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Longitudinal Quantification of *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus* Target DNA in Ripening Raw-Milk Cheese Using Single-Copy Gene qPCR and Agar Plating

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Abstract

Indigenous lactic acid bacteria (LAB) drive the fermentation and maturation of artisanal raw-milk cheeses, yet tracking individual species over several months of ripening remains difficult when relying solely on conventional agar media. Extending our earlier work on molecular LAB detection, we developed intercalating dye-based quantitative PCR (qPCR) assays directed at single-copy chromosomal loci (*yjaE* for *Lactococcus lactis* subsp. *lactis* and *ACRRRJ_03990* for *Streptococcus thermophilus*) to follow target DNA levels across 180 days of raw cow's milk cheese maturation (sampled at 0, 30, 60, 90, 120, and 180 days) in parallel with routine enumeration on M17 agar. Selecting single-copy targets eliminated the copy-number variation typical of 16S or 23S rRNA operons. According to qPCR measurements, *S. thermophilus* gene copies increased up to day 90 (reaching 6.32–6.89 $\log_{10}$ gene copies/g) and subsequently dropped to 2.74–3.51 $\log_{10}$ gene copies/g by day 180, reflecting progressive loss and degradation of amplifiable chromosomal DNA. By contrast, *L. lactis* subsp. *lactis* target DNA accumulated throughout the entire ripening period, reaching 7.26–7.90 $\log_{10}$ gene copies/g at day 180. Routine plate counts diverged substantially from qPCR data during mid- and late ripening: colonies of presumptive thermophilic streptococci were no longer recovered by day 90 (<1.0 $\log_{10}$ CFU/g), whereas presumptive mesophilic lactococci decreased to roughly 1.95 $\log_{10}$ CFU/g at day 90 and fell below the detection limit from day 120 onwards. Because standard DNA-qPCR amplifies total intact template regardless of physiological state, these data alone cannot separate viable-but-non-culturable (VBNC) cells from intact dead cells or residual matrix-bound DNA. Nevertheless, the high abundance of target gene copies detected long after colony formation ceased shows that culture-based monitoring alone markedly under-represents the persistence of autochthonous LAB target DNA during extended cheese ripening.

Keywords: *Lactococcus lactis* subsp. *lactis*; *Streptococcus thermophilus*; single-copy genetic markers; qPCR; culture-independent enumeration; raw-milk cheese; ripening kinetics

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1. Introduction

Raw-milk artisanal cheeses represent a profound segment of the global dairy heritage, distinguished by their unique sensory attributes, complex flavor profiles, and deep connection to their geographical regions of origin [1]. Unlike industrial cheeses ISO 7218

manufactured from pasteurized milk using standardized commercial starter cultures, the fermentation and maturation processes of artisanal cheeses are driven primarily by the indigenous (autochthonous) microbiota naturally present in the raw milk and the dairy environment [2,3]. The core of this autochthonous microflora is constituted by Lactic Acid Bacteria (LAB), which play an indispensable role in shaping both the technological and organoleptic properties of the final product. Recent advances in microbial ecology have emphasized that the complex interactions within these wild microbial consortia—including acidification, proteolysis, lipolysis, and the synthesis of volatile organic compounds (VOCs)—are fundamentally responsible for the characteristic complexity of artisanal cheese flavors [4–7].

The continuous globalization of the food market has driven the dairy industry towards standardization, predominantly relying on a limited pool of commercial starter cultures. While this ensures product uniformity and safety, it concomitantly leads to a severe loss of regional cheese typicity and microbial biodiversity [8]. Consequently, there is a growing scientific and industrial consensus regarding the vital need to explore, isolate, and characterize autochthonous LAB strains from traditional dairy products. These wild strains often exhibit high adaptability to local cheesemaking conditions, distinct acidification kinetics, and diverse flavor-forming enzymatic activities compared to standardized commercial starters [8–10]. Harnessing this natural biodiversity to develop novel, customized starter and adjunct cultures presents a highly promising perspective for the dairy sector. It offers a strategic pathway to improve industrial cheese quality, provide bio-protection against spoilage and pathogenic microorganisms, and simultaneously preserve the authentic, terroir-driven characteristics of regional cheeses [8,11–14].

Among the myriad of microorganisms involved in dairy fermentations, *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus* represent two of the most technologically crucial LAB species. As primary acidifiers, their rapid metabolism of lactose into lactic acid is essential for initial curd formation, moisture expulsion, and the inhibition of undesirable adventitious microflora [15]. However, their population dynamics and physiological behavior during the prolonged maturation of hard and semi-hard cheeses are highly species-dependent [10]. As the cheese matrix undergoes drastic physicochemical shifts—characterized by severe carbohydrate starvation, decreasing water activity, and increasing osmotic stress from salt diffusion—the microbial ecology diverges significantly [16]. Thermophilic species, such as *S. thermophilus*, are particularly susceptible to these low-temperature and high-salt stress conditions, resulting in a substantial population decline and subsequent cellular autolysis during late maturation [17]. Conversely, highly adaptable autochthonous mesophiles, such as specific strains of *L. lactis* subsp. *lactis*, demonstrate remarkable resilience. Rather than declining, these populations can persistently proliferate and dominate the cheese ecosystem even after 180 days of cold ripening, continuously releasing intracellular peptidases and esterases that are cornerstones of flavor development [15,17,18]. Therefore, accurately monitoring the distinct survival kinetics and proliferation dynamics of these contrasting microbial populations throughout the entire ripening period is of paramount importance for understanding and ultimately controlling the cheese maturation process.

Historically, the evaluation of microbial population dynamics in cheese has relied heavily on conventional, culture-dependent techniques, primarily utilizing selective agar media such as M17 or de Man, Rogosa and Sharpe (MRS) agar [2,19]. Despite their widespread use, these classical methodologies possess critical and well-documented limitations. Primarily, solid culture media severely lack taxonomic resolution; for instance, M17 agar facilitates the concurrent growth of various coccoid LAB, rendering the phenotypic differentiation between *L. lactis* subspecies or closely related streptococci virtually impossible without extensive downstream biochemical testing [2,20]. Furthermore, traditional plating meth-

ods frequently result in massive underestimations of the true active microbial biomass. Prolonged exposure to environmental stresses (cold storage, low pH, and high salinity) naturally forces significant sub-populations of dairy LAB, particularly thermophiles, into a Viable But Non-Culturable (VBNC) state [15,16,21,22]. In the VBNC state, bacterial cells lose their ability to divide and form visible colonies on synthetic media—leading to false-negative enumerations—yet their cellular envelopes remain intact, and their enzymatic machinery continues to actively participate in the biochemical transformation of the cheese [15,16]. Consequently, relying solely on classical agar plating fundamentally misrepresents the ecological reality and the true functional biomass within long-ripened artisanal cheeses.

To circumvent the inherent biases of cultivation-based methods, modern dairy microbiology has increasingly shifted towards culture-independent, DNA-based approaches. High-throughput amplicon sequencing (e.g., 16S rRNA metataxonomics) has revolutionized our understanding of cheese microbial ecology by unveiling vast networks of previously unculturable taxa [23–25]. However, standard Next-Generation Sequencing (NGS) heavily relies on the amplification of the 16S rRNA gene, which often lacks the evolutionary divergence required to accurately resolve closely related taxa at the subspecies level, frequently clustering distinct strains into ambiguous phylotypes (e.g., *Lactococcus lactis*/*cremoris*) [20,26,27]. Moreover, metataxonomic approaches generally provide relative abundances rather than the absolute quantification of microbial cells, limiting their utility for precise kinetic monitoring.

Quantitative real-time PCR (qPCR) offers a practical way to overcome the low taxonomic resolution of selective agars and the relative-abundance nature of 16S rRNA amplicon sequencing. Still, many dairy qPCR protocols rely on ribosomal operons (16S or 23S rRNA), which occur in variable copy numbers across LAB species and even between strains of the same taxon (from 1 to 9 copies per chromosome), complicating the direct estimation of genome equivalents. In an earlier study [28], our group designed hydrolysis probe (TaqMan) qPCR assays targeting species-specific genes to quantify *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Lactococcus lactis* subsp. *cremoris* in artisanal raw-milk cheeses at three ripening points (0, 90, and 180 days).

The present work expands on that earlier study [28] in several practical ways by introducing intercalating dye-based (EvaGreen) chemistry, doubling the temporal sampling resolution, and incorporating parallel culture-dependent control. Specifically, the primary aim of this research was to develop and validate subspecies- and species-specific qPCR assays targeting single-copy chromosomal protein-coding loci—*yjaE* for *Lactococcus lactis* subsp. *lactis* (a subspecies not covered in our previous report) and an alternative single-copy locus (*ACRRRJ_03990*) for *Streptococcus thermophilus*—and to monitor their longitudinal target DNA abundance ($\log_{10}$ gene copies/g) across three independent batches of artisanal raw cow's milk cheese at six ripening intervals (0, 30, 60, 90, 120, and 180 days). A concurrent objective was to directly compare these culture-independent gene copy profiles with routine colony counts on selective M17 agar ($\log_{10}$ CFU/g) at each maturation stage. Because standard DNA-qPCR amplifies any intact target sequence—whether originating from culturable cells, non-culturable dormant cells, or unlysed dead biomass—this side-by-side evaluation aimed to quantify the divergence between agar plating and target DNA persistence while defining the interpretive scope of single-copy gene qPCR in long-ripened artisanal cheeses.

2. Results and Discussion

2.1. Assay Specificity, In Silico Copy-Number Verification, and Analytical Validation

Monitoring individual bacterial taxa in ripening raw-milk cheese requires molecular targets that distinguish closely related subspecies while avoiding the copy-number variability of 16S or 23S rRNA operons [17,20,27]. In this study, we targeted two chromosomal

protein-coding loci identified through the Genome-Specific Marker (GSMer) database: the yjaE gene for *Lactococcus lactis* subsp. *lactis* and the ACRRRJ_03990 locus for *Streptococcus thermophilus* [29].

