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Occurrence and diversity of Shiga toxin-producing *Escherichia coli* (STEC) in Italian Alpine raw milk cheeses and their development in the earlier stages of different cheese-making processes

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens implicated in diseases including hemorrhagic colitis and hemolytic uremic syndrome. In the last years, an increasing number of STEC infections associated with the consumption of raw milk cheeses have been reported, contributing to raise the public awareness. The aim of this study was to assess the occurrence of STEC biotypes in raw milk cheeses from Italian Alpine region and evaluate the influence of different scalding temperatures on their development. Six out 82 (7.3%) cheeses led to the isolation of *E. coli* biotypes with different virulence traits (*stx1*, *stx2* and *eae* genes). To evaluate the impact of scalding temperatures on STEC growth, three *E. coli* biotypes were spiked into milk (<50 CFU/mL) according to the FAO and WHO recommendation. An increase in STEC counts of 3 log occurred in uncooked and semi-cooked cheeses (scalding temperature of 38 and 45 °C) while in the cooked-cheeses (54 and 56 °C) *E. coli* content does not exceed the 2.5 $\log_{10}$ CFU/g. These findings showed that raw milk cheese safety is strictly related to their production technology, highlighting the importance of the control measures at farm and dairy level to preserve the safety of these products.

1. Introduction

Escherichia coli is a Gram-negative, facultative anaerobe microorganism, belonging to the Enterobacteriaceae family, that normally inhabits the lower intestinal tract of warm-blooded animals, including humans. Different *E. coli* clones have acquired virulence factors that have allowed them to adapt to new niches and in some cases to cause serious disease. To date, six pathogenic *E. coli* categories have been recognized: Shiga-toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAEC); enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Among them STEC are frequently involved in a variety of clinical outcomes in humans, ranging from diarrhoea to haemorrhagic colitis, and in some severe cases can they cause haemolytic uremic syndrome (HUS) and even death (Farrokh et al., 2013; FAO and WHO, 2022). In particular, the strains belonging to the "big 7″ STEC serogroups (O26, O45, O103, O111, O121, O145 and O157) are increasingly being reported as causes of severe diseases and outbreaks (Han et al., 2022). *E. coli* STEC strains are of major concern to the dairy chain since they are characterized by low infective dose (5–50 cells) and high pathogenicity (Farrokh et al., 2013).

The main virulence factor of STEC strains is the production of Shiga toxin Stx1 and Stx2 encoded by the genes *stx1* and *stx2*. Moreover, other reported virulence traits have be found in *E. coli* STEC, such as the *eae* and *aggR* adherence genes. The risk to contract severe disease, including HUS, is generally associated with the concurrent presence of the *stx2* and *eae* or *aggR* genes (FAO and WHO, 2018).

Cattle is a natural asymptomatic reservoir of STEC, representing a vehicle for human infections through direct contact or food products. Consequently, dairy products and in particular raw milk cheeses, are documented to be associated with STEC infections (Farrokh et al., 2013). In the 2020, 14 outbreaks linked to raw milk and raw milk cheeses consumption were reported in the EU (EFSA and ECDC, 2020). Raw milk contamination can occur through different ways, like animal infections, faecal matter, milking equipment, farm environment and staff (FAO and WHO, 2022). Recently, three outbreaks related to the consumption of dairy products were detected in Europe (EFSA and ECDC, 2021; 2022) and, the European Rapid Alert System for Food and Feed (RASFF) reported four “alerts” concerning the presence of this pathogen in raw milk cheeses in the first five months of 2024 (https://webgate.ec.europa

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.eu/rasff-window/portal/?event=searchResultList).

To avoid the risk of outbreaks from the dairy products consumption, it is therefore essential to control and prevent the milkborne STEC (Miszczycha, Mazuy-Cruchaudet, Thollet, & Sergentet-Thevenot, 2023). A regular cleaning and disinfection of milking and dairy equipment, process monitoring, and stringent hygiene of operators can be considered fundamental measures to increase the safety of raw milk cheeses (FACE network, 2016). Several studies have demonstrated that *E. coli* STEC strains belonging to different serogroups can grow and survive during the entire cheese-making process (Bellio et al., 2018; Miszczycha et al., 2013; Otero, Santos, Rodríguez-Calleja, & García-López, 2022). These investigations were performed using different levels of pathogen inoculum (from 10^2 to 10^6 CFU/mL) and were focused mainly on the ripening stage. The aims of the present study were, instead, to assess the *E. coli* STEC presence in Italian Alpine raw milk cheeses (cow and goat cheeses), to characterize the eventually recovered STEC and to investigate the influence of different scalding temperatures (38, 45, 54 and 56 °C) on the development of these STEC strains. In particular, we addressed the FAO and WHO recommendations on the need to study the growth of STEC, in the early stages of cheese-making (before salting) starting from a low *E. coli* content (<50 CFU/mL) necessary to reduce the risk of HUS from STEC (FAO and WHO, 2022).

