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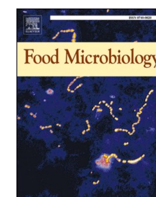

Development of a new prototypal natural whey starter production system to study biodiversity and technological features fluctuations during back-slopping practice used in Parmigiano Reggiano PDO cheese production: penicillin G as a case study

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ABSTRACT

Natural whey starter (NWS) is an undefined and variable microbial community of starter lactic acid bacteria (SLAB), traditionally produced by back-slopping and used in the manufacture of several hard-cooked Italian cheeses, including Parmigiano Reggiano PDO. Despite its importance, the parameters influencing the microbial composition and technological performance of NWS remain poorly investigated, largely due to the lack of controlled studies that do not interfere with actual cheese production. Although the Parmigiano Reggiano PDO regulations strictly forbid the use of selected starters and require NWS to be obtained exclusively through spontaneous whey fermentation following curd extraction, we developed a freeze-dried prototypical natural starter culture (NSC) system that simulates, under laboratory conditions, the back-slopping process used in Parmigiano Reggiano dairies to ferment sweet whey into NWS. This system (referred to as NSC_0) effectively replicated fresh NWS and was successfully propagated through three consecutive rounds of laboratory-scale cheese-making. Using this validated system, we demonstrated that raw cow milk spiked with penicillin G at or below the maximum residue limits (MRL) negatively impacted NSC fermentative performance during the first round of caseification and significantly reduced microbial counts during the second round, suggesting a cumulative inhibitory effect of penicillin G, non-observable in a single back-slopping cycle. As SLAB declined, spoilage yeasts increased. qPCR analysis revealed that 2 and 4 ppb penicillin G impaired NWS biodiversity by inhibiting *Lactobacillus delbrueckii* and streptococci, whereas *Lactobacillus helveticus* remained unaffected. The validated NSC system developed in this study offers a robust platform for future investigations into the abiotic and biotic factors affecting NWS, advancing our ability to predict the behavior of undefined starter cultures under realistic cheese-making conditions.

1. Introduction

Natural whey starter (NWS) is a thermophilic, undefined bacterial community used in the production of Parmigiano Reggiano (PR) PDO cheese through acid–rennet coagulation of partially skimmed, unpasteurized cow's milk obtained from animals fed a silage-free diet. Beyond lactose fermentation to lactate, various metabolic reactions associated with microbial growth and autolysis within NWS contribute to the development of the characteristic flavor and texture of PR cheese (Klaenhammer et al., 2005; Dea Lindner et al., 2008; Gatti et al., 2008; Bottari et al., 2010; Bertani et al., 2020).

While the usage of lyophilized NWS is permitted in the production of other hard-cooked cheeses, PR-NWS must be freshly produced daily by a back-slopping process. This involves fermenting the sweet whey (SW) of

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the previous cheese-making batch, following curd cooking and syneresis. Within each dairy in the PR production area, SW is incubated overnight in bioreactors under a gradually decreasing temperature regime, which promotes the development of a complex community enriched in thermophilic, homofermentative starter lactic acid bacteria (SLAB), such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Streptococcus thermophilus* (Bertani et al., 2020).

NWS composition and technological performance vary over time and between dairies. Recently, Sola et al. (2022) distinguished PR-NWS types enriched in *Lactobacillus delbrueckii* subsp. *lactis* and *Streptococcus thermophilus* (referred to as type-D) from those dominated exclusively by *L. helveticus* (referred to as type-H). Seasonal variations in cow feed and milk quality, differences in the cooling rate during fermentation, and the type of bioreactor used have been empirically associated with fluctuations in the quality and microbial composition of NWS. However, the inherent variability of the back-slopping process under real production conditions limits the ability to investigate NWS dynamics in controlled and reproducible environments without interfering with PR cheese production. Consequently, systematic data on the intrinsic and extrinsic factors shaping NWS biodiversity remain largely unavailable.

Among factors influencing NWS communities, is the presence of antibiotic residues in raw milk. The growing global demand for animal-derived food has promoted the widespread use of antibiotics in livestock production systems (Aminzare et al., 2024). In dairy cattle, the most used antibiotics, such as penicillin G (PEN G), macrolides, aminoglycosides, sulfonamides, and tetracyclines, are primarily administered to treat mastitis (Chiesa et al., 2020; Xu et al., 2022). Since antibiotics can move from the mammalian glands into milk, treated cows were submitted to a "withdrawal period", defined as the minimum time that must elapse between the last administration of a veterinary drug and the use of the animal or its products for human consumption (European Council, 2019). The European Union has defined both withdrawal periods and maximum residue limits (MRLs) for any pharmacologically active substances in milk and animal-origin foods (European Council, 2010).

A recent EU report demonstrated that raw milk is more frequently contaminated with veterinary antibiotic residues than previously expected (EFSA, 2019), posing potential risks not only to consumer safety but also to the technological performance of dairy processing (Li et al., 2019; Virto et al., 2022; Climova et al., 2024). Several studies have documented the risks associated with the spread of antibiotic resistance through the food chain (Bahmani et al., 2020; Founou et al., 2016; Khalifa et al., 2024). In addition, antibiotic residues can interfere with fermentation processes and alter the organoleptic features of dairy products, resulting in economic losses for the dairy industry (Quintanilla et al., 2019; Beltrán et al., 2023). Research investigating the effect of antibiotics on the acidification activity of dairy starters has generally followed two main approaches. One set of studies has evaluated the effect of antibiotics intentionally added to raw milk on the viability and acidification capacity of individual SLAB strains (Berruga et al., 2008; Chiesa et al., 2020; Navrátilova et al., 2022). Other studies have investigated the behavior of LAB cultures inoculated into milk samples obtained from cows previously treated with antibiotics after the mandatory withdrawal period (Beltrán et al., 2023; Morandi et al., 2024). In the latter case, the residual antibiotic activity is often due not only to the parent compound but also to its metabolites formed in vivo. Findings from both approaches suggest that although MRLs for antimicrobials are essential to ensure the safety of foods of animal origin, they may not be sufficient to guarantee the technological suitability of raw milk for dairy fermentation. For instance, Paba et al. (2019) demonstrated that oxytetracycline added to ovine milk at its MRL of 1 ppb significantly impaired the acidification ability of thermophilic SLAB and negatively affected the growth of *S. thermophilus* and *L. helveticus*, while *L. delbrueckii* subsp. *lactis* remained unaffected.

Gamba et al. (2020) were the first to raise concerns about whether

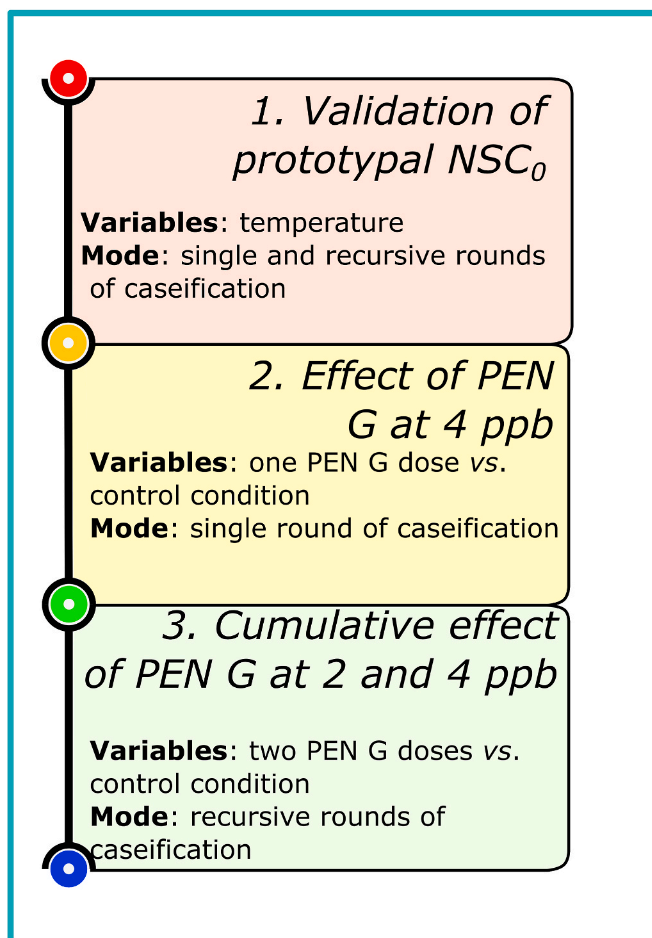


Fig. 1. Experimental plan used in this study. Abbreviations: NSC, natural starter culture; PEN G, penicillin G.

antibiotic residues at MRL in unpasteurized cow milk can impair the acidifying activity of fresh NWS used in the production of Grana Padano, another Italian hard-cooked cheese made with NWS. The reduced acidification ability of the resulting NWS, coupled with the low quality of the milk, has been associated with the development for late blowing defects in ripened cheese (Carminati et al., 2023). More recently, Morandi et al. (2024) have proved that lactams and sulfonamides, even at concentrations below the MRL, inhibit acidification curves of axenic cultures of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and, to a lesser extent, *L. helveticus.* However, no studies to date have examined the effects of repeated exposure to sub-MRL levels of antibiotics on the technological properties and microbial composition of NWS consortia during back-slopping.

In this study, we developed a prototypical natural starter culture (NSC) production system that simulates the back-slopping procedure of PR-NWS at laboratory–pilot scale. This system was then used to investigate the effects of penicillin G (PEN G), at both the MRL (4 ppb) and sub-MRL (2 ppb) concentrations, on the technological performance and microbial community structure of PR-NWS.