To verify that these markers occur as single copies per chromosome and do not exist as multiple paralogs or pseudogene remnants, we performed an in silico self-BLASTn analysis of each target locus and its 50-nt GSMer signature against its respective reference genome (*L. lactis* subsp. *lactis* IL1403, GenBank: CP033607.1; and *S. thermophilus* UCCSt50, GenBank: CP194174.1). In the *L. lactis* subsp. *lactis* IL1403 chromosome (CP033607.1), self-BLASTn returned a single unique match (Number of Matches: 1, 100% query coverage, 100.00% sequence identity, identical Max and Total alignment scores of 93.5) located within the *yjaE* gene (positions 904,023–906,422; Protein ID: AYU52619.1). Similarly, in the *S. thermophilus* UCCSt50 chromosome (CP194174.1), self-BLASTn confirmed strictly a single chromosomal locus (Number of Matches: 1, positions 747,254–747,303, 100% query coverage, 100.00% sequence identity, identical Max and Total alignment scores of 91.5) within the *ACRRRJ_03990* gene (Protein ID: YAB83324.1). Comparative BLASTn searches across complete reference chromosomes of *L. lactis* subsp. *lactis* and *S. thermophilus* in the NCBI GenBank database likewise confirmed a single hit per genome (100% query coverage and 100.00% sequence identity across reference strains). Importantly, when screening the target markers and primer pairs against non-target cheese-associated lactic acid bacteria in the NCBI nr/nt database—including *Lactococcus lactis* subsp. *cremoris*, *Lactococcus garvieae*, *Lactococcus raffinolactis*, non-target *Streptococcus* spp., *Enterococcus* spp., *Leuconostoc* spp., and *Lacticaseibacillus* spp.—no significant sequence alignments were detected under standard BLASTn parameters (0% significant query coverage; no hits returned outside the target taxa).

Primer specificity was next evaluated experimentally against genomic DNA isolated from 34 bacterial reference strains representing target and non-target LAB commonly encountered in raw milk and artisanal cheeses (Table 1). The LlacL-F/LlacL-R primer pair selectively amplified DNA from all eight *L. lactis* subsp. *lactis* reference strains, yielding consistent quantification cycle (Cq) values ranging from 15.56 ± 0.08 to 16.64 ± 0.14. No amplification (nd, Cq > 40) was observed for the closely related subspecies *L. lactis* subsp. *cremoris* or for any other tested non-target species. Likewise, the Sthe-F/Sthe-R primer pair amplified all five *S. thermophilus* reference strains (mean Cq values from 16.21 ± 0.08 to 17.41 ± 0.11) with no cross-reactivity toward non-target LAB (Table 1). Because the assays employed the intercalating dye EvaGreen, amplicon specificity was verified following each qPCR run via melting curve analysis (65 °C to 95 °C), which consistently produced a single, sharp dissociation peak for each target product (135 bp for *L. lactis* subsp. *lactis* and 81 bp for *S. thermophilus*) and confirmed the absence of primer-dimers or non-specific amplification in negative controls and no-template controls (NTC).

Following specificity verification, the quantitative performance of the qPCR protocols was validated using serial dilutions of linearized recombinant plasmid standards. As shown in the calibration curves in Figures 1–4, both assays exhibited strong linearity (R^2 ranging from 0.995 to 1.000) and amplification efficiencies between 89.9% and 105.8% across 10^1 to 10^7 target gene copies per reaction well. Low-copy replicate testing established both the formal limit of detection (LOD) and limit of quantification (LOQ) at 10 gene copies per reaction well (corresponding to 5.0×10^2 gene copies/g, or 2.70 $\log_{10}$ gene copies/g of cheese), with replicate Cq coefficients of variation (CV) remaining below 2.5% across the dynamic range. Furthermore, post-extraction internal positive control (IPC) spiking experiments confirmed that the DNA purification procedure (incorporating CTAB/PVP40 lysis and chloroform extraction) effectively removed dairy matrix PCR inhibitors [16,20,28]. However, because the IPC was introduced after DNA isolation rather than as a pre-extraction

cellular process control, potential variations in absolute DNA extraction recovery across progressively aged cheese matrices were not directly quantified and should be recognized as a methodological limitation, although minor differences in extraction efficiency are unlikely to account for the multi-log target gene trajectories observed over ripening.

Table 1. Quantification cycle (C_q) values obtained from qPCR assays using DNA isolated from *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus* (positive controls) and closely related or non-target bacterial species (negative controls).

Name of the Tested Bacteria [1]	*Lactococcus lactis* subsp. *lactis* Primer Pairs	*Streptococcus thermophilus* Primer Pairs	Mean Cq Value in Positive Control
Lactococcus lactis subsp. *lactis* NCTC 6681	Positive control	Negative control	15.56 ± 0.08
Lactococcus lactis subsp. *lactis* ZFX-2	Positive control	Negative control	16.23 ± 0.12
Lactococcus lactis subsp. *lactis* L21	Positive control	Negative control	16.32 ± 0.09
Lactococcus lactis subsp. *lactis* FD-56	Positive control	Negative control	16.64 ± 0.14
Lactococcus lactis subsp. *lactis* 125	Positive control	Negative control	15.59 ± 0.11
Lactococcus lactis subsp. *lactis* M1	Positive control	Negative control	16.36 ± 0.08
Lactococcus lactis subsp. *lactis* 9155	Positive control	Negative control	15.78 ± 0.09
Lactococcus lactis subsp. *lactis* NCDO 1007	Positive control	Negative control	15.67 ± 0.08
Streptococcus thermophilus NCDO 573	Negative control	Positive control	17.36 ± 0.11
Streptococcus thermophilus LMD-9	Negative control	Positive control	16.78 ± 0.12
Streptococcus thermophilus LMG 18311	Negative control	Positive control	16.21 ± 0.08
Streptococcus thermophilus IDCC 2201	Negative control	Positive control	17.36 ± 0.12
Streptococcus thermophilus PCI 1327	Negative control	Positive control	17.41 ± 0.11
Lactococcus lactis subsp. *cremoris* SK11	Negative control	Negative control	nd [2]
Lactococcus lactis subsp. *cremoris* HP	Negative control	Negative control	nd
Lactococcus lactis subsp. *cremoris* NCDO 607	Negative control	Negative control	nd
Lactococcus lactis subsp. *cremoris* DSM 20069	Negative control	Negative control	nd
Lactococcus garvieae YT-3	Negative control	Negative control	nd
Lactococcus garvieae NCDO 2155	Negative control	Negative control	nd
Lactococcus garvieae S-1477	Negative control	Negative control	nd
Lactococcus raffinolactis NCDO 617	Negative control	Negative control	nd
Lactococcus raffinolactis DSM 20443	Negative control	Negative control	nd
Enterococcus faecalis NCTC 775	Negative control	Negative control	nd
Enterococcus faecalis OG1RF	Negative control	Negative control	nd
Enterococcus faecalis V583	Negative control	Negative control	nd
Enterococcus faecium NCTC 2699	Negative control	Negative control	nd
Enterococcus faecium MMC4	Negative control	Negative control	nd
Enterococcus faecium TEX16	Negative control	Negative control	nd
Leuconostoc mesenteroides NRRL B-512	Negative control	Negative control	nd
Leuconostoc mesenteroides NCIB 8023	Negative control	Negative control	nd
Leuconostoc mesenteroides NRRL B-512F	Negative control	Negative control	nd
Lacticaseibacillus casei IDCC 3451	Negative control	Negative control	nd
Lacticaseibacillus casei IAM 12473	Negative control	Negative control	nd
Lacticaseibacillus casei L 944	Negative control	Negative control	nd

[1] Each reaction contained 2 µL of clarified thermal cell lysate standardized to approximately 5 ng/µL (~10 ng of genomic DNA per reaction); [2] nd, not detected (Cq > 40).

2.2. Longitudinal Target Gene Abundance of Autochthonous LAB Evaluated by Single-Copy Gene qPCR

Following analytical validation, the longitudinal dynamics of *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus* target DNA were monitored across three independent batches of artisanal raw cow's milk cheese. Targeting the single-copy chromosomal loci *yjaE* and *ACRRRJ_03990* allowed us to track target gene copy numbers without the multi-copy bias inherent to rRNA operons [17,20]. Because synthetic plasmid calibration curves quantify target gene copies entering the PCR reaction rather than correcting for absolute cell recovery or distinguishing viable from non-viable intact genomes, quantitative results are expressed as target gene copies per gram of cheese ($\log_{10}$ gene copies/g). Cheese samples were analyzed at six ripening intervals: immediately after curd molding prior to brining (day 0, representing baseline autochthonous populations) and after 30, 60, 90, 120, and 180 days of maturation at 12 °C.

The calibration curves and corresponding sample quantifications for *Lactococcus lactis* subsp. *lactis* during early-to-mid ripening (days 0, 30, and 60) are shown in Figure 1, while late ripening stages (days 90, 120, and 180) are presented in Figure 2.