2. Materials and methods

2.1. Sampling and microbiological analysis

A total of 82 raw milk uncooked cheeses with 30 days of ripening were collected from 12 artisan cheese factories in the Alpine region of Italy (6 producing cow's milk cheese and 6 producing goat's milk cheese) (Table 1). All cheeses were produced in different days and after collection were transferred to the laboratory under refrigerated conditions. Microbiological analyses were performed within 24 h of sample arrival.

Ten g of cheese were homogenized in 90 mL of a 2% (w/v) sterile K_2HPO_4 buffer solution (Sigma-Aldrich, St. Louis, MO USA) for 2 min in a Stomacher BagMixer (Interscience, St. Nom, France). Samples were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology, Barcelona, Spain) and inoculated into 3M™ Petrifilm Enterobacteriaceae and 3M™ Petrifilm *E. coli* Count Plate (3M, Minneapolis, MN, USA) (3M) for the enumeration of Enterobacteriaceae and *E. coli*, respectively. All plates were incubated at 37 °C for 24–48 h.

2.2. Isolation of presumptive E. coli STEC

Twenty-five g of cheese samples were added to 225 mL of sterile Buffered Peptone water (Biolife Italiana, Milan, Italy) and incubated at 37 °C for 24 h. After incubation, a loopful of culture was streaked onto CHROMagar STEC agar plates (CHROMagar Microbiology, Paris, France). This medium allows to differentiate between presumptive STEC (mauve/pink colonies) and other Enterobacteriaceae (blue colonies) and inhibits growth of Gram-positive bacteria. CHROMagar STEC agar plates were incubated at 37 °C for 24 h (Hoyle et al., 2021). Five mauve/pink colonies from each positive samples were picked and sub-cultured in Brain Heart Infusion (BHI) broth (Biolife Italiana) at 37 °C. The purity of the isolates was checked by streaking repeatedly on CHROMagar STEC agar plates. After purification, the isolates were stored at - 20 °C in Litmus milk (Biolife Italiana) for further genotypic characterization.

Table 1
Enterobacteriaceae, *E. coli* and presumptive Shiga toxin-producing *E. coli* in cow and goat raw milk cheeses.

Samples	n.	Cheese factories	Enterobacteriaceae $\log_{10}$ CFU/g	*E. coli* $\log_{10}$ CFU/g	Samples Positive on CHROMagar STEC
Cow cheese	38	7	4.9 ± 1.0[a]	3.0 ± 1.1	8 (21.1 %)
Goat cheese	44	6	3.4 ± 1.1[b]	2.3 ± 0.8	12 (27.3 %)
Total	82	12[a]	4.1 ± 1.3	2.6 ± 1.0	20 (24.4 %)

Means with different lowercase letters in the same column are significantly different ($P < 0.05$).
[a] A dairy produced both goat and cow raw milk cheeses.

2.3. DNA extraction

All presumptive *E. coli* STEC were propagated in BHI broth and incubated at 37 °C for 24 h. Genomic DNA was isolated from 1 mL of an overnight culture by the Microlysis kit (Aurogene, Rome, Italy) following the manufacturer's instructions. Yield and purity of DNA were evaluated using the Infinite F200 PRO microplate reader (Tecan, Mannedorf, Switzerland).

2.4. Randomly amplified polymorphic DNA-PCR (RAPD-PCR)

RAPD-PCR method was applied to explore the biodiversity and genetic relatedness within the presumptive *E. coli* STEC strains isolated from the cheese samples. This assay was performed with primers M13 according to Morandi, Silvetti, Miranda Lopez, and Brasca (2015) and resulting fingerprints were compared with the BioNumeric 5.0 software package (Applied Maths, Sint-Martens-Latem, Belgium), using the UPGMA (unweighted pair group method with arithmetic averages) cluster analysis. Strains with a similarity coefficient equal to or higher than 90% were considered belonging to the same biotype (Morandi et al., 2024).

2.5. Identification and detection of E. coli serotypes, stx1, stx2 and eae genes

Representative strains of each RAPD profile were identified by 16S rRNA sequencing as previously described by Decimo, Morandi, Silvetti, and Brasca (2014). Amplification products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing and the sequences analysis was performed by the Basic Local Alignment Search Tool (BLAST) software (National Center for Biotechnology Information, MD, USA). Species names were assigned whenever the degree of homology was higher than 98%. All strains were successively examined for the presence of *stx1*, *stx2* and *eae* genes, in the conditions reported by Idland, Granquist, Aspholm, and Lindbäck (2022). The identification of stx2 subtypes (stx2a and stx2d) was carried out according to Scheutz et al. (2012) and *E. coli* serotypes (O26, O45, O103, O111, O121, O145 and O157) were determined using a multiplex PCR assay, as previously described by Idland et al. (2022). The primer used in this study are listed in Table S1.