2. Materials and methods

2.1. Experimental plan

This study comprised three experimental phases (Fig. 1). In the first step, we validated the *in vitro* prototypal system for replicating PR-NWS under controlled conditions and applied it in a pilot scale cheese-making trials. In the second step, the validated prototypal NSC system was used

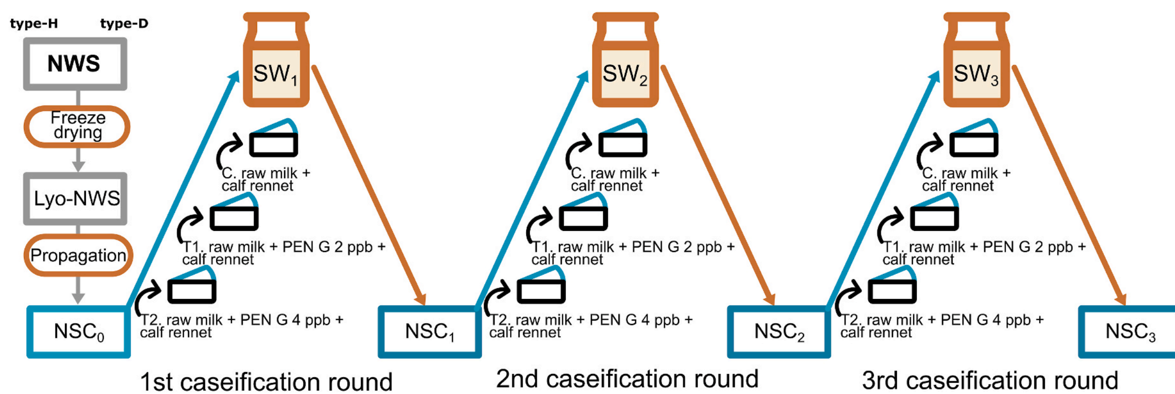


Fig. 2. Workflow for the recursive propagation of prototypical natural starter culture (NSC) system. Blue arrows indicate caseification steps, while orange arrows indicate fermentation of sweet whey (SW_x) into the corresponding NSC_x. "X" represents round of caseification. "C" refers the control condition (raw milk plus calf rennet), while "T1" and "T2" indicate the treatments involving raw milk with calf rennet supplemented with 2 ppb and 4 ppb PEN G, respectively. Abbreviations: NWS, natural whey starter; Lyo-NWS, freeze-dried natural whey starter; NSC, natural starter culture; SW, sweet whey; PEN G, penicillin G. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Main abbreviations used in this study.

Abbreviation	Description
NWS	Fresh natural whey starter collected in Parmigiano Reggiano PDO cheese dairy farm
Lyo_NWS	Freeze-dried powder obtained from NWS by freeze-drying
SW_x	Sweet whey collected at *x* round of caseification
NSC_x	Natural starter culture produced at laboratory scale after *x* round of SW fermentation.

to perform cheese-making trials with raw milk spiked with PEN G at 4 ppb. In the third step, we assessed the cumulative effect of PEN G at 2 and 4 ppb on NWS quality over three successive rounds of caseification. The workflow for the recursive propagation of prototypal NSC was described in Fig. 2. A list of abbreviations used in this work is provided in Table 1.

2.2. Materials, samples, and reference strains

All media and anaerobic systems were purchased from Oxoid (Basingstoke, Hampshire, United Kingdom), while chemicals from Sigma-Aldrich (St. Louis, MO, United States), respectively, except where differently indicated. Molecular biology reagents were purchased from Thermo Fisher Scientific (Waltham, MA, United States). Penicillin sodium salt was from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Oligonucleotides were provided by BMR Genomics (Padua, Italy).

Two fresh PR-NWS samples, namely C4 and C9, were collected from two PR-producing farms belonging to the consortium of PR PDO cheese production and used as initial stocks. These NWS represented PR-NWS type-D and type-H, respectively, according to Sola et al. (2022). They were submitted to lyophilization by Alce srl (Novara, Italia), a process in which the product is frozen and placed under a vacuum to remove about 99 % of water through sublimation, resulting in Lyo_NWS samples. The lyophilization process was not part of the research study. This activity was done by a commercial firm that carried out the lyophilization with its own internal protocol. The freeze-dried stocks were properly aliquoted and stored at −20 °C for the duration of the experiments.

The type strains used in this study were the following: *Lactobacillus helveticus* DSM20075^T, *Streptococcus salivarius* subsp. *Tthermophilus* DSM20617^T, and *Lactobacillus delbrueckii* subsp. *lactis* DSM20072^T. All the strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Deutschland) and cultivated according to DSMZ culture condition specifications.

2.3. Propagation of freeze-dried PR-NWS under laboratory conditions

Lyo_NWS samples were reconstituted using whey powder as revitalizing substrate. In detail, whey powder (fat 1.1 % (w/w), carbohydrates 75 % (w/w), proteins 11 % (w/w), pH 6.6; Reire, Reggio Emilia, Italy) was dissolved to the final concentration of 60 g/L (corresponding to a final lactose percentage of app. 5 % w/v) in sterile mineral H_2O previously brought to boiling point. Rehydrated whey was cooling down to 38 °C before adding lyo_NWS under sterile conditions at the dosage of 5 g/L, respectively. NSC propagation was carried out using the following conditions: 1) variable thermic curve (VT) consisting in 1 h at 48 °C, followed by 3 h at 46 °C, 3 h at 44 °C, 3 h at 42 °C, and gradual cooling down until the room temperature for the final 11h (25 °C ± 3 °C); 2) constant temperature of 42 °C for 21 h (CT42); 3) constant temperature of 45 °C for 16 h (CT45). Temperature and pH were continuously monitored by a multi-parameter meter (CyberScan PC 650, Eutech Instruments, Thermo Fisher Scientific, Milan, Italy). After incubation, the resulting metabolically active microbial community, referred to as NSC_0, was submitted to microbiological, technological, and molecular characterization as reported below.

2.4. Prototypal cheese-making procedure

The cheese-making process was performed at laboratory-scale as previously reported (Bortolazzo et al., 2014; Franceschi et al., 2022), with minor modifications. In details, cheese production was carried out in 4 vats (Elettronica Veneta, Italy), each containing approximately 7 L of fresh raw cow's milk from a dairy farm belonging to the PR PDO consortium. The volume of milk in each trial was registered and used to calculate the standard cheese yield. When required, fresh milk was spiked with PEN G to achieve the final concentrations of 2 and 4 ppb, respectively. Prior to inoculation with the NSC, milk baches were pre-heated to 30 °C. NSC was added to each batch to reach a titratable acidity of 4.4 °SH/50 mL (approximately corresponding to pH 6.44), according to the following equation:

$$W[NSC] = W[milk]*\frac{(4.4 - TA\ [milk])}{(TA\ [NSC] - 4.4)} \quad 3.1$$

Where: W, W: weight (g), TA, titratable acidity (°SH/50 mL), NSC, natural starter culture.

The temperature was maintained at 30 °C for 20 min, then increased to 34 °C. After that, milk was clotted with calf rennet (1000 IMCU - International Milking Clotting Unit, Caglificio Clerici, Italy) added to the final concentration of 40 IMCU/kg milk. Coagulation and curd firming occurred in about 12 min. The optimal time for cutting the curd was

determined using an Optigraph according to manufacturer's instructions (AMS, Frépillon, France). The Optigraph is an analytical instrument designed to measure the absorbance of a near-infrared light beam passing through a milk sample. As coagulation progresses, the aggregation of casein micelles increases the opacity of the sample, resulting in a measurable change in absorbance. The Optigraph records this change as a signal, expressed in volts (V), which is directly proportional to the consistency of the curd. The system also calculates the first derivative of the signal curve, providing real-time insights into the rate of coagulation. This dual-curve output enables accurate identification of the inflection point, which corresponds to the moment the curd reaches the desired firmness. Thus, the Optigraph allows for precise determination of the optimal time to cut the curd in the vat, ensuring consistent curd structure and improved process control during cheesemaking. The curd was then broken into small granules of about 0.5 cm, that were immediately cooked by increasing the temperature to 55 °C for 12 min with continuous agitation. When the temperature reached 55 °C the agitation stopped maintaining the temperature stable. At this moment two portions of SW were collected in sterilized bottles which had undergone temperature-controlled fermentation to produce NSC. Subsequently, the curd particles were left to settle naturally on the bottom of the vat for about 1 h, at constant temperature and, then, the curd was extracted. Temperature and pH were continuously monitored as reported above.

Standard cheese yield (SCY) was calculated as follows:

$$SCY = \left(\frac{CW}{MW}\right) * \left(\frac{DM}{60}\right) * 100 \qquad 3.2$$

where: SCY is the standardized cheese yield at 60 % of dry matter, CW is the cheese weight (expressed in g), MW is the milk weight (expressed in g), and DM is the cheese dry matter (expressed in g/100 g).

2.5. Temperature-controlled SW fermentation

For each cheese-making round (x), SW samples from experimental dairy making were incubated in sterilized bottles in a programmable heating-cooling thermostat (BFT5, Huber Kältemaschinenbau AG, Offenburg, Germany) by progressively cooling down the temperature as follows: 1 h at 48 °C, 6 h at 46 °C, the remaining 14 h at 42 °C from the one derived from NWS type-H. For NWS type-D the cooling curve was as follows: 46 °C for 4h, 43 °C for 4 h, and 38 °C until use (about 14 h). In step 2 of the experimental plan, the protocol was repeated for three consecutive rounds (Fig. 2). Each SW_X and the resulting starter culture (NSC_X) were subjected to technological and microbiological characterization, as detailed below. Temperature and pH were monitored as reported above.