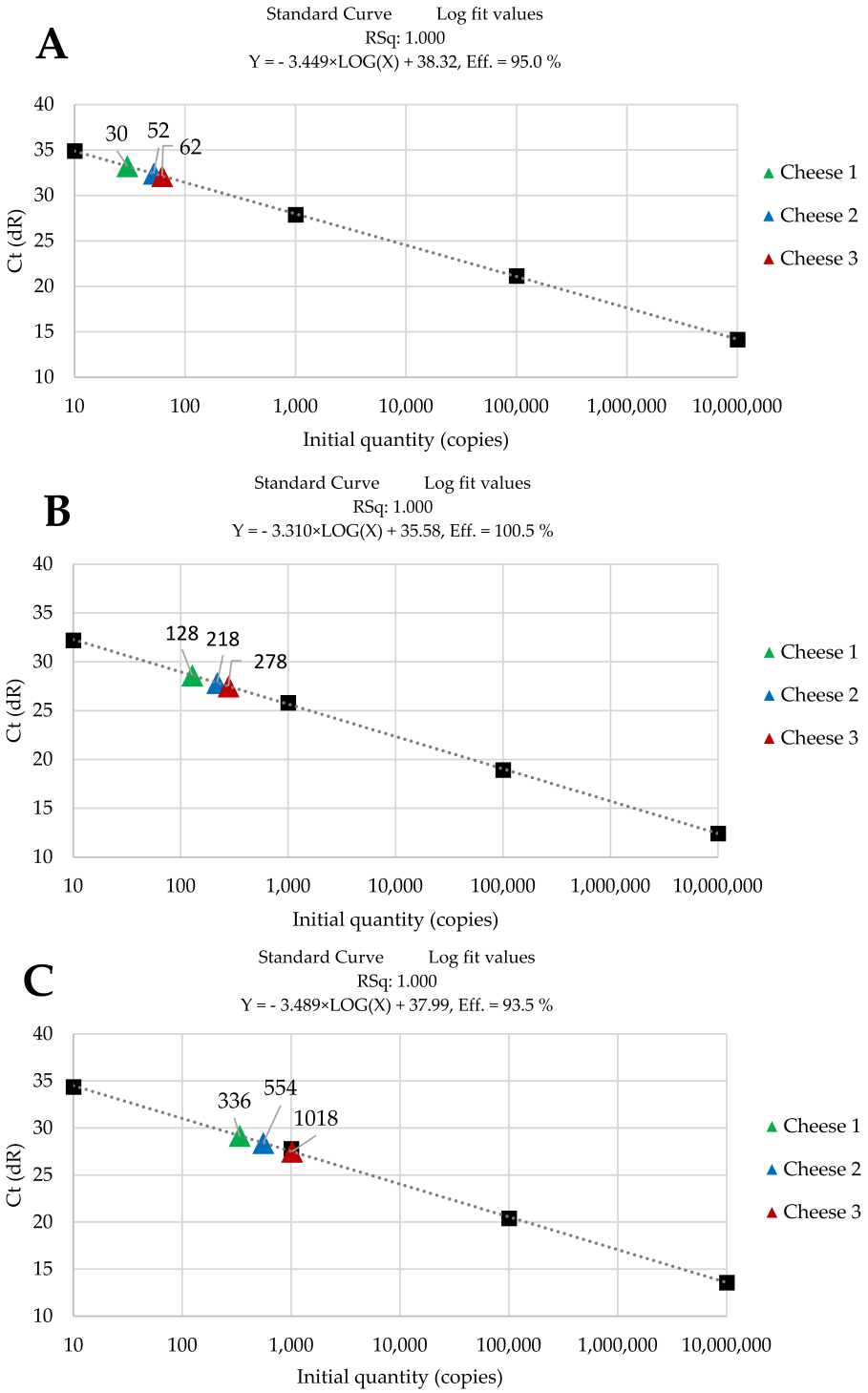

Figure 1. Calibration curves and qPCR quantification of *Lactococcus lactis* subsp. *lactis* target DNA in raw cow's milk cheese: (**A**) fresh curd sampled directly after coagulation and molding (day 0, prior to brine salting); (**B**) after 30 days of ripening; (**C**) after 60 days of ripening. The horizontal axis (initial quantity, copies) represents the *yjaE* target gene copy number per qPCR reaction well prior to conversion to $\log_{10}$ gene copies/g of cheese. Black squares represent linearized plasmid standards (plotted at 100-fold intervals from 10^1 to 10^7 copies per well) delineating the calibration curve; colored triangles represent the mean values obtained for each quantified cheese batch (Cheese 1–3).

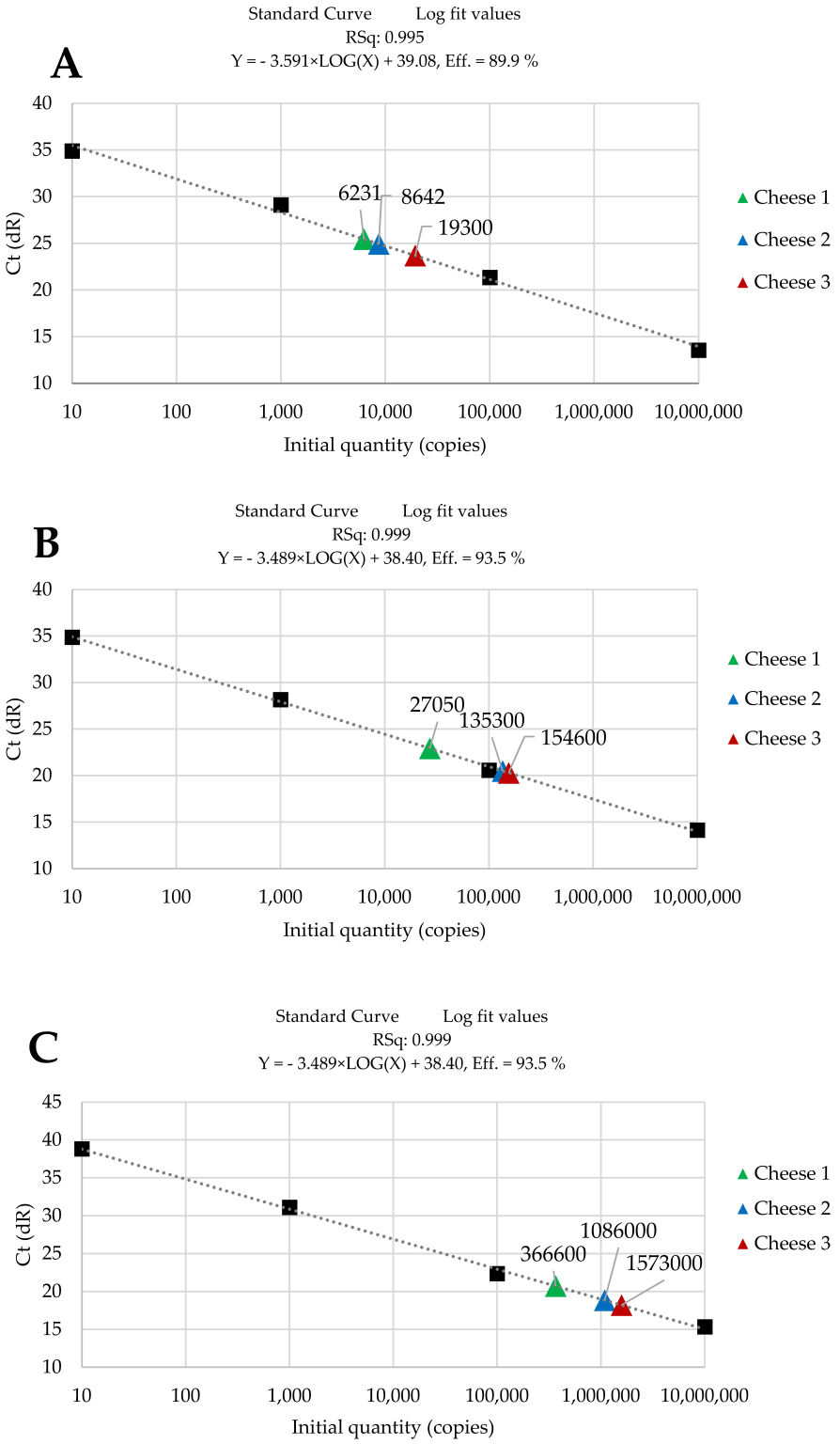

Figure 2. Calibration curves and qPCR quantification of *Lactococcus lactis* subsp. *lactis* target DNA in raw cow's milk cheese: (**A**) after 90 days of ripening; (**B**) after 120 days of ripening; (**C**) after 180 days of ripening. The horizontal axis (initial quantity, copies) represents the *yjaE* target gene copy number per qPCR reaction well prior to conversion to $\log_{10}$ gene copies/g of cheese. Black squares represent linearized plasmid standards (plotted at 100-fold intervals from 10^1 to 10^7 copies per well) delineating the calibration curve; colored triangles represent the mean values obtained for each quantified cheese batch (Cheese 1–3). Samples from days 120 (**B**) and 180 (**C**) were amplified within the same 96-well qPCR plate run against the shared plate-specific calibration curve.

Similarly, the qPCR calibration curves and sample quantifications for *Streptococcus thermophilus* during the first 60 days of maturation (days 0, 30, and 60) are presented in Figure 3, and the late-stage measurements (days 90, 120, and 180) are shown in Figure 4.

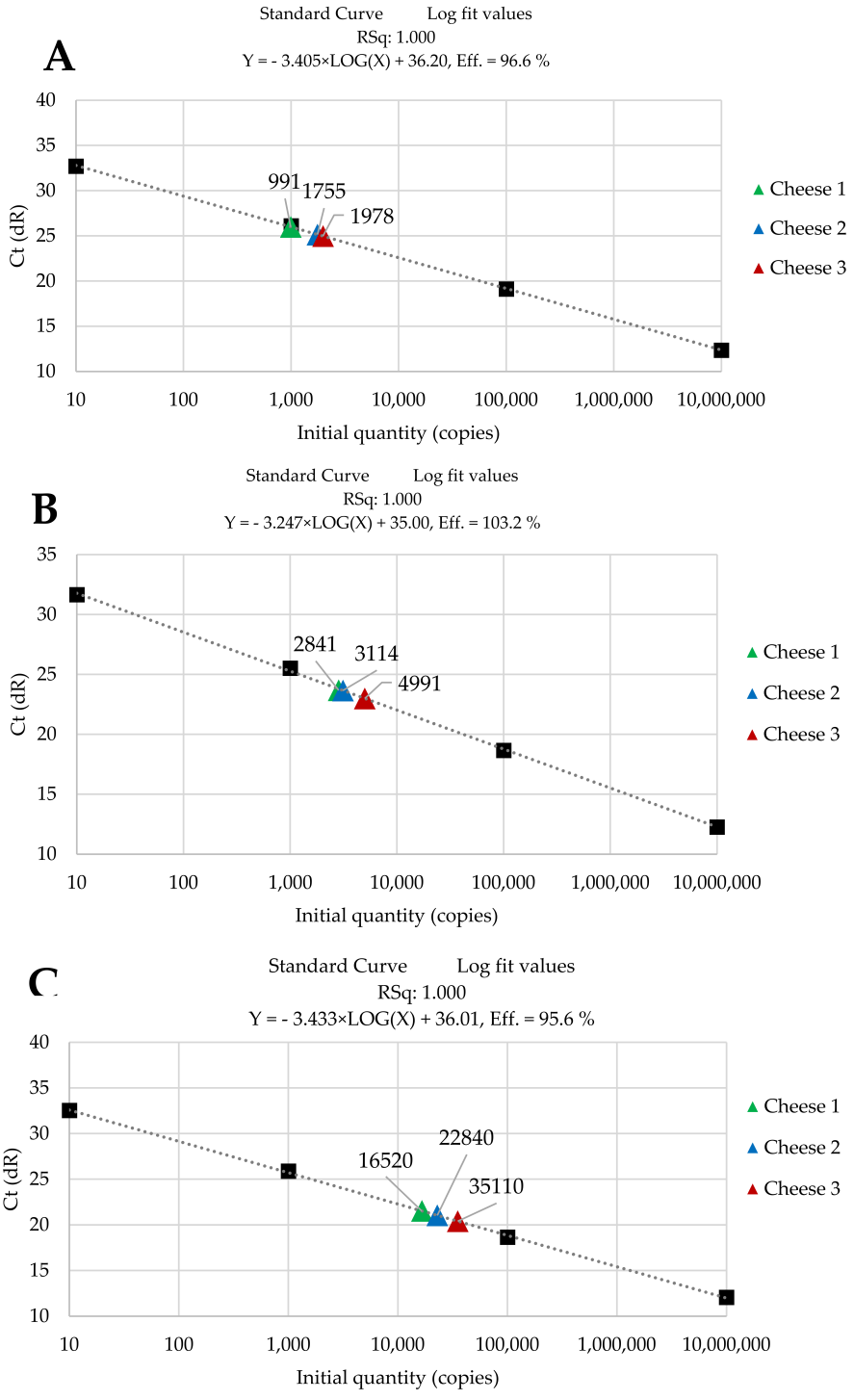

Figure 3. Calibration curves and qPCR quantification of *Streptococcus thermophilus* target DNA in raw cow's milk cheese: (**A**) fresh curd sampled directly after coagulation and molding (day 0, prior to brine salting); (**B**) after 30 days of ripening; (**C**) after 60 days of ripening. The horizontal axis (initial quantity, copies) represents the *ACRRRJ_03990* target gene copy number per qPCR reaction well prior to conversion to $\log_{10}$ gene copies/g of cheese. Black squares represent linearized plasmid standards (plotted at 100-fold intervals from 10^1 to 10^7 copies per well) delineating the calibration curve; colored triangles represent the mean values obtained for each quantified cheese batch (Cheese 1–3).

