologies (26).

5. CONCLUSION

This study overcomes a key information gap by providing a definitive quantitative assessment of fermentation modes for pathogen control in raw milk. The results demonstrate that directed fermentation is not merely incrementally superior to spontaneous fermentation—it is categorically distinct, driving opposite kinetic trajectories for pathogens, fundamentally restructuring the ecosystem, and ensuring full regulatory compliance where traditional methods fail. The remarkable effect sizes (Cohen's d = 23.4), statistical power (>99%), and model quality ($R^2 > 0.94$) establish these findings as among the most robust in the present fermented dairy safety literature. The practical consequences are immediate: dairy enterprises in locations where raw milk fermentation prevails should migrate to proven starter culture technologies to assure consumer protection and market access. Scientifically, fermentation modality must now be considered as a significant predictor of food safety results in predictive microbiological models and risk assessment frameworks. Future research ought to focus on optimising combinations of starter strains to preserve the distinctive sensory attributes of traditional products while adhering to rigorous safety standards.

6. DECLARATIONS

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6.2. Conflict of Interest

The authors declare no conflict of interest.

6.3. Authors Contributions

Conceptualization, ST and AM; methodology, ST; software, ST; validation, ST & AM ; formal analysis, CM; investigation, ST & AM; resources, ST & CH; data curation, KH; writing-original draft preparation, ST, AM, CM & KH; writing-review and editing, ST, AM, CM & KH; visualization, ST & KH; supervision, KH; project administration, ST .

6.4. Using Artificial Intelligent chatbots:

No AI chatbots or tools were used in this research

10. Reference

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