2.6. Technological analyses

NWS, SW, and NSC samples were analyzed for pH and titratable acidity (TA; recorded in Soxhlet-Henkel degrees as °SH/50 mL), as previously reported (Sola et al., 2022). The fermentative activity (FA) of NSC samples was determined by mixing 3 % of microbial starter to a commercial UHT semi-skimmed milk (fat 1.7 % (w/w), carbohydrates 4.5 % (w/w), proteins 3.5 % (w/w), pH 6.6), purchased in a local supermarket, preheated to 55 °C, and incubated at the same temperature for 4 h in a thermostatic bath. TA values were determined at the beginning (T_0) and the end of incubation (T_1). FA was calculated as follows:

$$FA = TA\left(\frac{^{\circ}SH}{50Ml}\right)T_1 - TA\left(\frac{^{\circ}SH}{50Ml}\right)T_0 \qquad 3.3$$

where: FA, fermentative acidity; TA, titratable acidity; SH, Soxhlet-Henkel.

2.7. Microbiological analyses

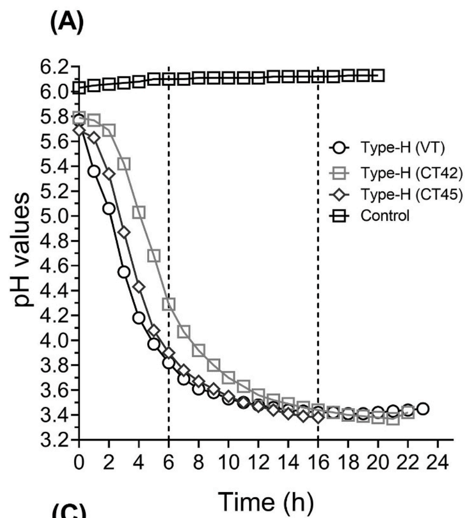
Total microbial counts (TMC) were determined with a Bürker haemocytometer (BT, Brand, Wertheim, Germany) using 10 μL of cell suspension prepared by triplicates as follows. One mL volume of sample was centrifuged at 8000 rpm for 10 min at 4 °C. After two washing steps in saline solution (9 g/L NaCl), cells were resuspended in 1 mL of saline solution. SW samples were counted without any dilution, whereas NSC samples were 1:10 diluted in saline solution prior to counting. Optical microscope (Nikon ECLIPSE 80i) equipped with 40× objective lens (400× final magnification) was used for TMC assay. All steps, with the exception of centrifugation, were carried out under a laminar-flow hood to prevent environmental contamination.

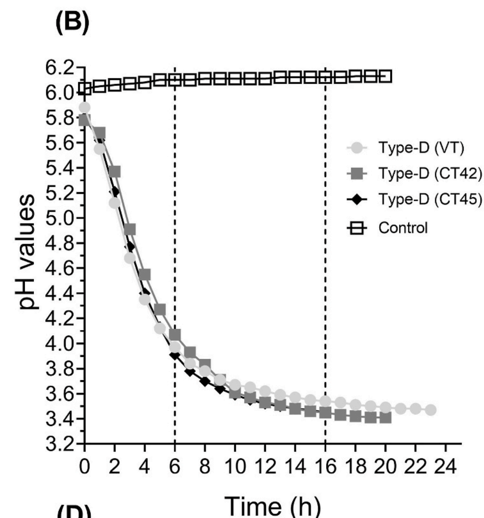
Microbial counts were determined by plating ten-folded diluted samples on MRS medium (pH 6.2 – pH 6.5) and MRS medium (brought to pH 5.4 with 1 N HCl) at 42 °C for 72 h under anaerobic conditions to estimate thermophilic lactobacilli; M17 medium supplemented with sterile skimmed whey (Reire, Reggio Emilia, Italy) (M17-SSW) at 42 °C for 72 h under aerobic conditions to estimate putative streptococci populations (Fornasari et al., 2006); YPDA medium supplemented with chloramphenicol (final concentration 50 μg/mL) at 26 °C for 48 h under aerobic conditions to determine generalist mesophilic yeast populations. MRS (pH 5.4), MRS (pH 6.5), and M17-SSW media were added with the antibiotic cycloheximide (final concentration 50 μg/mL) to inhibit yeasts. Viable cell counts were recorded as number of colony-forming units (CFU)/mL recovered from plates with CFU/mL ranging from 20 to 200 and expressed as Log_{10} CFU/mL.

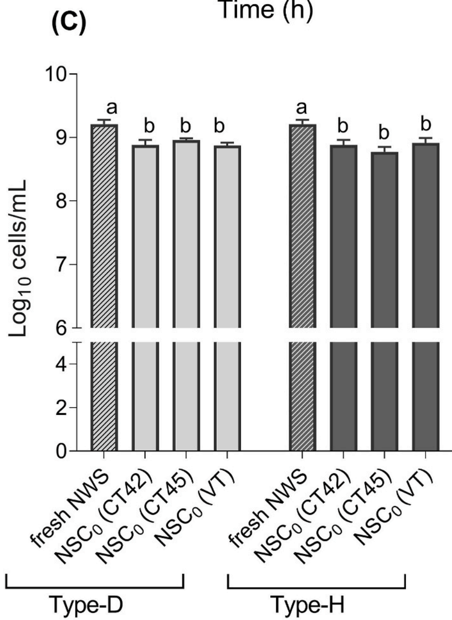
LIVE/DEAD BacLight Bacterial Viability Kit (cat.no. L7012; Thermo Fisher Scientific) was used to assess bacterial viability in SW and NSC samples according to manufacturer's instructions. Briefly, 500 μL of the cell suspension prepared as reported above for TMC determination were stained with SYTO 9 and propidium iodide (PI) dyes (75 μL each) in a 1:1 ratio. Samples were vortexed for 1 min at room temperature and incubated in the dark for 15 min at room temperature. Cell viability assay was performed using a fluorescence microscope (Nikon DIGITAL SIGHT DS-U1) equipped with a 40× objective lens and filters TRITC (Tetramethylrhodamine Isothiocyanate) and BV-2A for PI and SYTO 9 staining, respectively. Green fluorescence was observed with a 488 nm excitation filter and 500–550 nm emission filter for Syto 9. Red fluorescence was observed with a 488 nm excitation filter and 570–620 nm emission filter for PI. Green cells have been considered viable, while red cells unviable. Ten photos have been acquired and analyzed with ImageJ software (NIH, Bethesda, MD, USA). The results were the means of the resulting counts and expressed as % of viable cells.

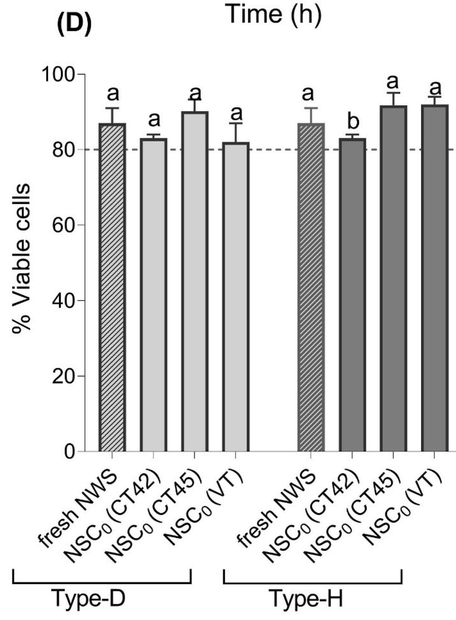
2.8. Species-specific qPCR assays

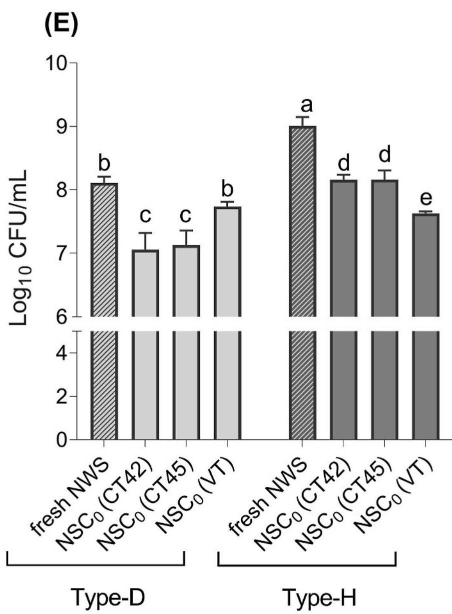
Total DNA was extracted from 1 mL of sample with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), according to manufacturer's specifications for Gram-positive microbes, and diluted in a final volume of 60 μL. Real Time quantitative PCR (qPCR) assays targeting the species *L. helveticus*, *L. delbrueckii* and *S. thermophilus*, respectively, were performed with SYBR green chemistry according to the absolute quantification method. Genomic DNA (gDNA) was extracted from 2 mL of late exponentially grown culture of each type strain, according to mechanical lysis and phenol:choloroform method described by Tagliazucchi et al. (2020). Primer pairs were designed based on the housekeeping gene *pheS*, according to Bottari et al. (2013). qPCR reactions were conducted with QuantStudio® 3 instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with the following thermal cycle: a first hold stage of 2 min at 50 °C followed by 2 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, during which fluorescence acquisition took place, and a final melting curve stage from 60 °C to 95 °C with a temperature gradient of 0.1 °C/s. For each primer pair, reaction mixture contained 10 μL of 2 × PowerUp SYBR Green Master Mix (Code: A25742, Thermo Fisher Scientific), forward and reverse primers at the