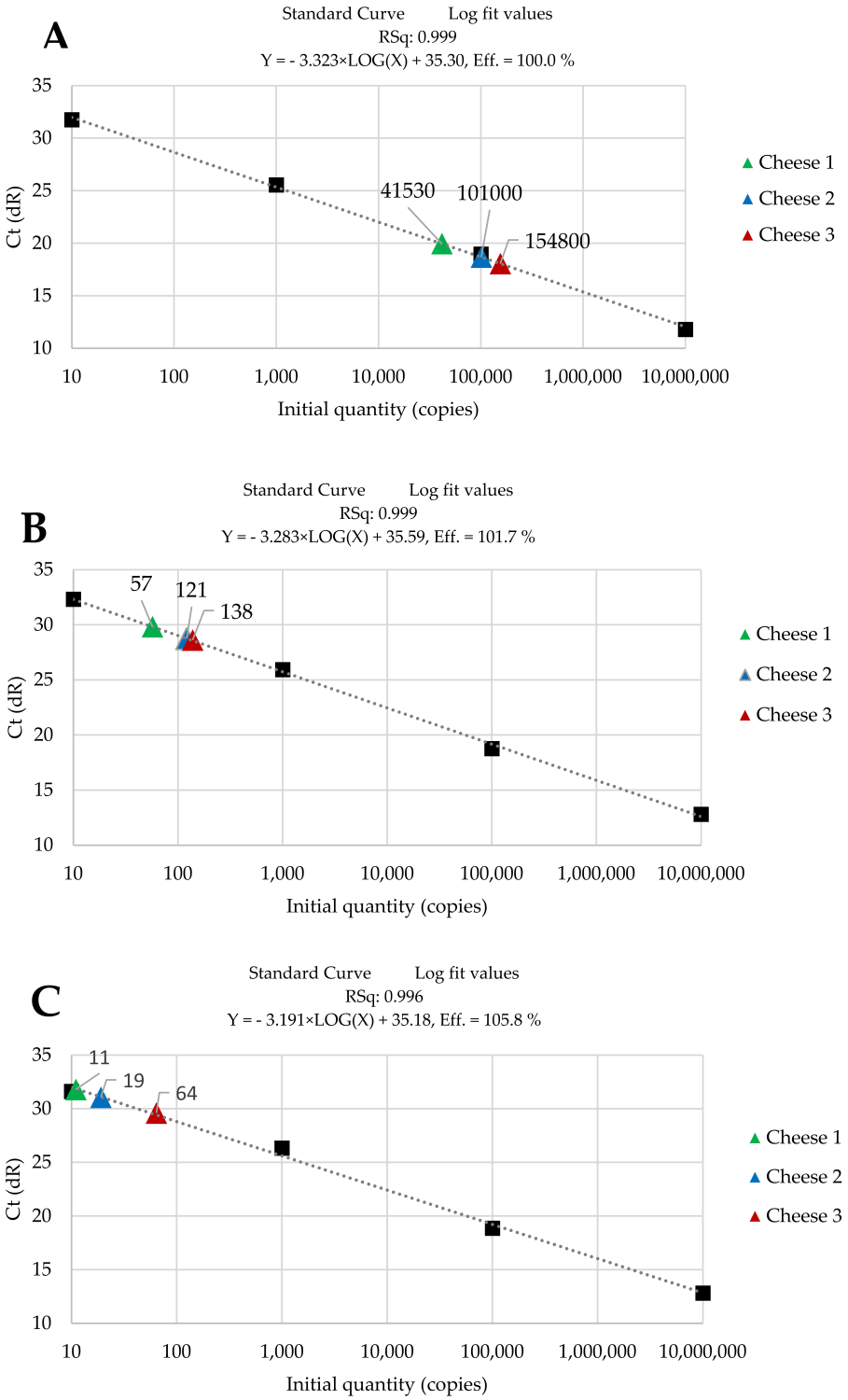

Figure 4. Calibration curves and qPCR quantification of *Streptococcus thermophilus* target DNA in raw cow's milk cheese: (**A**) after 90 days of ripening; (**B**) after 120 days of ripening; (**C**) after 180 days of ripening. The horizontal axis (initial quantity, copies) represents the *ACRRRJ_03990* target gene copy number per qPCR reaction well prior to conversion to $\log_{10}$ gene copies/g of cheese. Black squares represent linearized plasmid standards (plotted at 100-fold intervals from 10^1 to 10^7 copies per well) delineating the calibration curve; colored triangles represent the mean values obtained for each quantified cheese batch (Cheese 1–3).

To compare the temporal trajectories of both bacterial populations, the mean target gene copy numbers (expressed in $\log_{10}$ gene copies/g) determined separately for each of the three independent cheese batches (Cheese 1, 2, and 3) are shown in Figure 5A–C.

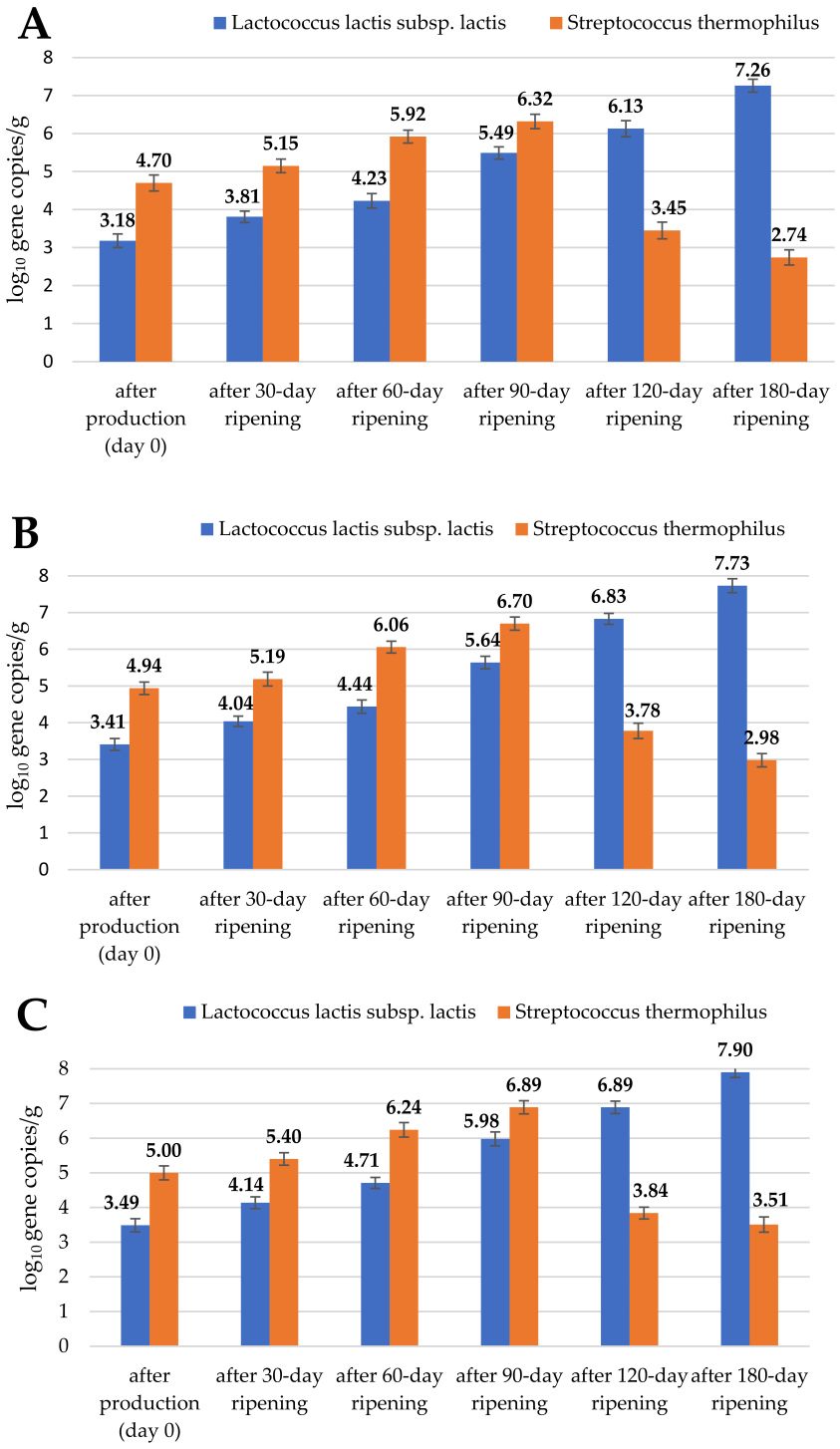

Figure 5. Target gene abundance of *Lactococcus lactis* subsp. *lactis* (yjaE) and *Streptococcus thermophilus* (ACRRRJ_03990) in (**A**) the first batch of cheese (Cheese 1); (**B**) the second batch of cheese (Cheese 2); (**C**) t-he third batch of cheese (Cheese 3). Abundance expressed as $\log_{10}$ gene copies/g of cheese determined by qPCR over 180 days of ripening. Sampling at day 0 represents unsalted fresh cheese blocks obtained after curd drainage and molding (prior to brine immersion and cold storage). The whiskers on the bars represent the standard deviation from three independent DNA extractions from the same composite sample.