2.6. MALDI-TOF MS analysis: sample preparation and identification

To confirm the 16S rRNA sequencing data, all strains that harboured virulence factor genes were successively identified by the MALDI-TOF mass spectrometry method. Overnight cultures were streaked on BHI agar (Biolife Italiana) plates and incubated at 37 °C for 24 h. One colony for each strain was selected and transferred onto a steel MALDI target plate containing inoculation spots. Two spots were assigned to each isolate. The inoculated spots were then overlaid with 1 μL of 70% formic acid (Sigma-Aldrich), air-dried and overlaid with 1 μL of 4HCCA matrix solution (10 mg/mL of alpha-cyano-4-hydroxycinnamic acid dissolved in a solution of 50% ACN and 2.5% trifluoroacetic acid, Sigma-Aldrich)

to permit sample ionization. The strains mass spectrum profile was acquired by Maldi Biotyper system (Bruker Daltonics, Bremen, Germany) previously calibrated using the Bruker Bacterial Test Standard (BTS, Bruker Daltonics) according to the manufacturer's instructions. The assessment of the bacterial spectra quality was carried out by FlexAnalysis (v 3.4; Bruker Daltonics), while the strain identification was carried out using the MBT Compass Explorer 4.100.1 module (Bruker Daltonics). Data were interpreted according to manufacturer interpretation, (log)score values between 2.00 and 3.00 or 1.70 and 1.99 indicated a high (species level) and low (genus level) confidence identification, respectively. Lower (log)score values meant that no microorganism identification was possible (Lebano et al., 2024).

2.7. Preparation of spiking and starter culture

Three *E. coli* STEC strains isolated from cow and goat raw milk cheeses were used to evaluate the effect of the scalding temperature on the pathogenic bacteria growth during cheese-making process. *E. coli* STECs were grown in BHI broth overnight at 37 °C, then diluted in Ringer to obtain a STEC concentration in milk equal to 20–50 CFU/mL according to FAO and WHO (2022). The indicated level was checked by plating inocula on CHROMagar STEC agar plates.

Autochthonous cultures of Valtellina Casera PDO cheese (starter 1) and Silter PDO cheese (starter 2) were used as starter in the laboratory cheese-making model (Morandi et al., 2015; Silvetti et al., 2017). These starters were composed by *Streptococcus thermophilus* and *Lactococcus lactis* and were added at a level of 10^7 CFU/mL into milk. The two starter cultures were chosen considering their different acidifying activity in the first hours of fermentation (ΔpH ($pH_{zero\ time}$ -$pH_{at\ 3h}$) 0.7 starter 1 and 1.2 starter 2, respectively).

2.8. Laboratory model cheese production

Cheeses were prepared on a laboratory scale as previously reported by Schwendimann et al. (2020) with slight modifications. The model was set up considering the parameters and conditions (temperature profile, pH, cultures, and rennet) encountered during the first 24 h of Alpine cheese production. Cheeses were obtained in a 100 mL Schott bottle containing 80 mL of fresh pasteurized homogenized whole milk (Latteria Sociale Valtellina, Delebio, Italy).

In each trial, cheeses inoculated with *E. coli* STEC and one negative control were produced at the same time, with one scalding step. The Schott bottles were placed in a water bath in which the temperature was controlled by a thermostat (Julabo Corio C, Seelbach, Germany). The bottles containing the pathogenic strains were kept closed, while one bottle (without pathogenic strains) was used to record the temperature