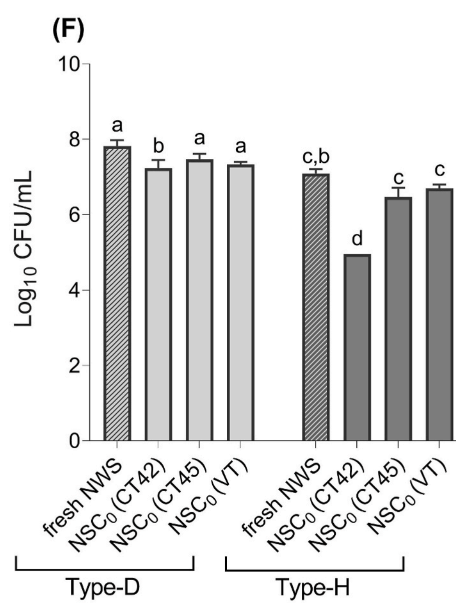


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Fig. 3. Effect of three different temperature regimes, namely 42 °C for 21 h (CT42), 45 °C for 16 h (CT45), and variable temperature (VT), on the development of prototypal type-D and type-H NSC_0. Each Lyo-NWS sample was added to sweet whey (60 g/L) at the final concentration of 5 g/L. Sweet whey without Lyo-NWS was used as negative control. (A) Acidification kinetics in type-H NSC_0. (B) Acidification kinetics in type-D NSC_0. (C) Total microbial cell (TMC) counts (expressed as Log_{10} cells/mL). (D) Viable cell percentages. (E) Lactobacilli counts scored on MRS pH 6.5 at 42 °C under anaerobiosis. (F) Streptococci counts scored on M17-SSW at 42 °C under aerobiosis. All the microbial counts were expressed as Log_{10} CFU/mL. All values are an average of at least three replicates and bars, when visible, indicate standard deviation. Different letters indicate significant differences ($p < 0.05$) (one-way ANOVA). Graphs were generated using GraphPad version 8 (San Diego, CA, USA). Abbreviations: CT, constant temperature; VT, variable temperature.

final concentration of 250 nM (each), 5 μL of template (5 ng/μL), and nuclease-free water up to the final volume of 20 μL per reaction. All the reactions were performed in triplicate, and no template controls were included in each experiment.

Standard curves were constructed using appropriately ten-fold diluted gDNAs to obtain from 10^8 to 10^3 total genome copies. For each species, the genome copy number was calculated according to Equation (3.4):

$$\textit{Genome copy number} = \frac{DNA\ amount\ (ng) \times Na}{N \times 660\left(\frac{g}{mol}\right) \times 10^9\ ng/g} \quad 3.4$$

where Na is the Avogadro's number (6.022×10^{23} molecules/mol), N is the length of dsDNA genome of each species, 660 g/mol is the average weight of a single base pair, and 10^9 is the conversion factor (Dhanasekaran et al., 2010).

The genome lengths were retrieved from NCBI database as follows: 2,020,582 for *L. helveticus* DSM 20075^T (GCF_000160855.1), 2,165,984 for *L. delbrueckii* subsp. *lactis* DSM70072^T (GCF_002278095.1), and 2,017,459 bp long for *S. thermophilus* DSM20617^T(GCF_019972875.1). The results were expressed as Log_{10}Genome copy number per mL of sample according to Ilha et al. (2015). Briefly, the microbial load was calculated as follows:

$$\text{Microbial load} = \frac{A \times B \times C}{D \times E} \quad 3.5$$

where: A is genome copy number per reaction well obtained from Ct of the DNA sample using standard curve (Ct *vs.* log genome copy number), B is the extracted DNA concentration (ng/μL), C is total volume of extracted DNA (μL), D is template DNA mass in reaction well (ng), and E is sample volume (mL) used for DNA extraction.

2.9. Statistical analysis

Statistical data are presented as mean ± standard deviation from at least three independent replicates. Statistical analyses and graph generation were performed using GraphPad Prism software, version 8.0.0 (GraphPad Software, La Jolla, CA, USA). Statistical differences among groups were assessed by using one-way analysis of variance (one-way ANOVA) followed by Tukey's post-hoc multiple comparisons test. Differences between treated samples and control were evaluated using Student *t*-test. Data from recursive rounds of caseification in the presence of 2 and 4 ppb PEN G were analyzed using two-way ANOVA, with antibiotic treatment and caseification rounds as independent factors. Statistical significance was defined as *p*-value <0.05.

3. Results

3.1. Validation of a laboratory scale method to rehydrate freeze-dried NWS

To identify valuable NWS cultures to be used as initial stock for all tests, two fresh NWS samples, previously described as type-H and type-D microbial consortia (Sola et al., 2022), were technologically and microbiologically characterized (Supplementary Table S1). Both NWS samples showed TMC values higher than 9 Log_{10} cells/mL and contained more than 80 % of cells viable. Microbiological analyses showed that

Table 2
Microbiological characterization of type-D and type-H NSC_0, SW_1 and NSC_1 samples. NSC_0 samples were obtained by propagating freeze-dried type-H or type-D NWS in sweet whey at a constant temperature of 45 °C for 16 h. Following one round of caseification and curd removal, SW_1 samples were fermented to produce the corresponding NSC_1 microbial communities. All cultivable microbial counts are expressed as Log_{10} CFU/mL. Values represent the mean of at least three replicates ± standard deviation. Statistical differences among samples for each parameter were assessed by two-way ANOVA ($p < 0.05$) and are indicated with different superscript letters.

Parameters	Type-H			Type-D		
	NSC_0	SW_1	NSC_1	NSC_0	SW_1	NSC_1
TMC (Log_{10} cells/mL)	9.08 ± 0.07^a	6.46 ± 0.03^d	8.76 ± 0.11^{ab}	8.96 ± 0.08^a	6.73 ± 0.02^c	8.78 ± 0.04^a
Viable cells (%)	92 ± 3.00^a	69 ± 19.00^a	95 ± 1.00^a	73 ± 11^a	87 ± 7^a	90 ± 10^a
Lactobacilli count (MRS, pH 6.5)	8.16 ± 0.08^b	5.76 ± 0.05^c	$8.35 \pm 0.15^{a,b}$	7.94 ± 0.11^b	5.38 ± 0.07^d	8.31 ± 0.01^a
Lactobacilli count (MRS, pH 5.4)	8.62 ± 0.13^a	6.49 ± 0.16^c	8.46 ± 0.2^a	7.76 ± 0.14^b	6.25 ± 0.08^c	8.72 ± 0.01^a
Streptococci count	6.49 ± 0.18^b	4.22 ± 0.21^d	6.61 ± 0.01^b	7.74 ± 0.14^a	5.7 ± 0.13^c	8.08 ± 0.05^a
Yeast count	0	0	0	0	0	1.6 ± 0.01

lactobacilli counts were higher in type-H than type-D samples, while streptococci and yeast counts were higher in type-D than type-H, consistent with previous findings (Sola et al., 2022) (Supplementary Table S1). Overall, the results indicated that the NWS samples were viable and representative of the type-H and type-D microbial consortia, respectively. Therefore, they were submitted to freeze-drying.

Preliminary lyo-NWS powders were revitalized under three different temperature regimes. One (VT) resembled the cooling curve typically used in PR dairy farms, while the other two consisted of stable temperature of 42 °C for 21 h (CT42) and 45 °C for 16 h (CT45), respectively. After 6 h of incubation, type-H CT45 sample exhibited pH value comprised between that of VT and CT42, whereas type-D CT45 sample reached a lower pH value compared to both VT and CT42 samples (Fig. 3A and B, respectively). NSC_0 samples were lower in TMC values compared to fresh NWS samples, regardless of the adopted temperature regime ($p < 0.05$) (Fig. 3C). In type-H NSC_0, viability percentages were comparable to the corresponding fresh NWS under both VT and CT45 conditions, while viability was lower under CT42 condition compared to VT and CT45 (Fig. 3D). In type-D NSC_0, viability percentages were comparable to the corresponding fresh NWS across all temperature regimes (Fig. 3D). All NSC_0 samples showed lower lactobacilli counts than their respective fresh NWS: in type-H NSC_0 lactobacilli counts were higher under CT42 and CT45 conditions than under VT, while in type-D NSC_0 VT performed better than CT42 and CT45 for this parameter (Fig. 3E). Streptococci counts decreased under CT42 compared to CT45 and VT in both type-H and type-D NSC_0 (Fig. 3F). The yeast population disappeared following freeze-drying in all samples, except for type-D NSC_0 under CT42 condition (data not shown). Based on pH values after 6 h of incubation, as well as viability and microbial counts of lactobacilli and streptococci, CT45 was identified as the optimal temperature regime for revitalizing both type-H and type-D lyo-NWS