The longitudinal qPCR measurements revealed contrasting temporal profiles between the two indigenous LAB taxa across the 180-day maturation period. Immediately after curd molding (day 0), both bacteria were readily detected in the fresh cheese blocks. *S. thermophilus* initially showed higher abundance in the fresh curd, ranging from 4.70 to 5.00 $\log_{10}$ gene copies/g, whereas *L. lactis* subsp. *lactis* was present at lower initial levels of 3.18 to 3.49 $\log_{10}$ gene copies/g. This initial distribution reflects the thermal conditions of early cheesemaking, where curd scalding at 42 °C and abundant lactose favor the rapid early growth of thermophilic streptococci [4,5,26].

Once the salted cheeses were transferred to the ripening chamber (12 °C, 80–85% relative humidity), progressive lactose depletion, salt diffusion, and low temperature affected the two species differently. As shown in Figure 5, *L. lactis* subsp. *lactis* target DNA levels increased steadily across all three cheese batches throughout the 180-day ripening period. By day 90, *L. lactis* subsp. *lactis* abundance reached 5.49 to 5.98 $\log_{10}$ gene copies/g, and continued to rise during late maturation to reach a maximum of 7.26 to 7.90 $\log_{10}$ gene copies/g at day 180. When interpreting microbial abundances expressed per gram of wet cheese over six months of ripening, gradual moisture evaporation (curd dehydration) must also be considered, as water loss concentrates solid-phase components [15,18,24]. Although dry matter content was not directly determined across sampling intervals in the present cheeses—representing a minor study limitation— even a hypothetical cumulative moisture loss of 20–35% over six months at 80–85% relative humidity would correspond mathematically to a mass concentration factor of less than 0.2 $\log_{10}$ units, which cannot explain the >4.0 $\log_{10}$ net increase recorded for *L. lactis* subsp. *lactis* between day 0 and day 180. Even allowing for unquantified variations in DNA extraction efficiency across aging matrices, a multi-log net increase in target gene copies of this magnitude is consistent with net growth of autochthonous *L. lactis* subsp. *lactis* during cold ripening. Previous studies have noted that wild mesophilic lactococci often display greater environmental tolerance than commercial starters, potentially utilizing alternative substrates such as citrate, arginine, or casein-derived peptides to sustain growth under carbohydrate starvation and cold stress [6,8,9,15,18].

By contrast, *Streptococcus thermophilus* exhibited a biphasic trajectory. Its target gene abundance increased during the first three months of ripening, reaching a peak of 6.32 to 6.89 $\log_{10}$ gene copies/g at day 90 across all three batches (Figure 5). Between day 90 and day 120, however, intact *ACRRRJ_03990* gene copies dropped sharply to 3.45–3.84 $\log_{10}$ gene copies/g, and declined further by day 180 to 2.74–3.51 $\log_{10}$ gene copies/g (corresponding to approximately 11–64 target copies per reaction well, approaching the assay LOQ of 10 copies per reaction, or 2.70 $\log_{10}$ gene copies/g).

Directly interpreted, this multi-log decrease in amplifiable ACRRRJ_03990 copies is consistent with progressive loss/degradation of amplifiable chromosomal target DNA, rather than directly proving a specific cellular lysis mechanism. From a physiological standpoint, *S. thermophilus*—an obligate thermophile with an optimal growth temperature near 42 °C—is poorly suited to prolonged storage at 12 °C, carbohydrate starvation, and reduced water activity [15,18,24]. In previous dairy microbiology studies, the late-stage decline of thermophilic streptococci has frequently been attributed to cell death followed by cellular autolysis and nuclease-mediated DNA clearance, processes hypothesized to release intracellular peptidases and esterases that support proteolysis and aroma development during extended ripening [6,8,15,24,30]. However, because cell-envelope rupture, autolytic enzyme release, and volatile flavor compounds were not experimentally measured in the present work, autolysis and its biochemical contributions remain literature-supported biological hypotheses rather than mechanisms directly demonstrated by our qPCR data.

Comparing these results with our 2024 investigation on raw cow's milk cheeses [28] reveals both consistent ecological trends and clearer late-stage resolution. In both studies, *S. thermophilus* was the most abundant target taxon in the fresh curd and reached its maximum gene copy number at 90 days (3 months), while indigenous mesophilic lactococci (*L. lactis* subsp. *cremoris* in our 2024 study and *L. lactis* subsp. *lactis* in the present work) accumulated progressively up to 180 days. However, increasing the sampling frequency to 30-day intervals and targeting the *ACRRRJ_03990* locus in the present cheese batches captured a much sharper decline in *S. thermophilus* DNA between days 90 and 180 than was observed in our earlier three-point study [28], highlighting batch- and marker-specific dynamics during late maturation.

2.3. Comparison Between Culture-Dependent Plate Counts and qPCR Target Gene Abundance

To evaluate how culture-independent DNA quantification compares with routine microbiological monitoring, cheese samples were analyzed in parallel at every ripening interval by surface plating on selective M17 agar (incubated at 30 °C for presumptive mesophilic *Lactococcus* spp. and at 42 °C under microaerophilic conditions for presumptive thermophilic *Streptococcus* spp.). When interpreting this comparison, an important methodological distinction must be recognized: M17 agar is an elective rather than strictly species-specific medium and therefore enumerates broader presumptive coccoid groups, whereas our qPCR assays target single-copy loci restricted to *L. lactis* subsp. *lactis* and *S. thermophilus*, respectively. Despite the broader taxonomic coverage of M17 agar, comparing the two methods over 180 days revealed a widening discrepancy between culturable colony counts ($\log_{10}$ CFU/g) and target gene copy numbers ($\log_{10}$ gene copies/g), as illustrated in Figures 6 and 7.

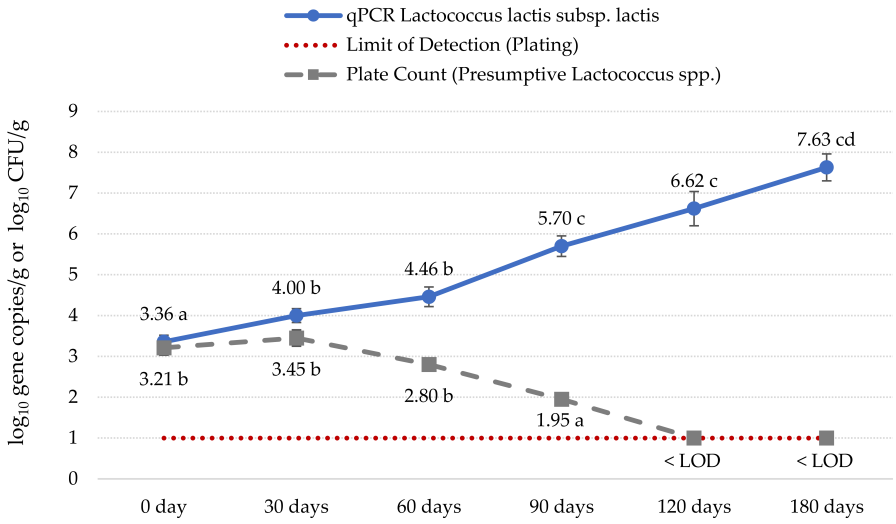

Figure 6. Comparison of *Lactococcus* longitudinal profiles during 180 days of raw cow's milk cheese ripening determined by single-copy gene qPCR versus culture-dependent plating. The solid blue line represents qPCR quantification of *Lactococcus lactis* subsp. *lactis* (*yjaE* gene) expressed as $\log_{10}$ gene copies/g of cheese (mean of three batches ± SD). The dashed grey line represents culture-dependent enumeration of presumptive mesophilic *Lactococcus* spp. on M17 agar at 30 °C expressed as $\log_{10}$ CFU/g, while the dotted red line indicates the plating limit of detection (LOD = 1.0 $\log_{10}$ CFU/g). Plate counts below the detection limit at days 120 and 180 are plotted at the LOD with an explicit "< LOD" label and were excluded from inferential analysis as censored observations. Within each individual trajectory, quantified time points bearing different lowercase letters differ significantly over ripening time according to repeated-measures ANOVA with Greenhouse–Geisser correction and Bonferroni post hoc adjustment across the three independent cheese batches ($n = 3$, $p < 0.05$).

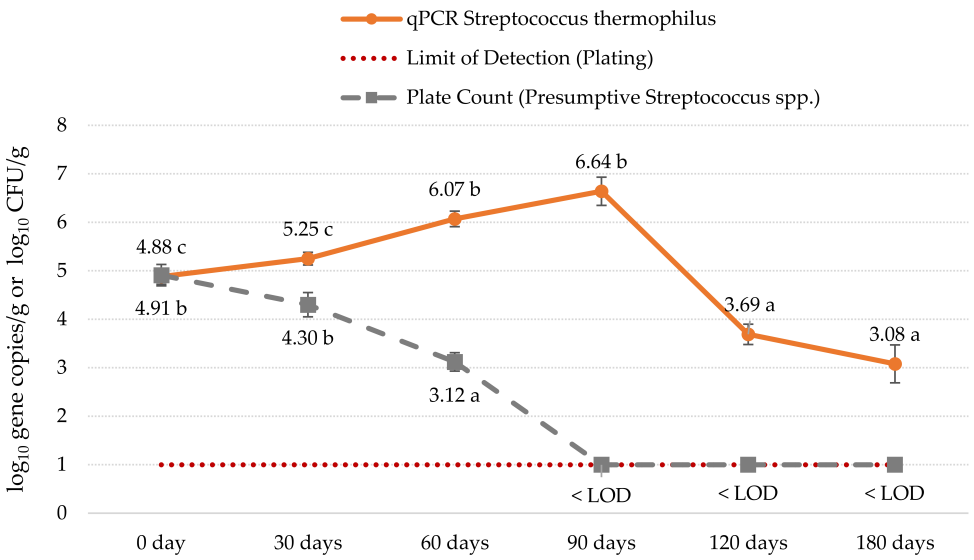

Figure 7. Comparison of *Streptococcus thermophilus* longitudinal profiles during 180 days of raw cow's milk cheese ripening determined by single-copy gene qPCR versus culture-dependent plating. The solid orange line represents qPCR quantification of *S. thermophilus* (*ACRRRJ_03990* gene) expressed as $\log_{10}$ gene copies/g of cheese (mean of three batches ± SD). The dashed grey line represents culture-dependent enumeration of presumptive thermophilic *Streptococcus* spp. on M17 agar at 42 °C expressed as $\log_{10}$ CFU/g, while the dotted red line indicates the plating limit of detection (LOD = 1.0 $\log_{10}$ CFU/g). Plate counts below the detection limit from day 90 through day 180 are plotted at the LOD with an explicit "< LOD" label and were excluded from inferential analysis as censored observations. Within each individual trajectory, quantified time points bearing different lowercase letters differ significantly over ripening time according to repeated-measures ANOVA with Greenhouse–Geisser correction and Bonferroni post hoc adjustment across the three independent cheese batches ($n = 3$, $p < 0.05$).