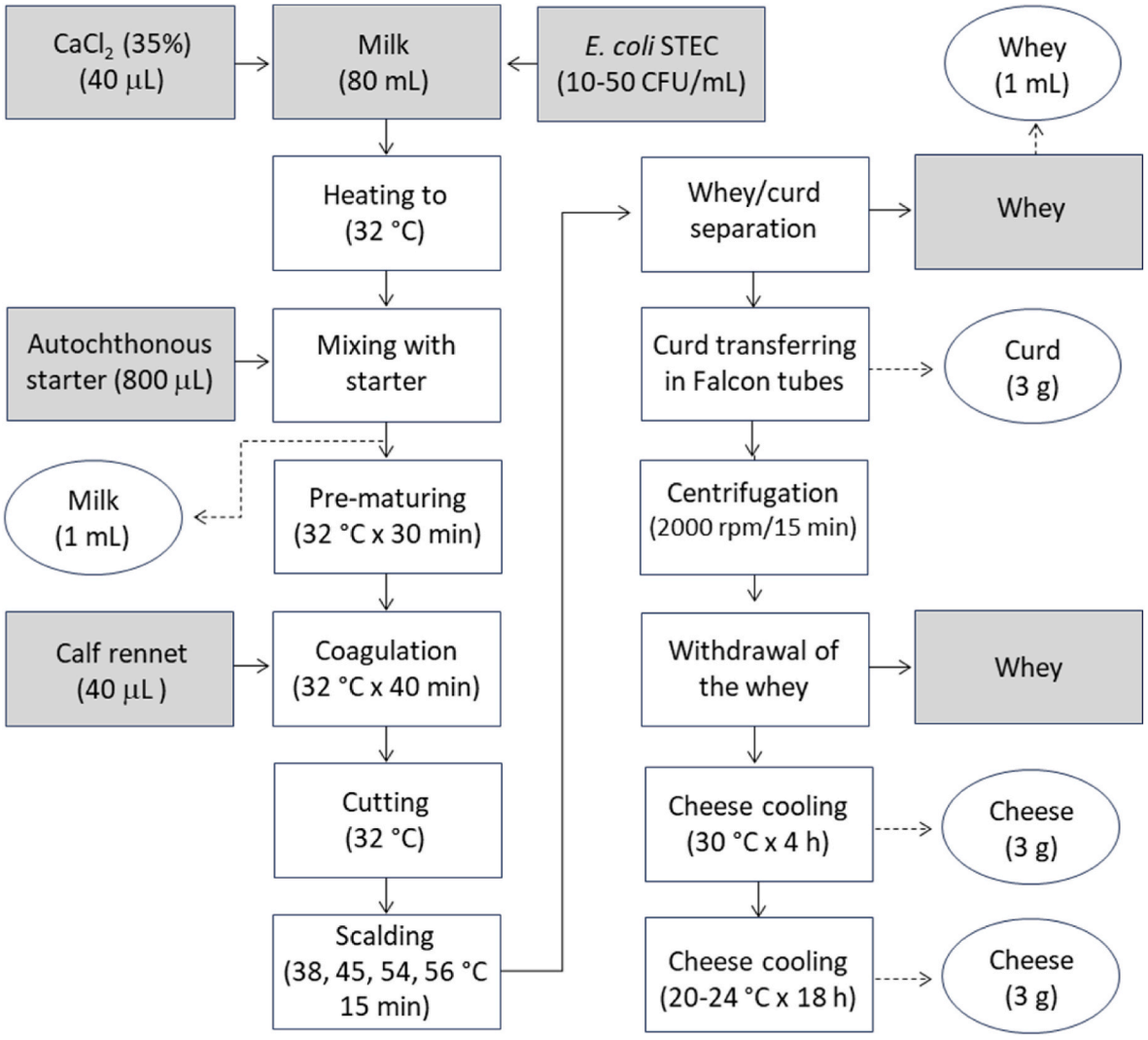


Fig. 1. Flow chart of the production of cheese in the laboratory cheese-making model. The white boxes indicate the processes applied, the grey boxes provide information on material added or removed during the cheesemaking process, and the elliptical boxes refer to the time points of microbiological analysis.

and pH during curdling and scalding. These parameters were automatically recorded at 10-min interval by a multi-channel pH meter (Acidification Monitoring System and Analyzer, Star Ecotronics) equipped with combined pH electrodes (In Lab Power 51343110, Mettler-Toledo).

In Fig. 1 is described the outline of the experiments. An appropriate volume of *E. coli* STEC (reaching ~50 CFU/mL in milk) and 40 μL of calcium chloride (35%) (Sigma-Aldrich) were added to each bottle containing 80 mL of milk. The mixture was then heated to 32 °C. Then 800 μL of autochthonous starter cultures (about 10^7 CFU/mL in milk) were added, and the milk was left for 30 min for prematuration. Subsequently, 40 μL of calf rennet (Naturen Extra 220, CHR Hansen, Hørsholm, Denmark) were added to induce mild coagulation. The coagulum was cut horizontally and vertically with a kitchen knife 40 min later. The resulting curd (size and homogeneity) was not the same as that in commercial facilities, but this difference was not expected to impact the outcome of the experiment. The curd was then heated to a scalding temperature (38, 45, 54 and 56 °C) for 15 min while gently stirring. After scalding, the whey and curd were separated, and the remaining curd was then placed in 50-mL Falcon tubes and centrifuged for 15 min at 2.000 rpm (ALC 4227R, Asal, Milan, Italy). After whey removal, the experimental cheeses were cooled to 30 °C and left at this temperature for 4 h. Later, the cheese models were incubated overnight at ambient temperature. Considering that the time and temperature profiles applied were like those in artisanal cheese factories, the experimental conditions reflected the situation in real cheeses peripheral zones (Schwendimann et al., 2020). The *E. coli* STEC development in cheese was evaluated at four different scalding temperatures (38, 45, 54, and 56 °C) and for each of the three *E. coli* strains the cheese-making process was replicated three times, employing two different starter cultures.