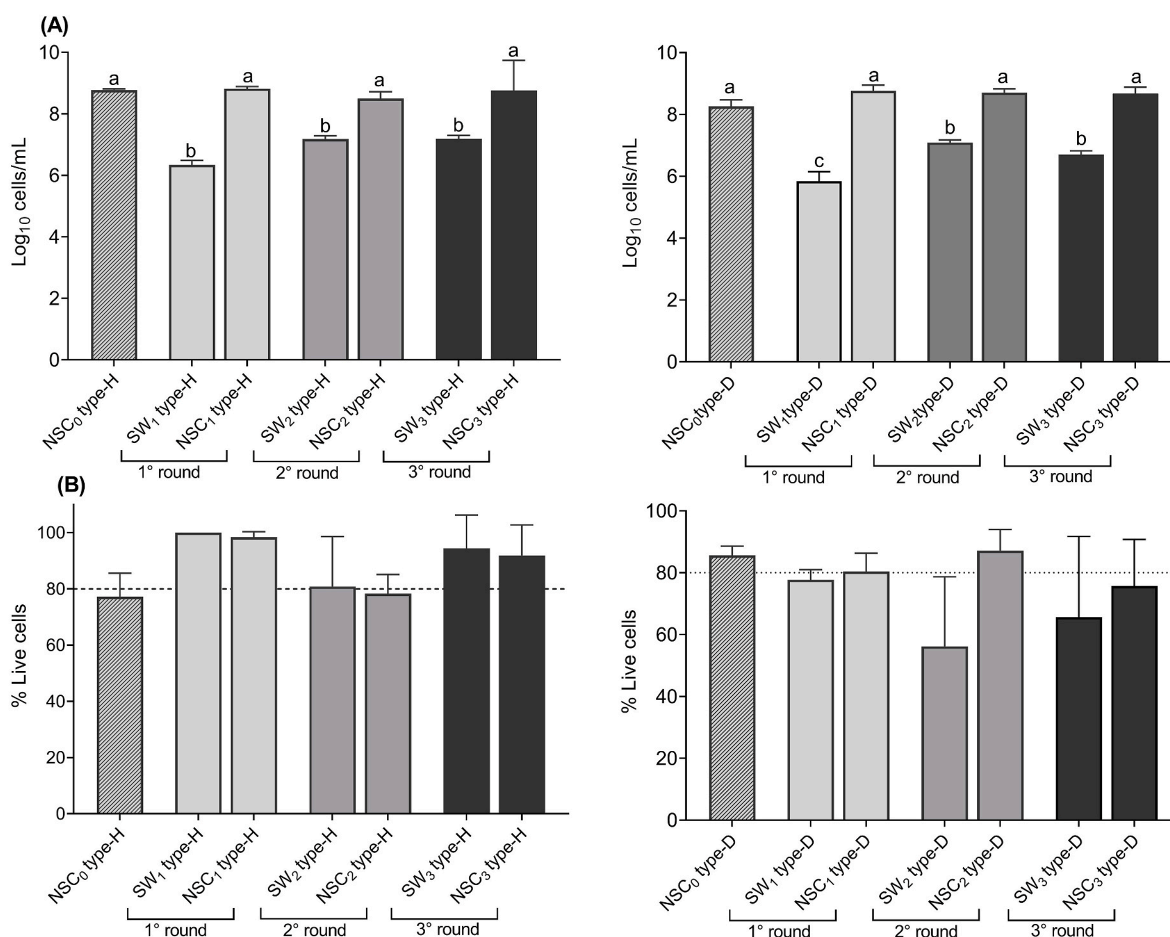

Fig. 4. Total microbial cells (TMC) (expressed as Log_{10} cells/mL) (A) and viability (%) (B) in type-H and type-D NSC communities obtained after three consecutive rounds of cheese-making. NSC_0 samples were obtained by propagating freeze-dried type-H or type-D NWS in sweet whey at a constant temperature of 45 °C for 16 h. Following each round of caseification, the resulting SW samples were fermented to produce the corresponding NSC. All values represent the mean of at least three replicates; error bars, when visible, indicate standard deviation. In panel B the dotted horizontal line represents the viability threshold of 80 %. For each variable, different letters indicate significant differences among samples ($p < 0.05$), as determined by one-way ANOVA. Graphs were created using GraphPad version 8 (San Diego, CA, USA).

powders into their corresponding NSC_0 viable microbial consortia.

3.2. SW fermentation into NSC under single and recursive cheese-making trials

Type-D and type-H NSC_0 microbial consortia were used as starters in a single cheese-making trial to simulate the PR cheese production at laboratory scale, according to Fig. 2. Addition of NSC_0 samples to milk resulted in two mixtures (vat milk + NSC_0) with pH 6.45 and TA of 4.3 °SH/50 mL for type-D and with pH 6.39 and TA value of 4.3 °SH/50 mL for type-H, respectively. SCY were 9.57 ± 0.01 kg cheese/100 kg milk and 9.58 ± 0.34 kg cheese/100 kg milk for type-D and type-H milk batches, respectively (Supplementary Table S2). After curd removal, the resulting two batches of SW samples (called type-D and type-H SW_1) exhibited pH values of 6.26 and 6.24, respectively. They were incubated under temperature-controlled conditions and, after 6 h of incubation, type-H SW_1 and type-D SW_1 exhibited a pH of 3.96 and 4.23, respectively (Supplementary Fig. S1). At the end of SW_1 fermentation, the resulting type-D and type-H NSC_1 exhibited FA values of 2.1 ± 0.28 and 1.6 ± 0.01 Δ°SH/50 mL, respectively (Supplementary Table S2).

As reported in Table 2, NSC_0 and NSC_1 exhibited comparable values across nearly all microbiological parameters in both type-H and type-D datasets. In contrast, the most significant pairwise differences were observed between NSC_0 and SW_1. As expected, curd cooking temperature (55 °C) significantly reduced TMC values and plate counts of at least 1.5 Log_{10} units in all SW samples ($p < 0.05$) (Table 2). In NSC_1 samples, cell viability remained above the 80 % threshold. Streptococci counts were higher in NSC_1 type-D than NSC_1 type-H, according to the previous classification (Sola et al., 2022). In the type-H batch, lactobacilli counts in NSC_1 were similar to that found in NSC_0, whereas in type-D batches NSC_1 showed significant higher lactobacilli counts than NSC_0 ($p < 0.05$). Overall, the results indicate that, in both type-H and type-D experiments, SW_1 fermentation led to the development of a viable NSC_1 microbial community that resembled, and in case of lactobacilli counts, even surpassed, that of the initial NSC_0.

Subsequently, the cheese-making process was repeated for three consecutive steps to mimic the back-slopping process in PR cheese production (Fig. 2). As result, 3 SW (namely SW_1, SW_2, and SW_3) were fermented into 3 NSC (namely NSC_1, NSC_2, and NSC_3) for both type-H and type-D NWS (Supplementary Fig. S2). TA values of NSC samples ranged from 21.57 ± 1.19 to 31.30 ± 0.56 in type-D samples and from 24.80 ± 1.13 to 34.53 ± 1.11 in type-H samples (Supplementary Table S3). FA values increased after the first round of caseification and remained higher than NSC_0 for the subsequent rounds (Supplementary Table S3). After each cheese-making cycle, both type-H and type-D NSC samples exhibited higher microbial counts compared to preceding SW samples and showed TMC values like those of NSC_0 (Fig. 4A). NSC samples exhibited a viability of approximately 80 %, with the exception of NSC_3 type-D, which showed lower value (75.66 % ± 15.10) (Fig. 4B). Microbial counts in NSC_1, NSC_2, and NSC_3 samples were generally

 Food Microbiology 133 (2026) 104861

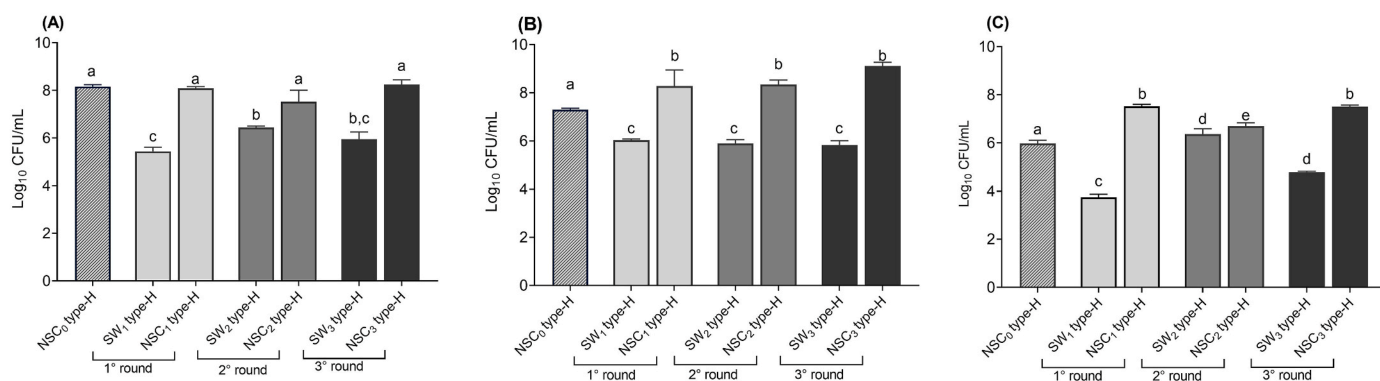

Fig. 5. Microbial counts (expressed as Log_{10} CFU/mL) in type-H NSC communities obtained after three consecutive rounds of cheese-making. NSC_0 samples were obtained by propagating freeze-dried type-H NWS in sweet whey at a constant temperature of 45 °C for 16 h. Following each round of caseification, the resulting SW samples were fermented to produce the corresponding NSC. (A) Lactobacilli counts scored on MRS pH 6.5 at 42 °C under anaerobiosis. (B) Lactobacilli counts scored on MRS pH 5.4 at 42 °C under anaerobiosis. (C) Streptococci counts scored on M17-SSW at 42 °C under aerobiosis. All values represent the mean of at least three replicates; error bars, when visible, indicate standard deviation. For each microbial count, different letters indicate significant differences among samples ($p < 0.05$), as determined by one-way ANOVA. Graphs were created using GraphPad version 8 (San Diego, CA, USA).