At the initial production stage (day 0), baseline levels in the fresh curds were 3.21 $\log_{10}$ CFU/g on M17 agar (30 °C) alongside 3.36 $\log_{10}$ gene copies/g by qPCR for *L. lactis* subsp. *lactis*, and 4.91 $\log_{10}$ CFU/g on M17 agar (42 °C) alongside 4.88 $\log_{10}$ gene copies/g by qPCR for *S. thermophilus*. Beyond day 30, however, culturable colony counts on M17 agar declined rapidly for *S. thermophilus* and steadily for *L. lactis*. Specifically, on M17 agar at 42 °C, presumptive thermophilic streptococci decreased from 4.30 $\log_{10}$ CFU/g at day 30 to 3.12 $\log_{10}$ CFU/g at day 60, and fell below the limit of detection (<1.0 $\log_{10}$ CFU/g) from day 90 through day 180 (Figure 7). On M17 agar at 30 °C, presumptive mesophilic *Lactococcus* spp. counts were 3.45 $\log_{10}$ CFU/g at day 30, 2.80 $\log_{10}$ CFU/g at day 60, and 1.95 $\log_{10}$ CFU/g (estimated count) at day 90, before dropping below the detection limit (<1.0 $\log_{10}$ CFU/g) at days 120 and 180 (Figure 6). In contrast, the qPCR assays detected a continuous increase in *L. lactis* subsp. *lactis* target DNA across all intervals up to day 180 (Figure 6) and rising copy numbers of *S. thermophilus* target DNA up to day 90, followed by a decline to 3.69 and 3.08 $\log_{10}$ gene copies/g at days 120 and 180 (Figure 7).

This pronounced divergence between qPCR-positive and plate-negative results during mid- and late ripening can be attributed to two main biological and methodological factors. First, prolonged exposure to ripening stresses—including carbohydrate exhaustion, low temperature (12 °C), lactic acid accumulation, and elevated NaCl concentration—is known to induce sublethal injury or force subpopulations of dairy LAB into a viable-but-non-culturable (VBNC) state [15,16,21,22]. Bacteria in a dormant or VBNC state lose the capacity to form visible colonies on standard rich agar media such as M17, yet retain intact cell envelopes and chromosomal DNA [15,16]. Second, because conventional DNA-based qPCR

amplifies any intact target sequence extracted from the cheese matrix, the measured gene copy numbers represent a composite signal comprising culturable cells, VBNC cells, recently dead cells with intact membranes, and potentially persistent extracellular DNA adsorbed onto casein or fat globules. Consequently, while the continuous multi-log increase in *L. lactis* subsp. *lactis* target DNA between day 90 and day 180 is consistent with net growth of stress-adapted subpopulations that fail to form colonies on standard M17 agar, the persistence of *S. thermophilus* DNA at days 60 and 90 likely reflects a combination of non-culturable dormant cells and unlysed dead biomass prior to late-stage DNA degradation.

A recognized limitation of conventional DNA-qPCR is that it cannot partition the total gene copy pool into viable versus dead fractions. Future studies incorporating viability-discriminating treatments—such as propidium monoazide (PMA-qPCR) or ethidium monoazide (EMA-qPCR) to block DNA from membrane-compromised cells, reverse-transcription qPCR (RT-qPCR) targeting labile mRNA transcripts, or fluorescence flow cytometry—will be essential to quantify the exact proportion of metabolically active VBNC cells in long-ripened artisanal cheeses [15,16,31]. Nevertheless, our side-by-side comparison demonstrates that relying solely on routine M17 agar plating misses major shifts in target DNA abundance during late ripening, underscoring the value of single-copy gene qPCR as a complementary monitoring tool.

3. Materials and Methods

3.1. Cheese Production

Three separate experimental batches of traditional raw-milk cheese were produced using unpasteurized bovine milk (4.8% fat content) of high hygienic and microbiological quality, sourced from a single certified organic dairy farm in the Podlasie region of Poland. To capture natural daily fluctuations in the native milk microflora, the raw material designated for each individual batch was harvested from morning milkings across three consecutive days. The manufacturing protocol intentionally omitted any commercial starter cultures or exogenous adjunct strains. Consequently, both lactic fermentation and the subsequent formation of sensory attributes relied exclusively on the spontaneous activity of the autochthonous microbiota present in the raw milk.

To begin the cheesemaking procedure, the unpasteurized milk was thermally conditioned to 32 °C and supplemented with liquid calf rennet (Chr. Hansen, Hørsholm, Denmark), resulting in complete enzymatic clotting within 30 min. Subsequently, the formed coagulum was diced into approximately 5 cm cubes and subjected to thermal scalding by gradually raising the temperature to 42 °C, which promoted curd shrinkage (syneresis) and whey separation. Once the heating step was completed, the curd particles were transferred into perforated colanders and maintained at 22 °C for 48 h to allow gravitational whey drainage and spontaneous structural consolidation. The resulting molded, unsalted fresh curd blocks served as the initial reference material (sampling day 0) prior to salting and chamber maturation.

Next, the consolidated cheese blocks underwent salting via 48 h submersion in an aqueous brine (15% NaCl, pH adjusted to 5.25) to achieve uniform salt diffusion within the matrix [32]. After brining, the cheeses were placed inside a climatic maturation unit (model KK 500, POL-EKO sp.k., Wodzisław Śląski, Poland) for a 180-day (6-month) ripening period under strictly regulated environmental conditions (constant temperature of 12 °C and 80–85% relative humidity). Over the course of this 6-month maturation, the metabolic succession of the indigenous microflora imparted the final product's characteristic compact body, yellowish hue, and distinctive organoleptic profile.

3.2. Cheese Sampling and Sample Preparation for DNA Isolation

To track longitudinal changes in target LAB gene abundance over the 6-month maturation cycle, cheese specimens were collected from all three independent experimental batches ($n = 3$ biological replicates) at six designated ripening intervals: day 0 (unsalted, freshly molded curd blocks sampled immediately after enzymatic clotting, cutting, and 48 h whey drainage) and after 30, 60, 90, 120, and 180 days of maturation at 12 °C.

Because bacterial distribution within artisanal cheese blocks can be spatially heterogeneous due to temperature and salt gradients between the core and outer layers, a standardized composite sampling protocol was applied at each time point to minimize spatial sampling bias. Under strictly aseptic conditions, three cylindrical plugs were extracted from distinct spatial zones (central core, mid-radius, and peripheral interior) of each cheese block using a sterilized stainless-steel cheese trier. Immediately after extraction, the outer rind layer (approximately 1 cm thick) of each plug was trimmed off with a sterile scalpel and discarded so that the analysis represented the interior cheese matrix. The three trimmed interior plugs from a given cheese batch and time point were then combined and thoroughly homogenized in a sterile blender to generate a single, uniform composite analytical sample per batch.

Directly prior to nucleic acid extraction, three independent analytical test portions of 200 mg (0.2 g) each were weighed from the homogenized composite cheese mass of each batch into separate sterile 2 mL microcentrifuge tubes (so that each individual DNA extraction was performed on a single 200 mg cheese aliquot) using an internally calibrated analytical balance (model AXIS ATA220, 0.001 g readability; Superwagi, Warsaw, Poland) and subjected to downstream enzymatic cell lysis and genomic DNA purification (Syngen Food DNA Mini Kit).

3.3. Microbial DNA Isolation from Cheese Samples

The extraction of total genomic DNA from the cheese matrix, collected both immediately after production and at defined ripening stages (30, 60, 90, 120, and 180 days), was performed using the Syngen Food DNA Mini Kit (Syngen Biotech, Wrocław, Poland). The procedure was optimized to handle the high lipid and protein content characteristic of long-ripening artisanal cheeses.

For each isolation, 200 mg of the homogenized cheese sample was precisely weighed into a 2 mL sterile microcentrifuge tube using an AXIS ATA220 electronic balance (precision 0.001 g; Superwagi, Warsaw, Poland). To initiate cellular lysis, 1 mL of DLF buffer (2% CTAB, 1% PVP40, and 2% β-mercaptoethanol) was added, followed by 30 µL of Proteinase K (Bioron GmbH, Römerberg, Germany). The samples were thoroughly vortexed and then subjected to enzymatic digestion at 60 °C for 30 min.

Following incubation, the lysate was centrifuged, and 700 µL of the supernatant was transferred to a new tube. To eliminate proteinaceous and lipid contaminants, 500 µL of chloroform was added. After phase separation, 350 µL of the clarified upper aqueous phase was recovered and mixed with 350 µL of DWF buffer (3 M guanidine chloride in 70% isopropyl alcohol). The mixture was then loaded onto a DF silica-membrane column and centrifuged.

The purification was completed through successive washing steps with 700 µL of DPF buffer (70% ethanol). Finally, the purified DNA was eluted using 100 µL of pre-warmed DE elution buffer (0.1 M Tris-EDTA, pH 7.5). The elution involved a 1 min incubation at room temperature followed by centrifugation at maximum speed (18,000× *g*).

The concentration and purity of the extracted DNA were verified via spectrophotometry using an Evolution 220 instrument (Thermo Fisher Scientific, Waltham, MA, USA). High-

quality DNA templates, characterized by A_{260}/A_{280} absorbance ratios between 1.8 and 2.0, were obtained and stored at −20 °C for subsequent qPCR quantification.

3.4. Selection of Target Microorganisms and Rationale

The indigenous microbiota of raw bovine milk forms a complex microbial consortium that distinguishes traditional artisanal cheeses from industrial products made with defined starters. Within this community, *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus* were selected for longitudinal monitoring over 180 days of ripening because they represent key mesophilic and thermophilic cocci with contrasting tolerances to ripening stress.

Lactococcus lactis subsp. *lactis* participates in initial curd acidification and is known for its adaptability to low temperatures and carbohydrate depletion during prolonged maturation, where wild strains can utilize alternative nitrogenous and carbon substrates. Conversely, *Streptococcus thermophilus* proliferates rapidly during warm curd manufacture (scalding at 42 °C) and early fermentation, but is considerably more sensitive to subsequent cold storage (12 °C), lactose exhaustion, and salt diffusion. Monitoring both taxa across six ripening points allowed us to compare their distinct target DNA trajectories during extended cold maturation.