2.9. Microbiological analysis

The experimental samples were examined at 5 time points (inoculated milk, curd after scalding, whey and cheese at 4 and 18 h) (Fig. 1). In brief, 1 mL of milk and whey samples were serially diluted in Ringer solution, while 3 g of curd or cheese samples were homogenized in 27 mL of a 2% (w/v) sterile K_2HPO_4 buffer solution and subjected to serial dilutions. *E. coli* STEC were enumerated both in 3M™ Petrifilm *E. coli* Count Plate and in CHROMagar STEC agar plates, while LAB content was determined by the 3M™ Petrifilm Lactic Acid Bacteria Count Plates (3M). Whey samples were analysed only for the presence of *E. coli* STEC. All plates were incubated at 37 °C for 24–48 h.

2.10. Statistical analysis

All data related to microbiological assays are presented as means ± standard deviation (SD). Significant differences ($P < 0.001$) among the data were calculated by one-way ANOVA using Minitab ver. 14.13 (Minitab Inc.).

3. Results and discussion

3.1. Enterobactericeae and E. coli enumeration

Enterobacteriaceae counts in cow and goat raw milk cheeses collected from twelve different dairy farms averaged at 4.9 ± 1.0 at 3.4 ± 1.1 $\log_{10}$ CFU/g, respectively (Table 1). However, 55.3% of the cheeses from cow exceeded the 5 log, with up to 6.7 $\log_{10}$ CFU/g in three samples. On the contrary, the 65.9% of goat cheeses showed Enterobacteriaceae load ranging from 2 to 3 log and only 1 sample exceeded 6 log (Fig. 2).

These differences depend upon many factors that hamper or support the development of these bacteria.

Usually, a high Enterobacteriaceae content in raw milk cheeses is related to problematic conditions that promote their development such as hygienic deficiencies during milking or milk storage, but also hygienic shortcomings throughout the cheese-making process (Metz, Sheehan, & Feng, 2020).

Escherichia coli counts in the examined raw milk cheeses ranged from <2.0 to 6.0 $\log_{10}$ CFU/g with a mean value of 2.6 ± 1.0 $\log_{10}$ CFU/g (Table 1). A high percentage of goat samples (65.9%) showed low *E. coli* contamination (<2 log) while in the 44.6% of cow dairy products the content of this microorganism exceeded the 3 log (Fig. 2). The great diversity of intrinsic characteristics of raw milk cheeses due to the many variables concurring during the milking and cheese-making process (farm and milking hygiene practices, milk storage temperature, starter cultures used, mode and extent of salting and conditions and duration of ripening), makes it difficult to generalize the fate of *E. coli* (Metz et al., 2020). Currently, no criteria were provided by the European legislation

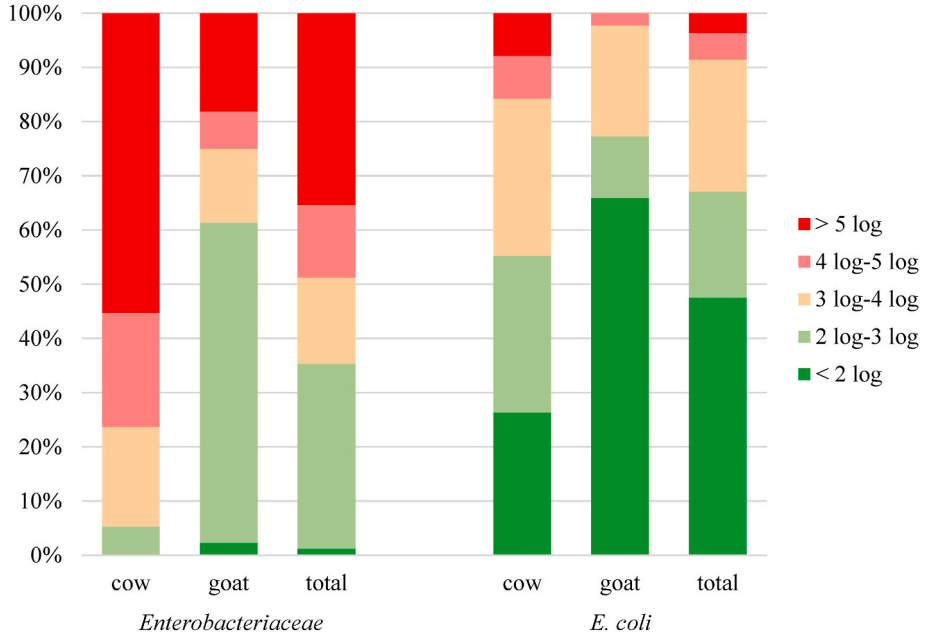


Fig. 2. Enterobacteriaceae and *Escherichia coli* concentration ranges in cow and goat raw milk cheeses.

for *E. coli* level in raw milk cheese, but different authors suggest that a content lower than 100 CFU/g could be a target for this product type, since high levels of *E. coli* in raw milk cheeses were significantly associated with the presence of STEC (Willis et al., 2022).