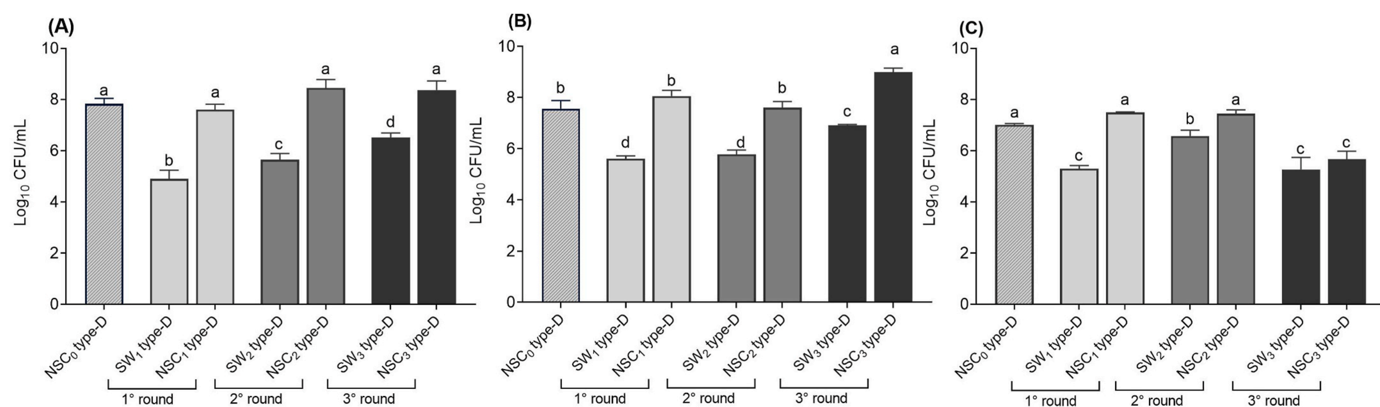

Fig. 6. Microbial counts (expressed as Log_{10} CFU/mL) of type-D NSC communities obtained after three consecutive rounds of cheese-making. NSC_0 samples were obtained by propagating freeze-dried type-D NWS in sweet whey at a constant temperature of 45 °C for 16 h. Following each round of caseification, the resulting SW samples were fermented to produce the corresponding NSC, respectively. (A) Lactobacilli counts scored on MRS pH 6.5 at 42 °C under anaerobiosis. (B) Lactobacilli counts scored on MRS pH 5.4 at 42 °C under anaerobiosis. (C) Streptococci counts evaluated on M17-SSW at 42 °C under aerobiosis. All values represent the mean of at least three replicates; error bars, when visible, indicate standard deviation. For each microbial count, different letters indicate significant differences among samples ($p < 0.05$), as determined by one-way ANOVA. Graphs were created using GraphPad version 8 (San Diego, CA, USA).

comparable to the corresponding NSC_0, with few exceptions (Figs. 5 and 6). In the type-H community, streptococci and lactobacilli (MRS pH 5.4) populations showed higher values than the initial NSC_0 after each round of back-slopping (Fig. 5B and C). In the type-D community, lactobacilli counts on MRS pH 6.5 remained stable across consecutive rounds of back-slopping ($p > 0.05$), while a significant increase was observed in the third round on MRS pH 5.4 ($p < 0.05$) (Fig. 6A and B). We also observed a decrease in streptococci count at the third round of fermentation compared to the corresponding type-D NSC_0 (Fig. 6C). Based on these results, we concluded that curd cooking reduces the number and viability of microbial cells. However, at each step, the survivors present in SW efficiently grow and replicate during fermentation, resulting in NSC samples enriched in viable SLAB cells. The back-slopping procedure was reproducible over three consecutive rounds.

3.3. Effect of PEN G on NSC in a single round of caseification

In experimental step 2, type-H and type-D NSC_0 cultures were used to inoculate raw milk supplemented with PEN G at the dosage of 4 ppb (corresponding to the MRL) for a single round of caseification. The resulting SW_1 samples were subsequently fermented to generate type-H and type-D NSC_1 through back-slopping (Fig. 2). PEN G did not affect the acidification curves of either the type-H or type-D SW_1 samples (Supplementary Fig. S3), nor did it alter the TA values of the resulting NSC_1 (Table 3). However, FA and the percentage of viable cells were negatively affected by the presence of 4 ppb PEN G in both type-H and type-D NSC_1, when compared to their respective controls (i.e., NSC_1 obtained from cheese-making trial with raw milk not supplemented with antibiotic) (Table 3). The observed reduction in viability may be linked to the decreased FA.

In type-H NSC_1, PEN G at the dosage of 4 ppb did not significantly affect TMC values or lactobacilli counts compared to the control (Table 3). However, we observed an increase in yeast contamination and a reduction in streptococci counts in NSC_1 derived from PEN G-spiked milk compared to the control (Table 3).

In the type-D trials, no differences were observed between treated and control NSC_1 samples in terms of microbiological parameters, except for a notable increase in yeast counts and a reduction in viable cells in treated type-D NSC_1, similarly to the effects observed in treated type-H NSC_1 (Table 3).

3.4. Effect of PEN G on NSC in multiple rounds of caseification

In experimental step 3, we evaluated the cumulative effect of PEN G

Table 3
Effect of a single round of caseification with milk spiked with 4 ppb PEN G on technological and microbiological parameters of type-H and type-D NSC_1 samples. Titratable acidity (TA) and fermentative activity (FA) were expressed as °SH/50 mL and Δ°SH/50 mL, respectively; total microbial count (TMC) as Log_{10} cells/mL; viable cells as percentage; lactobacilli, streptococci, and yeast counts as Log_{10} CFU/mL. For the sake of brevity, only lactobacilli counts obtained on MRS at pH 6.5, incubated at 42 °C under anaerobic conditions, are reported. Raw cow's milk without PEN G was used as control. Data are the means of at least four replicates. Significant differences between control (raw milk without antibiotic) and treatment (milk spiked with 4 ppb PEN G) in NSC_1 samples were calculated with Student's t-test ($p < 0.05$) and are indicated with different superscript letters. Abbreviations: TA, titratable acidity; FA, fermentative acidity; NSC, natural whey starter.

Sample	Parameters	Control		4 ppb PEN G		*p*-value
		Mean	SD	Mean	SD	
NSC_1 type-H	TA	30.34[a]	2.80	29.40[a]	1.83	0.4536
	FA	2.28[a]	0.11	1.63[b]	0.35	**0.0122**
	TMC	8.29[a]	0.04	8.24[a]	0.05	0.1024
	Viable cells	91.69[a]	9.60	52.91[b]	7.76	**<0.0001**
	Lactobacilli count	8.34[a]	0.18	8.23[a]	0.32	0.4799
	Streptococci count	6.74[a]	0.18	5.99[b]	0.13	**<0.0001**
	Yeast count	0[a]	0	2.83[b]	0.10	**<0.0001**
NSC_1 type-D	TA	29.65[a]	0.68	29.00[a]	0.36	0.4536
	FA	3.023[a]	0.201	2.378[b]	0.262	**<0.0001**
	TMC	8.29[a]	0.04	8.24[a]	0.05	0.0657
	Viable cells	96.00[a]	7.47	65.83[b]	8.97	**<0.0001**
	Lactobacilli count	8.34[a]	0.18	8.23[a]	0.32	0.5697
	Streptococci count	6.74[a]	0.18	5.99[b]	0.13	0.3794
	Yeast count	1.24[a]	0.34	2.92[b]	0.14	**<0.0001**

at dosages ≤ MRL on technological parameters, viability, and microbial composition of 3 consecutive NSC samples. For this purpose, type-H or type-D NSC_0 samples were inoculated in milk spiked with PEN G at the fortification level of 2 and 4 ppb (corresponding to 0.5 and 1 MRL, respectively) and submitted to 3 consecutive rounds of caseification (Fig. 2).

In experiments carried out with type-H NSC_0, FA values were significantly lower in 4 ppb PEN G treatment compared to control condition at first and third round of caseification, while TA values did not significantly change in presence of PEN G (Supplementary Fig. S4A). We observed a moderate decrease in TMC values and a strong decrease in viability at the second and third rounds of caseification in presence of 2 and 4 ppb PEN G compared to control condition (Fig. 7A and B). At the first round of caseification, the lactobacilli count decreased in NSC_1 obtained from milk spiked with 4 ppb PEN G, while the decline of lactobacilli population was more evident in both NSC_3 samples at 2 and 4 ppb PEN G (Fig. 8A). In response to both PEN G dosages, the streptococci count decreased starting from the first round of caseification compared to the control ($p < 0.05$) (Fig. 8B). A progressive increase in yeast load was observed over time from NSC_1 to NSC_3 obtained with spiked milk. The increase in yeast count was greater at the highest PEN G dosage, indicating a positive relationship between antibiotic concentration and yeast count (Fig. 8C).