Implementing single-copy gene qPCR alongside routine M17 agar plating was motivated by the known limitations of conventional culture media. Selective agars generally enumerate broad phenotypic groups rather than specific subspecies and cannot detect bacterial cells that have lost culturability due to sublethal environmental stress. Combining subspecies- and species-specific DNA quantification with routine plate counts thus enabled a direct evaluation of the divergence between culturable colony counts and total intact target DNA abundance over 180 days of cheese ripening.

3.5. Designing Primer Sets for Real-Time PCR Assays

To achieve species- and subspecies-level specificity and avoid cross-reactivity with closely related taxa (such as *Lactococcus lactis* subsp. *cremoris*), a bioinformatic pipeline based on the GSMer (Genome-Specific Marker) database was employed [29]. Candidate 50-nucleotide (50-nt) sequences unique to *Lactococcus lactis* subsp. *lactis* (TaxID: 1360) and *Streptococcus thermophilus* (TaxID: 1308) were identified using GSMer and inspected with the glogg tool (v. 24.11.0). The resulting 50-nt markers—5′-ACAACTGGCGACGAGTGCAGGAACTT ACCCCTTACAACTTGCGCCTAAAA-3′ for *L. lactis* subsp. *lactis* and 5′-GCTCGTCCATC TTGTCACTAGGTCTAATAACGAGCCTATAACGGGGCTTT-3′ for *S. thermophilus*—were used as templates for primer design.

The selected 50-nt markers were validated in silico using NCBI BLASTn against reference chromosomes (*L. lactis* subsp. *lactis* IL1403, GenBank: CP033607.1; and *S. thermophilus* UCCSt50, GenBank: CP194174.1) and the non-redundant (nr/nt) database. Selection criteria required 100% query coverage and 100% sequence identity across target reference strains, strictly single-copy chromosomal status (Number of Matches: 1 per chromosome in self-BLASTn analysis, with identical Max and Total alignment scores), and the absence of significant similarity in non-target cheese-associated LAB.

For *L. lactis* subsp. *lactis*, the *yjaE* gene (locus tag EFV54_04745, positions 904,023–906,422; encoding a YhgE/Pip domain-containing protein, Protein ID: AYU52619.1) on the IL1403 chromosome (GenBank: CP033607.1) was selected as the single-copy molecular target. For *S. thermophilus*, the marker mapped to a single locus (positions 747,254–747,303) within the *ACRRRJ_03990* gene (encoding a primase alpha-helix C-terminal domain-containing protein, Protein ID: YAB83324.1) on the UCCSt50 chromosome (GenBank: CP194174.1).

Primers were designed within these genomic coordinates using NCBI Primer-BLASTn (LlacL-F: 906,115–906,134; LlacL-R: 906,249–906,230 on CP033607.1; Sthe-F: 747,294–747,314;

Sthe-R: 747,374–747,355 on CP194174.1). The design was optimized for the Quantum EvaGreen PCR kit (Syngen Biotech) under the following parameters: primer length of 20–21 nucleotides, melting temperature (T_m) of 60.0 ± 1.0 °C, GC content between 47.6% and 55.0%, short amplicon sizes (135 bp and 81 bp) suitable for DNA recovered from aged cheese matrices, and low self- and 3′-end complementarity to prevent primer-dimer formation (Table 2).

Table 2. Characteristics of the optimized species-specific primer pairs designed for quantitative real-time PCR detection of *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*.

Target Species	Target Gene (Locus Tag; Protein ID)	Primer Name	Oligonucleotide Sequence (5′ → 3′)	Tm (°C)	GC Content (%)	Amplicon Size (bp)
Lactococcus lactis subsp. *lactis*	*yjaE* (EFV54_04745; AYU52619.1)	LlacL-F LlacL-R	TCAGCTTCGCTTACATGGCA TAGGCGCAAGTTGTAAGGGG	60.04 60.04	50.0 55.0	135
Streptococcus thermophilus	*ACRRRJ_03990* (ACRRRJ_03990; YAB83324.1)	Sthe-F Sthe-R	ACGGGGCTTTTCTTTTGTGTG GCTAGGGTCGTCCATGAAGT	59.86 59.18	47.6 55.0	81

Oligonucleotide primers were synthesized by Eurofins Genomics (Ebersberg, Germany), and fluorescence signals were monitored using the integrated optical detection system of a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). The detailed characteristics of the optimized species-specific primer pairs, including their sequences, melting temperatures (Tm), GC contents, and generated amplicon sizes, are comprehensively summarized in Table 2.

3.6. Bacterial Strains, Culture Conditions, and Specificity Validation

The bacterial strains used in this study (Table 1) were employed exclusively for the in vitro specificity validation of the newly designed primer pairs. The primary objective of these culture-dependent procedures was to verify that the primers targeting the *yjaE* gene (for *L. lactis* subsp. *lactis*) and the *ACRRRJ_03990* gene (for *S. thermophilus*) do not exhibit cross-reactivity with closely related taxa or other non-target lactic acid bacteria (LAB) commonly associated with the raw milk and cheese ecosystem.

Lactococcus lactis subsp. *lactis* reference strains, along with non-target lactococcal species and subspecies (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus garvieae*, and *Lactococcus raffinolactis*), were propagated in 10 mL of M17 broth (Merck KGaA, Darmstadt, Germany) and incubated statically at 30 °C for 24 h under aerobic conditions. *Streptococcus thermophilus* strains were cultured in 10 mL of M17 broth (Merck KGaA) and incubated statically at 42 °C for 24 h under microaerophilic conditions. Other non-target lactic acid bacteria were grown to the stationary phase under species-tailored conditions: *Lacticaseibacillus casei* strains were cultured in 10 mL of de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA) and incubated at 37 °C for 48 h under anaerobic conditions (GasPak EZ Anaerobe Container System, Becton, Dickinson and Company, Sparks, MD, USA); *Leuconostoc mesenteroides* strains were cultured in 10 mL of MRS broth (Merck KGaA) at 30 °C for 24 h under microaerophilic conditions; and *Enterococcus* spp. (*Enterococcus faecalis* and *Enterococcus faecium*) were propagated in 10 mL of Brain Heart Infusion (BHI) broth (Merck KGaA) at 37 °C for 24 h under aerobic conditions.

Bacterial cell growth was monitored by measuring the optical density at 650 nm (OD_{650}) using an Evolution 220 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Biomass from 2 mL of late exponential growth-phase cultures (OD_{650} = 0.7–0.8) was harvested by centrifugation at 3000× *g* for 5 min at 4 °C and stored at −20 °C until processing.

For the preparation of DNA templates for specificity validation, 1 mL of each enriched culture was centrifuged at 13,000× *g* for 5 min. The culture supernatant was discarded, and

the cell pellet was resuspended in 200 µL of Fast Lysis Buffer (Syngen Biotech, Wrocław, Poland). Thermal cell lysis was carried out in a thermo-shaker (800 rpm) at 100 °C for 10 min, followed by cooling to room temperature for 2 min. After a final centrifugation step at 13,000× *g* for 5 min to sediment cellular debris, the clarified supernatant was spectrophotometrically checked and adjusted to approximately 5 ng/µL, and 2 µL (~10 ng of genomic DNA) was used as the template for the qPCR specificity assays.

The absence of non-specific amplification and confirmation of species-specific detection were evaluated based on quantification cycle (Cq) values (with Cq > 40 classified as not detected, nd; Table 1) together with post-amplification melting curve profiles to verify amplicon identity.

3.7. Construction of Standard Curves and Analytical Performance Validation

To quantify target gene copy numbers within the cheese DNA extracts, calibration curves were constructed using recombinant plasmid standards containing the target sequences from *Lactococcus lactis* subsp. *lactis* IL1403 (*yjaE*, GenBank: CP033607.1, locus tag EFV54_04745, Protein ID: AYU52619.1) and *Streptococcus thermophilus* UCCSt50 (*ACRRRJ_03990*, GenBank: CP194174.1, Protein ID: YAB83324.1). Targeting single-copy chromosomal loci rather than multi-copy rRNA operons ensured a 1:1 correspondence between target gene copies and bacterial chromosome equivalents.

The target dsDNA fragments were custom-synthesized, cloned into a standard pEX-A128 plasmid vector (~2.45 kb backbone), sequence-verified, and supplied in lyophilized form by Eurofins Genomics (Ebersberg, Germany). Following the manufacturer's certificate of analysis, the exact molecular weight of each complete recombinant plasmid construct (vector backbone plus target insert) was used to reconstitute the lyophilized DNA in sterile TE buffer (Tris-EDTA, pH 8.0) to a standardized master stock concentration of 1.0×10^9 gene copies/µL. Stock concentrations were verified spectrophotometrically at 260 nm (Evolution 220, Thermo Fisher Scientific, Waltham, MA, USA), and plasmid constructs were enzymatically linearized outside the target amplicon sequence prior to dilution to prevent supercoiling bias during amplification.

From the master stocks (10^9 copies/µL), serial dilutions were freshly prepared in sterile DNase-free water, covering a dynamic range of 10^7 to 10^1 target gene copies per reaction well (using 10 µL of template per reaction). Standard curves were constructed by plotting Cq values against the decimal logarithm ($\log_{10}$) of initial target gene copy numbers per reaction well, and amplification efficiencies (Eff., %) were automatically generated by the Stratagene Mx3005P system software. The resulting calibration curves showed strong linearity (R^2 ranging from 0.995 to 1.000) and amplification efficiencies between 89.9% and 105.8%. Replicate analysis of low-copy standards (10^2, 10^1, and 5 copies per reaction; six replicates each) was used to determine the formal limit of detection (LOD, defined as the lowest copy number detected in ≥95% of replicates with the characteristic amplicon melting peak) and limit of quantification (LOQ, defined as the lowest copy number within the linear range quantified with a replicate coefficient of variation CV ≤ 25%).

3.8. Culture-Dependent Enumeration of Lactic Acid Bacteria

To evaluate and compare the quantitative performance of the targeted qPCR assays against conventional microbiological methods, classical culture-dependent enumerations were performed concurrently at each specified ripening interval (days 0, 30, 60, 90, 120, and 180) across all three independent cheese batches. For each batch and time point, three independent 10 g analytical test portions of the homogenized composite cheese sample were aseptically transferred into sterile stomacher bags, diluted 1:10 (*w*/*v*) with sterile

physiological saline solution (0.85% NaCl), and homogenized for 2 min using a laboratory paddle blender (Stomacher 400 Circulator, Seward, Worthing, UK).