3.2. Identification, typing and characterization of E. coli STEC from raw milk cheeses

Twenty out of 82 samples (24.4%) (8 from cow and 12 from goat cheese samples) from 6 different cheese factories (named from A to F) presented typical mauve colonies on CHROMagar STEC agar plates (Table 1). Five colony from each positive sample were isolated and after purification were typed by RAPD-PCR. This assay was able to distinguish 8 different clusters characterised by a high similarity level (>90.0%) (Table 2). This observation provides the evidence that CHROMagar STEC is an effective media for selective isolation of the potential *E. coli* STEC strains and that these microorganisms persist over time on the farm, being isolated from cheeses produced in different days. Segura et al. (2018) reported that the persistence over time of *E. coli* STEC is due to both the ability of these bacteria to survive in the farm environment and the duration and magnitude of fecal shedding by individual animal. The large number of colonization factors, the capacity to produce biofilms and to activate stress fitness genes, support the *E. coli* STEC survival in the farm environment, favouring the contamination- and recontamination of the raw milk and raw milk cheeses.

One strain representative for each cluster and cheese was identified by partial 16S rRNA sequencing. All the isolates from the eight samples of the dairy A belonged to *Citrobacter freundii*, while the strains from the other 12 cheeses (dairies B, C, D, E and F) were identified as *E. coli* (Table 2). Six out of 20 strains harboured the virulence genes, in particular, two *E. coli* (S13 and S19) from cow cheeses were positive for *stx1* gene, while the isolates from goat samples (S9, S10, S11 and S12) carried the *stx1*, *stx2* and *eae* genes (Table 2). No virulence determinants were detected in any of the *C. freundii* strains. Generally, *E. coli* strains are considered to be the principal carriers of Shiga toxins, but recently *stx* genes have been detected also in other bacterial species including *Citrobacter* (Seliga-Gąsior et al., 2024). According to Multiplex PCR analyses, the *E. coli* from goat cheeses were found to belong to O26 serotype, while the S13 and S19 strains were not included in the top −7 serogroups (Table 2). The identification obtained by MALDI-TOF system was in agreement with 16S sequencing in fact, all the strains that harboured the virulence gens were found to belong to *E. coli* species ((log) scores between 2.34 and 2.50).

In summary, six out 82 (7.3%) raw milk cheeses were positive for *E. coli* STEC and three different STEC strains were isolated in these samples. These results can be compared with previous data available about the *E. coli* STEC percentage in raw milk cheeses from different Alpine European countries like Italy (5.3%) (Cortimiglia, Borney, Bassi, & Cocconcelli, 2021), Switzerland (ranging from 3.7 to 6.3%) (Stephan et al., 2008) and France (5.5 and 13%) (Madic et al., 2011; Vernozy-Rozand, Montet, Berardin, Bavai & Beutin, 2005). *E. coli* strains isolated from goat cheeses produced in dairy B harboured the *stx1*, *stx2* and *eae* genes. Currently, the presence of the *stx2* gene in relation with adherence gene (*eae*) is deemed to be a reliable predictor of STEC that pose a risk of severe disease (FAO and WHO, 2018). Moreover, several researchers have indicated that subtypes Stx2a or Stx2d are significantly associated with the risk of bloody diarrhoea, HUS, or both, since they were at least 25 times more potent than other subtypes in cytotoxicity assays (FAO and WHO, 2019). In this study, isolates carrying *stx2a* and *stx2d* were not identified, even if *E. coli* strains from dairy B belonged to the O26 serotype, the second most frequently reported STEC serogroup in clinical cases of European countries (Hoyle et al., 2021). Recent outbreaks of O26 serotype strains have caused multiple HUS cases in young children in Italy (2013), Romania (2016) and France (2019).

Table 2
Origin, RAPD-PCR typing, identification, genotypic characterization and potential risk of disease of the isolates recovered in cow and goat raw milk cheeses. In the brackets are reported the number of the isolates that presented mauve colonies on CHROMagar STEC agar plates.