The results obtained in caseification rounds with NSC_0 type-D were consistent with those observed for type-H NSC_0, supporting the conclusion that repeated exposure to 2 and 4 ppb PEN G negatively impacts FA but not TA values (Supplementary Fig. S4). In the type-D microbial community, viability was impaired by 4 ppb PEN G starting from the second round of caseification (Supplementary Fig. S5). Specifically, 4 ppb PEN G led to a reduction in lactobacilli starting from the first round of caseification, while both 2 and 4 ppb PEN G negatively affected streptococci counts in NSC_1 and NSC_3. Additionally, both PEN G

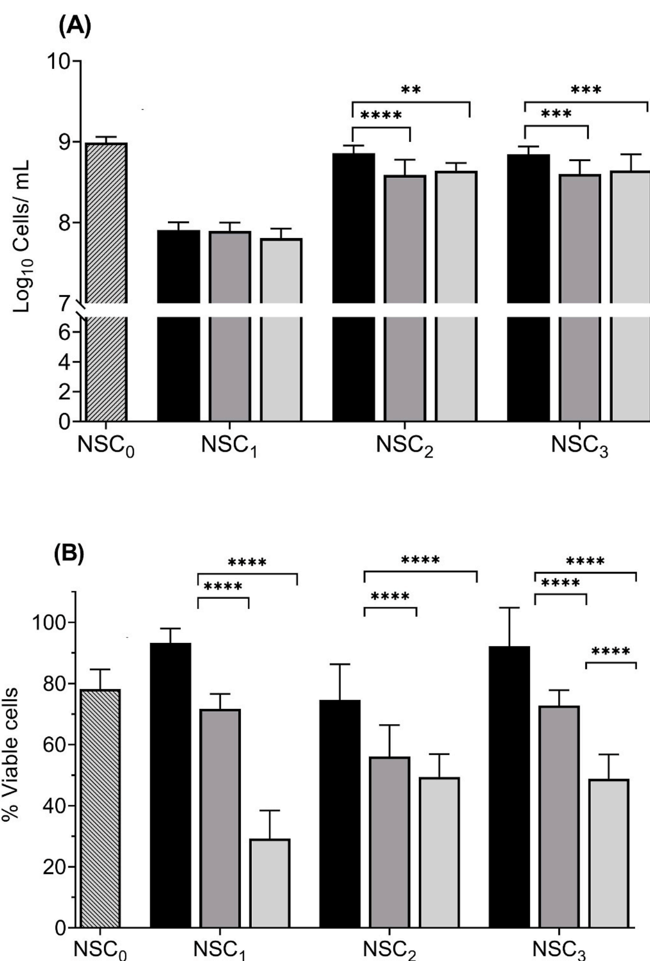

Fig. 7. Total microbial cell (TMC) counts (expressed as Log_{10} cells/mL) (A) and viable cell percentage (B) in type-H NSC communities obtained after three consecutive rounds of cheese-making in presence of PEN G at either 1X LMR (4 ppb) or 0.5X LMR (2 ppb). NSC_0 samples were obtained by propagating freeze-dried type-H NWS in sweet whey at a constant temperature of 45 °C for 16 h. Following each round of caseification, the resulting SW samples were fermented to produce the corresponding NSC, respectively. For each round of cheese-making three different raw milk batches were used: control (CTRL) milk (black), raw milk spiked with 2 ppb PEN G (grey), and raw milk spiked with 4 ppb PEN G (light grey). All values represent the mean of at least three replicates; error bars, when visible, indicate standard deviation. Significant differences among samples in function of treatment were determined by two-way ANOVA ($p < 0.05$) and indicated as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Graphs were created using GraphPad version 8 (San Diego, CA, USA).

concentrations promoted the growth of lactose-consuming spoilage yeasts in all type-D NSC samples (Supplementary Fig. S5).

3.5. Species-specific bacteria quantification by qPCR

The three main species present in NWS, namely *S. thermophilus*, *L. helveticus*, and *L. delbrueckii*, were quantified by qPCR to confirm the cumulative inhibitory effect of PEN G in recursive rounds of caseification. The assay was designed using species-specific primer pairs for the amplification and quantification of the single copy *pheS* gene, coding for α-subunit of bacterial phenylalanyl-tRNA synthase in *S. thermophilus*, *L. helveticus*, and *L. delbrueckii* genomes, respectively. In the last case, the primers were not able to discriminate between *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* (Bottari et al., 2013). The standard curves were created by diluting the gDNA of type strains *L. helveticus* DSM20075[T], *S. salivarius* subsp. *thermophilus* DSM20617[T], and

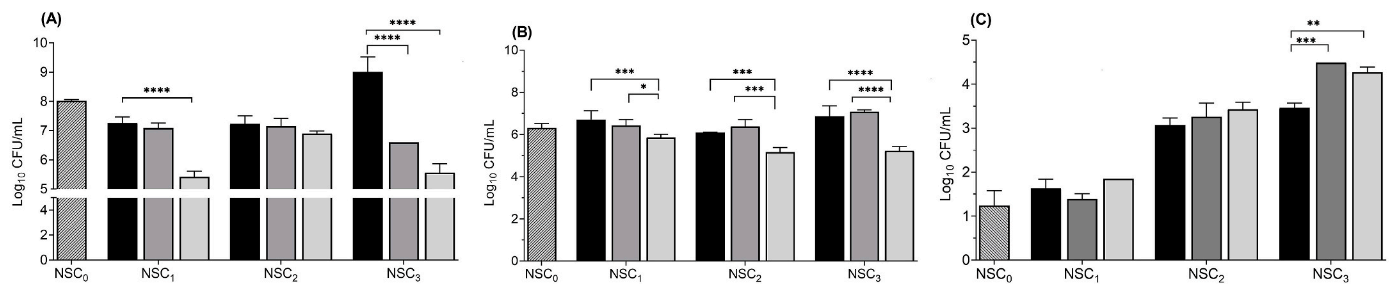

Fig. 8. Microbial counts (expressed as Log_{10} CFU/mL) in type-H NSC communities obtained after three consecutive rounds of cheese-making in presence of PEN G at either 1X LMR (4 ppb) or 0.5X LMR (2 ppb). NSC_0 samples were obtained by propagating freeze-dried type-H NWS in sweet whey at a constant temperature of 45 °C for 16 h. Following each round of caseification, the resulting SW samples were fermented to produce the corresponding NSC. For each round of cheese-making three raw milk samples were used: control (CTRL) milk (black), milk spiked with 2 ppb PEN G (grey), and milk spiked with 4 ppb PEN G (light grey). (A) Lactobacilli counts scored on MRS pH 6.5 at 42 °C under anaerobiosis. (B) Lactobacilli counts evaluated on MRS pH 5.4 at 42 °C under anaerobiosis. (B) Streptococci counts evaluated on M17-SSW at 42 °C under aerobiosis. (C). All values represent the mean of at least three replicates; error bars, when visible, indicate standard deviation. Significant differences among samples in function of treatment were determined by two-way ANOVA ($p < 0.05$) and are indicated as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Graphs were created using GraphPad version 8 (San Diego, CA, USA).

L. delbrueckii subsp. *lactis* DSM20072[T] and plotting the Ct values of all dilutions as a function of the concentration of genome copies (Supplementary Fig. S7). As expected, all three species were stable over time in control condition regardless of the type of NSC (Fig. 9). Milk spiked with 2 and 4 ppb PEN G did not affect *L. helveticus* counts in both the experiments (Fig. 9A and B). In type-H experiments, *S. thermophilus* genome counts significantly decreased in NSC_2 obtained using milk spiked with 4 ppb PEN G (Fig. 9A). Subsequently, during the third round of caseification, even 2 ppb PEN G resulted in a significant reduction of *S. thermophilus* counts ($p < 0.05$). In type-D NSC, *S. thermophilus* also decreased in NSC_3 derived from milk spiked with 4 ppb PEN G, however, this reduction was not statistically significant ($p > 0.05$) (Fig. 9B). Remarkably, *L. delbrueckii* genome counts significantly declined from the first round of caseification (NSC_1) in both type-H and type-D systems when milk spiked with 4 ppb PEN G was used. This negative trend appeared to be proportional to both the antibiotic concentrations and the number of caseification rounds (Fig. 9A and B).

4. Discussion

Thermophilic and undefined microbial consortia used as dairy starters have only rarely been compared across different stages of their reproduction cycles. Most studies have followed a bottom-up experimental approach, involving the analysis of a wide range of undefined starter cultures from various dairies in an attempt to retrospectively correlate technological parameters (e.g., cooling curve) or other variables (e.g., milk quality) with the composition and acidification capacity of these cultures.

In case of antibiotics, prior research has shown that antibiotic residues in milk can accumulate in SW used for NWS propagation (Giraldo et al., 2017). However, few studies have investigated the impact of antibiotics on the microbial communities inhabiting undefined starter cultures such as NWS. This gap in knowledge is mainly due to the instability of these complex starter cultures, which vary in composition and functionality depending on environmental conditions and dairy practices (Bertani et al., 2020; Sola et al., 2022; Morandi et al., 2024). Consequently, conducting controlled and reproductible experiments on NWS presents considerable challenges.

Chiesa et al. (2020) evaluated how milk contaminated with antibiotics concentrations affected the acidification ability of fresh NWS used for Grana Padano cheese-making, concluding that antibiotic residues generally compromise technological parameters. However, no studies have yet elucidated the specific effects of antibiotic residues on the microbial composition of NWS. In this study, we established a prototypal NSC system, which replicates, at laboratory scale, the back-slopping procedure used to propagate NWS in PR dairies. Freeze-drying effectively stabilized the microbial consortia, yielding two prototypal initial NSC systems, designed as NSC_0 type-H and type-D, that were microbiologically and functionally comparable to their fresh NWS counterparts. An incubation step at 45 °C for 16 h assured a reliable revitalization of both NSC_0 systems. When subjected to three successive rounds of pilot-scale cheese-making, NSC_0 samples generated first-, second-, and third generation-cultures, namely NSC_1, NSC_2, and NSC_3, that were comparable to NSC_0 in terms of technological and microbiological features. The system provided reproducible and stable microbial consortia as model to study the abiotic parameters affecting NWS without the interferences of natural fluctuations existing in real back-sloping process.