Subsequent decimal dilutions were prepared in sterile saline solution. From the primary suspension (10^{-1}) and subsequent decimal dilutions, 0.1 mL aliquots were surface-plated in duplicate on elective M17 agar (Merck KGaA, Darmstadt, Germany) supplemented with 0.5% (*w*/*v*) lactose. Additionally, to achieve a formal plating limit of detection (LOD) of 10 CFU/g (1.0 $\log_{10}$ CFU/g) from the 1:10 (10^{-1}) homogenate in accordance with standard microbiological guidelines (ISO 7218) [33], a total volume of 1.0 mL of the 10^{-1} suspension per analytical test portion was surface-plated by distributing equal aliquots across three M17 agar plates (approximately 0.33 mL per plate). For presumptive mesophilic lactococci (*Lactococcus* spp.), plates were incubated aerobically at 30 °C for 48–72 h. For presumptive thermophilic streptococci (*Streptococcus* spp.), M17 agar plates supplemented with 0.5% (*w*/*v*) lactose were incubated under microaerophilic conditions (GasPak™ EZ Campy Container System, Becton Dickinson, Franklin Lakes, NJ, USA) at 42 °C for 48 h.

Following incubation, plates yielding between 30 and 300 colonies were preferentially selected for standard enumeration. In late-ripening samples with low culturable cell densities where even the lowest dilution yielded fewer than 30 colonies per plate (such as presumptive *Lactococcus* spp. at day 90), visible colonies above the detection threshold (≥1 colony per 1.0 mL of the 10^{-1} homogenate, corresponding to ≥1.0 $\log_{10}$ CFU/g) were counted and calculated as low-level estimated counts in accordance with ISO 7218, whereas the absence of colonies across the 1.0 mL plated 10^{-1} homogenate was recorded as below the limit of detection (<1.0 $\log_{10}$ CFU/g). Results were converted and expressed as $\log_{10}$ CFU/g of cheese. Notably, this conventional methodology quantifies broader presumptive coccoid groups (*Lactococcus* spp. and *Streptococcus* spp.) based on morphological and thermal growth characteristics, lacking the genetic resolution required to differentiate specific subspecies (e.g., *L. lactis* subsp. *lactis* vs. *L. lactis* subsp. *cremoris*) within the cheese matrix.

3.9. Real-Time PCR Conditions, Inhibition Control, and Data Processing

Quantitative real-time PCR assays were performed using the Quantum EvaGreen PCR Kit (Syngen Biotech, Wrocław, Poland) in a total reaction volume of 20 µL on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). At each of the six sampling intervals (days 0, 30, 60, 90, 120, and 180), genomic DNA extracted from each of the three independent cheese batches was analyzed in technical triplicate. Each 20 µL reaction mixture contained 4 µL of 5× Quantum EvaGreen Mix, 0.5 µL of forward primer (10 µM; final concentration 0.25 µM), 0.5 µL of reverse primer (10 µM; final concentration 0.25 µM), 5 µL of PCR-grade water, and 10 µL of purified DNA template. Every qPCR plate included calibration standards in triplicate and no-template controls (NTC, containing 10 µL of PCR-grade water instead of DNA) in triplicate to confirm the absence of reagent contamination or primer-dimer artifacts.

The thermal cycling program comprised an initial hot-start DNA polymerase activation at 95 °C for 15 min, followed by 40 amplification cycles of denaturation at 94 °C for 30 s, primer annealing and fluorescence acquisition at 60 °C for 30 s, and elongation at 72 °C for 30 s. Immediately following amplification, a continuous melting curve (dissociation) analysis was recorded by heating the reaction products from 65 °C to 95 °C (ramp rate 0.2 °C/s) to verify that fluorescence signals originated from a single, specific amplicon with the expected melting temperature (T_m).

To verify that residual cheese matrix components did not inhibit polymerase amplification when quantifying unknown samples against plasmid standards, PCR inhibition was monitored using two approaches, as described in our previous work [28]: (i) spiking representative cheese DNA extracts across ripening stages with a known copy number

(10^4 copies) of linearized plasmid standard as an internal positive control (IPC) and confirming that Cq shifts relative to spiked water controls remained <0.3 cycles; and (ii) evaluating individual amplification plot slopes and dilution linearity. No evidence of post-extraction PCR inhibition was detected in the purified cheese extracts. However, because spiking was performed after DNA purification rather than prior to cell lysis, this control verified amplification efficiency rather than pre-extraction DNA recovery across ripening stages.

Quantification cycle (Cq) values were converted into target gene copy numbers per reaction well using the corresponding standard curve. Because single-copy chromosomal genes were targeted (*yjaE* and *ACRRRJ_03990*), the measured copy numbers represent intact target gene copies (genome equivalents) recovered from the cheese matrix. Target gene abundance per gram of cheese (gene copies/g) was calculated according to Equation (1):

$$\text{Gene copies/g} = \left[\frac{\text{Copies per well}}{\text{Template volume in well } (\mu\text{L})} \times 100\ \mu\text{L} \right] \times 5 \quad (1)$$

where *Copies per well* represents the target gene copy number determined in the reaction well from the standard curve; Template volume in well is the volume of purified DNA extract added to each reaction (10 µL); 100 µL corresponds to the total elution volume of isolated genomic DNA; and 5 is the sample mass normalization factor derived from the ratio of 1 g of cheese to the initial sample mass used for extraction (200 mg = 0.2 g; 1.0 g/0.2 g = 5). Quantitative data were subsequently log-transformed and expressed as $\log_{10}$ gene copies/g of cheese.

3.10. Statistical Analysis

The experimental design followed a hierarchical structure across three independently manufactured cheese batches ($n = 3$ true biological replicates) sampled longitudinally at six ripening intervals (days 0, 30, 60, 90, 120, and 180). Each DNA extract was amplified in technical triplicate by qPCR. In the calibration curve plots (Figures 1–4), replicate measurements were averaged to display the mean initial target copy number per reaction well for each individual cheese batch (Cheese 1, Cheese 2, and Cheese 3).

All quantitative data were converted to $\log_{10}$ gene copies/g (for qPCR) or $\log_{10}$ CFU/g (for plate counts), and data normality was verified using the Shapiro–Wilk test. For the overall longitudinal evaluation across the study (Figures 6 and 7), technical replicates and within-batch analytical subsamples were first averaged within each batch at each time point to avoid pseudoreplication, so that the three independent cheese batches ($n = 3$) served as the experimental units (data expressed as the grand mean of three batches ± between-batch SD). Longitudinal changes across the repeated maturation time points were evaluated separately for each quantitative trajectory (qPCR and culture-dependent enumeration) using a one-way repeated-measures analysis of variance (RM-ANOVA) with cheese batch ($n = 3$) treated as the repeated subject factor and ripening time as the within-subject factor. Because Mauchly's test of sphericity cannot be computed when the number of repeated time points exceeds the number of biological replicates, degrees of freedom for within-subject effects were adjusted directly using the Greenhouse–Geisser epsilon correction, and pairwise comparisons across ripening intervals were performed using the Bonferroni post hoc procedure for repeated measures.

Culture-dependent plate counts falling below the limit of detection (<1.0 $\log_{10}$ CFU/g; days 120–180 for presumptive Lactococcus spp. and days 90–180 for presumptive Streptococcus spp.) were treated as left-censored observations (<LOD), excluded from inferential RM-ANOVA testing (which was restricted to the quantified intervals: days 0–90 for Lactococcus spp. and days 0–60 for Streptococcus spp.), and plotted at the LOD threshold with an explicit "< LOD" marker. Because single-copy gene qPCR ($\log_{10}$ gene copies/g)

and elective M17 agar plating ($\log_{10}$ CFU/g) measure different physical quantities and encompass different taxonomic scopes, method-to-method divergence across ripening was evaluated descriptively.

Statistical significance was established at $p < 0.05$. All statistical analyses were conducted using IBM SPSS Statistics software (Version 26.0, IBM Corp., Armonk, NY, USA).

4. Conclusions

In this work, we expanded our earlier molecular approach for studying raw-milk cheese microbiota by validating EvaGreen qPCR assays based on single-copy chromosomal targets (yjaE for *L. lactis* subsp. *lactis* and ACRRRJ_03990 for *S. thermophilus*) and comparing their performance against routine M17 agar plating over 180 days of ripening. Using single-copy loci avoided the copy-number ambiguity associated with rRNA genes and allowed reproducible tracking of species- and subspecies-specific DNA in the cheese matrix.

Over the six-month maturation period, the two bacteria showed distinct DNA abundance profiles: *S. thermophilus* gene copy numbers rose until day 90 and dropped sharply thereafter, whereas *L. lactis* subsp. *lactis* copy numbers increased steadily until day 180. While enumerable colonies on elective M17 agar were readily recovered from the fresh curd at day 0, culture-based counts diverged markedly from qPCR target DNA trajectories as ripening progressed, disappearing after day 60 for presumptive thermophilic streptococci and after day 90 for presumptive mesophilic lactococci. While standard DNA-qPCR cannot by itself determine whether the remaining non-culturable DNA pool originates from metabolically active VBNC cells, intact dead cells, or persistent matrix-bound DNA—a distinction that requires viability-PCR (e.g., PMA-qPCR) or RNA-level assays—our data clearly show that conventional agar plating overlooks substantial persistence of indigenous LAB target DNA during cold ripening. Combining single-copy gene qPCR with classical microbiology therefore offers a more complete picture of microbial target DNA dynamics alongside culturability changes in traditional long-ripened cheeses.

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Abbreviations

ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
BLASTn	Basic Local Alignment Search Tool for Nucleotides

CFU	Colony-Forming Units
Cq	Quantification Cycle
CTAB	Cetyltrimethylammonium Bromide
CV	Coefficient of Variation
dsDNA	Double-Stranded Deoxyribonucleic Acid
EMA	Ethidium Monoazide
GSMer	Genome-Specific Marker
IPC	Internal Positive Control
LAB	Lactic Acid Bacteria
LOD	Limit of Detection
LOQ	Limit of Quantification
MRS	de Man, Rogosa and Sharpe
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
NTC	No-Template Control
OD	Optical Density
PMA	Propidium Monoazide
PVP40	Polyvinylpyrrolidone 40
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RM-ANOVA	Repeated-Measures Analysis of Variance
rRNA	Ribosomal Ribonucleic Acid
RT-qPCR	Reverse-Transcription Quantitative Real-Time PCR
SD	Standard Deviation
TE	Tris-EDTA
Tm	Melting Temperature
VBNC	Viable But Non-Culturable
VOCs	Volatile Organic Compounds

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