Sample	Cheese factory	Cheese sample	RAPD cluster	Similarity level [a]	Strains	16S rRNA identification	log score [b]	Strains characterization				Risk potential for severe disease [c]
								stx1	*stx2*	*eae*	serotype	
Goat cheese	A	A1 (5)	1	93.2%	S1	*C. freundii*		–	–	–		none
		A2 (5)	1		S2	*C. freundii*		–	–	–		none
		A3 (5)	1		S3	*C. freundii*		–	–	–		none
		A4 (5)	1		S4	*C. freundii*		–	–	–		none
		A5 (5)	1		S5	*C. freundii*		–	–	–		none
		A6 (5)	1		S6	*C. freundii*		–	–	–		none
		A7 (5)	1		S7	*C. freundii*		–	–	–		none
		A8 (5)	1		S8	*C. freundii*		–	–	–		none
	B	B9 (5)	2	95.2%	S9	*E. coli*	2.41	+	+	+	O26	high
		B10 (5)	2		S10	*E. coli*	2.36	+	+	+	O26	high
		B11 (5)	2		S11	*E. coli*	2.41	+	+	+	O26	high
		B12 (5)	2		S12	*E. coli*	2.34	+	+	+	O26	high
Cow cheese	C	C13 (5)	3	95.6%	S13	*E. coli*	2.34	+	–	–	n.i.	low
		C14 (5)	4	90.3%	S14	*E. coli*		–	–	–	–	none
		C15 (5)	4		S15	*E. coli*		–	–	–	–	none
	D	D16 (5)	5	97.2%	S17	*E. coli*		–	–	–	–	none
	E	E17 (5)	6	96.6%	S19	*E. coli*	2.50	+	–	–	n.i.	low
	F	F18 (5)	7	98.0%	S20	*E. coli*		–	–	–	–	none
		F19 (5)	8	96.0%	S21	*E. coli*		–	–	–	–	none
		F20 (5)	8		S22	*E. coli*		–	–	–	–	none

n.i.: not identified.
[a] Strains with a RAPD similarity coefficient equal to or higher than 90.0% were considered belonging to the same biotype (Morandi et al., 2024).
[b] Maldi Biotyper identification: specie identification (log score between 2.0 and 3.0; genus identification (between 1.7 and 1.9); non reliable identification (<1.7).
[c] FAO and WHO (2018).

Different investigations identified an association between the above-mentioned O26 STEC infections and consumption of artisanal dairy products (Germinario et al., 2016; Jones et al., 2019; Severi et al., 2016). The other two *E. coli* strains isolated in our study harboured only the *stx1* gene and were not included in the seven serotypes commonly involved in clinical cases. Biotypes possessing these characteristics (positive for *stx1* and negative for *eae* genes) are present in many food and environmental sources and they may not be linked to infections or symptoms of diarrhoea, therefore are rarely involved in outbreaks (FAO and WHO, 2018).

3.3. Fate of E. coli STEC during different cheese-making processes

Although the high risk of *E. coli* STEC for cheese consumers is known, few studies have addressed the development of this pathogen during the cheese-making process (Bellio et al., 2018; Centorotola et al., 2021; Miszczycha et al., 2013, 2016; Otero et al., 2022). The investigations available were performed using high levels of pathogen inoculum (from 10^2 to 10^6 CFU/mL) and were mainly focused on the *E. coli* STEC survival during the cheese ripening. In the present study we considered the FAO and WHO recommendation on the need to investigate the development of STEC strains in the first cheese-making phases (before salting) of various cheese types, starting from low *E. coli* content (<50 CFU/mL) (FAO and WHO, 2022).

The scalding temperatures used in the laboratory cheese-making model (38, 45, 54 and 56 °C) were comparable to those employed in the production of uncooked, semi-cooked and cooked curd cheeses found in Alpine dairies such as Formaggella del Luinese PDO, Valtellina Casera PDO and Gruyère AOP (Donnelly & Kehler, 2016).

No statistically differences in pH values were found during cheese-making among the cheese samples obtained with the addition of two different starter cultures in fact, an average pH of 5.1 ± 0.1 was recorded in all batches after 18 h (Table 3). These values correspond to the acidity normally detect in the production of the three reference Alpine cheeses considered (Morandi et al., 2021; Moser et al., 2018). The autochthonous cultures used in this study were composed mainly by thermophilic (*St. thermophilus*) and mesophilic (*Lc. lactis*) strains and none of them showed antimicrobial activity against *E. coli* STEC strains (data not shown).