This system was then employed to assess the effect of PEN G at 2 and 4 ppb (0.5 × and 1 × MRL). The concentrations did not significantly reduce overall microbial counts and TMC values in NSC_1. The only technological parameter negatively affected was the FA, while TA, the most routinely monitored in PR dairies, remained unaffected. This suggests that PEN G residues at or below the legal MRL may slightly impair NWS microbiological quality, potentially escaping standard quality control.

Significant decreases in microbiological parameters were observed in NSC_2 and NSC_3, indicating a cumulative, delayed effect of PEN G over successive propagation cycles. This recurrent exposure is likely to occur in PR dairy farms where daily back-slopping converts SW from the previous cheese-making session into fresh, active NWS highly enriched in thermophilic SLAB. In NSC type-H, culturomics revealed a greater decline in streptococci than in lactobacilli at both PEN G levels. qPCR confirmed a marked reduction in *S. thermophilus* in NSC_2 (4 ppb) and NSC_3 (2 and 4 ppb). qPCR analysis also revealed that *L. delbrueckii*, present at low abundance in type-H community, was the most sensitive species to PEN G, showing a decline starting from NSC_1 at 4 ppb of PEN G.

In the type-D consortium, qPCR analysis confirmed that *L. delbrueckii* is the species most negatively affected by legally admissible levels of PEN G, while *S. thermophilus* were less affected than in the type-H consortium. In contrast, *L. helveticus* appeared insensitive to PEN G at both 0.5X and 1X MRLs in both consortia.

These findings align with Morandi et al. (2024), who observed antibiotic-induced declines in *L. delbrueckii* subsp. *bulgaricus* in NWS for Grana Padano cheese production. The observed differences in *S. thermophilus* susceptibility between type-H and type-D could reflect strain-specific responses (Temmerman et al., 2003; Morandi and Brasca, 2012; Nunziata et al., 2022) or distinct microbial interaction networks within the two consortia. Although *S. thermophilus*, *L. delbrueckii*, and *L. helveticus* strains were generally susceptible to the inhibitors of cell wall synthesis like PEN G (Katla et al., 2001; Nawaz et al., 2011;

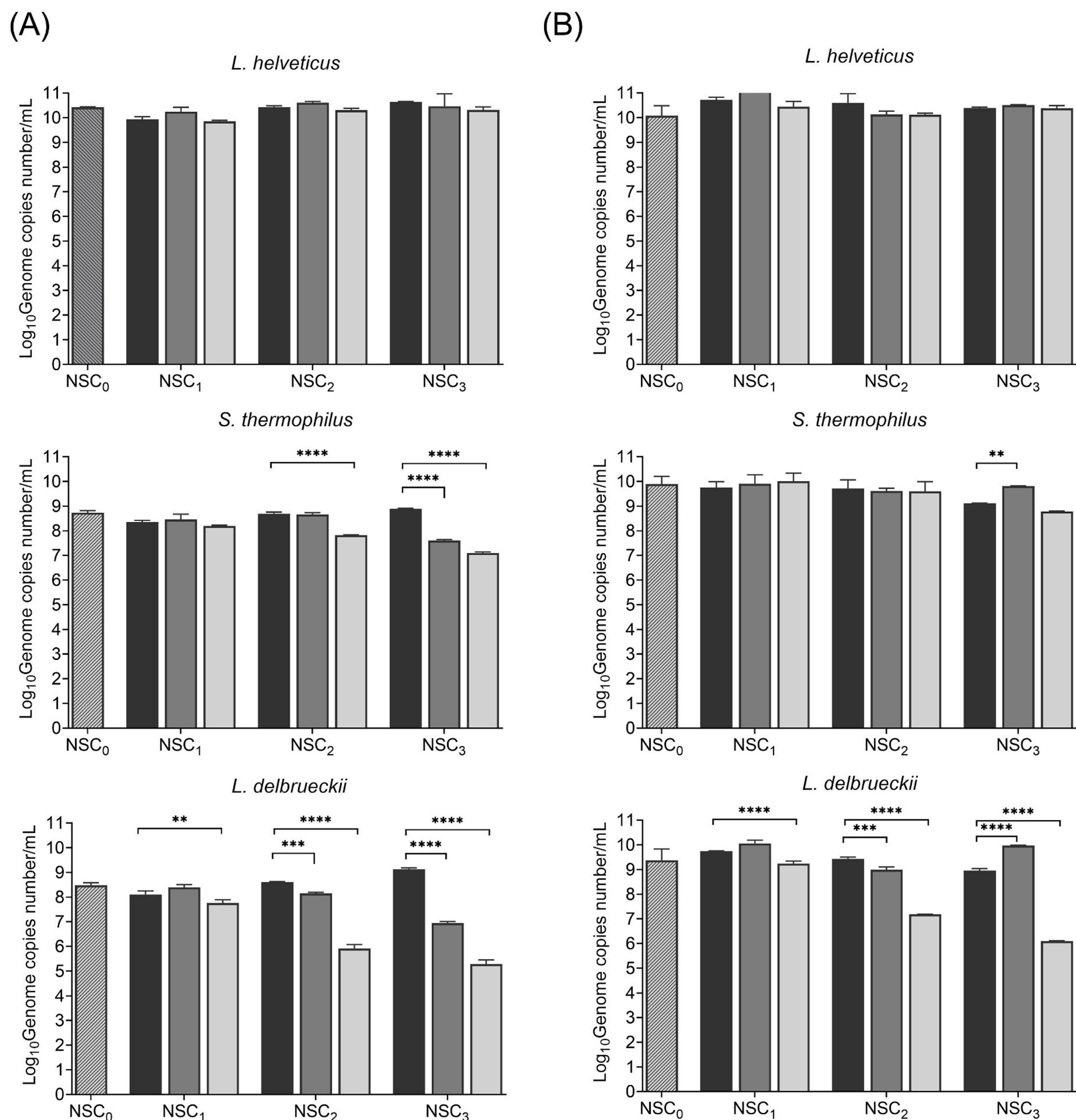

Fig. 9. Species-specific quantification of *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, and *Streptococcus thermophilus* in type-H (A) and type-D (B) NSC samples over three consecutive rounds of cheese-making. Quantification of each species was carried out by real time qPCR and expressed as log (genome copy number)/mL. NSC_0 samples were obtained by propagating either type-H or type-D freeze-dried NWS in sweet whey at a constant temperature of 45 °C for 16 h; after each round of caseification the resulting SW samples were fermented into corresponding NSC, respectively. For each round of cheese-making three raw milk samples were used: control (CTRL) milk (black), raw milk spiked with 2 ppb PEN G (grey), and raw milk spiked with 4 ppb PEN G (light grey). All values represent the mean of at least three replicates; error bars, when visible, indicate standard deviation. Significant differences among samples in function of treatment were determined by two-way ANOVA ($p < 0.05$) and are indicated as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Graphs were created using GraphPad version 8 (San Diego, CA, USA).

Morandi and Brasca, 2012; Abriouel et al., 2015), the inhibitory effects observed here at sub-MIC concentrations suggest more complex mechanisms requiring further investigation.

Metabarcoding analyses of other NWS samples suggest a progressive loss of biodiversity over successive NWS propagations, primarily due to reductions in *L. delbrueckii* (Morandi et al., 2019; Mancini et al., 2021). Recently, Sola et al. (2022) classified PR NWS into two groups, one enriched in *L. helveticus* and the other in *L. delbrueckii* subsp. *lactis*. It can be speculated that sub-MRL antibiotic residues may be one of the selective pressure driving community shifts between these two NWS types.

Finally, both NSC type-H and type-D showed significant yeast overgrowth when SLAB declined. Yeasts from the genera *Kluyveromyces*, *Wickerhamomyces*, *Saccharomyces*, and *Torulaspora*, have frequently been detected in PR-NWS, typically at concentrations not exceeding 4 Log_{10} CFU/mL (Coloretti et al., 2017; Martini et al., 2021). Some of these species are lactose-fermenting and may outcompete LAB for lactose utilization. Other species lack genes encoding for β-galactosidase in their genome but can easily consume the glucose and galactose moieties released by β-galactosidase-positive microbes (Martini et al., 2021). When LAB species decline due to antibiotic inhibition, yeasts may overgrow, thereby reducing the overall NWS acidification capability.

In conclusion, we developed and validated two reproducible NSC propagation systems that effectively mimic the back-slopping process of type-H and type-D communities under controlled and reproducible

conditions. We demonstrated that PEN G residues at or below the MRL can impair NWS biodiversity and drive compositional shits in undefined microbial consortia over successive stages of the back-slopping and across different dairy farms, with implication for PR cheese quality. This prototypal system offers a valuable tool for studying additional abiotic and biotic variables that influence undefined starter cultures, thereby enhancing the predictability of traditional cheese-making processes.

CRediT authorship contribution statement

Marianna Cristofolini: Writing – review & editing, Investigation, Data curation. **Maria Anna Ronsivalle:** Writing – review & editing, Investigation. **Davide Manicardi:** Writing – review & editing, Investigation. **Valentina Pizzamiglio:** Writing – review & editing, Supervision, Conceptualization. **Valeria Musi:** Writing – review & editing, Investigation. **Elena Bortolazzo:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Lisa Solieri:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

Data are available on request.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lisa Solieri reports financial support was provided by Ministry of Education and Merit, Italy. Davide Manicardi reports a relationship with Consorzio del formaggio Parmigiano Reggiano that includes: employment. Valentina Pizzamiglio reports a relationship with Consorzio del formaggio Parmigiano Reggiano that includes: employment. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. V.P. and D. M. are employed by Parmigiano Reggiano PDO cheese Consortium. This does not alter the authors' adherence to all the journal policies on sharing data and materials. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104861.

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