A significant increase ($P < 0.05$) in LAB content was observed in the curds obtained with the lowest scalding temperatures (38 and 45 °C). In these cheeses, the two starter cultures showed a similar trend and their concentration gradually increased reaching about 9 $\log_{10}$ CFU/g after 18 h (Table 3). In the cheese-making where the curd was cooked at 54 °C the LAB content increased slowly, achieving 7.7 log at the end of the trials, while at 56 °C a significant reduction of the starter loads was detected ($P < 0.05$) (Table 3). In our experimental conditions, the average level of STEC in milk was 1.4 ± 0.2 $\log_{10}$ CFU/mL (25 CFU/mL), 1.3 ± 0.1 $\log_{10}$ CFU/mL (19 CFU/mL) and 1.5 ± 0.1 $\log_{10}$ CFU/mL (31 CFU/mL) for S11, S13 and S19 cheese-making trials, respectively. During the laboratory cheese-making model, the pathogenic strains were enumerated both in CHROMagar STEC agar plates and in 3M™ Petrifilm *E. coli*. In the first medium *E. coli* strains grew with the typical mauve colonies while in the second one three different morphologies were detected: S11 appeared as red colonies with gas, S13 form blue colonies without gas and S19 showed blue colonies associated with gas. A substantial agreement was found in both methods considered ($P > 0.001$).

Considering the cheese-making process of uncooked cheese (scalding temperature of 38 °C), a significant increase of STEC content occurred (from 1.5 ± 0.1 $\log_{10}$ CFU/mL in milk to 3.4 ± 0.4 $\log_{10}$ CFU/g; $P < 0.05$ in cheese after 18 h) (Fig. 3). As reported in Fig. 4, the level of S11 and S13 increased constantly during the cheese production, while *E. coli* S19 grew more slowly reaching about 2.9 log at the end of the manufacturing. A similar scenario was observed with a scalding temperature of 45 °C, where the *E. coli* strains grew regularly increasing their concentration approximately 2 log (Figs. 3 and 4). Comparable results were obtained by Bellio et al. (2017) and Otero et al. (2022), who observed an important increase in STEC content during the early stages of production of Fontina (semi-cooked cheese) and Zamorano type cheese (uncooked cheese). The growth and survival of pathogenic *E. coli* in the first hours of cheese-making depend on several intrinsic and extrinsic factors, such as temperature, pH and water activity (Farrokh et al., 2013). As reported in Table 3, the temperature (from 32 to 45 °C)

Table 3
Change in pH, temperature and LAB content in laboratory model cheeses obtained with different scalding temperatures (38, 45, 56 and 58 °C). The data were expressed as means ± standard deviation.

Parameter	Starter	Sample	Scalding temperature			
			38 °C	45 °C	54 °C	56 °C
pH	Starter 1	milk	6.7 ± 0.0	6.7 ± 0.0	6.7 ± 0.0	6.7 ± 0.0
		curd	6.6 ± 0.0	6.5 ± 0.0	6.5 ± 0.0	6.5 ± 0.0
		cheese at 4 h	6.2 ± 0.2	6.0 ± 0.1	6.0 ± 0.2	5.7 ± 0.2
		cheese at 18 h	5.1 ± 0.3	5.0 ± 0.1	5.1 ± 0.2	5.2 ± 0.3
	Starter 2	milk	6.7 ± 0.0	6.7 ± 0.0	6.7 ± 0.0	6.7 ± 0.0
		curd	6.7 ± 0.0	6.6 ± 0.0	6.6 ± 0.0	6.5 ± 0.0
		cheese at 4 h	6.3 ± 0.2	5.8 ± 0.1	6.0 ± 0.2	5.8 ± 0.3
		cheese at 18 h	5.0 ± 0.2	4.9 ± 0.1	5.1 ± 0.2	5.1 ± 0.2
Temperature (° C)	Starter 1	milk	31.7 ± 0.1	31.8 ± 0.0	31.8 ± 0.0	31.8 ± 0.0
		curd	37.6 ± 0.1	44.7 ± 0.0	53.8 ± 0.1	55.8 ± 0.1
		cheese at 4 h	28.3 ± 1.7	30.6 ± 0.4	27.7 ± 0.2	31.5 ± 1.5
		cheese at 18 h	23.0 ± 0.3	22.2 ± 2.0	20.5 ± 0.3	22.7 ± 0.7
	Starter 2	milk	31.7 ± 0.0	31.8 ± 0.0	31.8 ± 0.0	31.8 ± 0.0
		curd	37.6 ± 0.1	44.7 ± 0.0	53.7 ± 0.0	55.8 ± 0.1
		cheese at 4 h	28.3 ± 1.7	30.6 ± 0.4	27.6 ± 0.3	31.5 ± 1.5
		cheese at 18 h	23.0 ± 0.3	22.2 ± 2.0	20.5 ± 0.3	22.7 ± 0.7
LAB content ($\log_{10}$ CFU/mL)	Starter 1	milk	7.3 ± 0.1 [a]	6.8 ± 0.6 [a]	7.1 ± 0.3	6.6 ± 0.3 [c]
		curd	8.1 ± 0.3 [a]	7.9 ± 0.3 [b]	7.2 ± 0.5	5.4 ± 0.3 [b]
		cheese at 4 h	8.4 ± 0.2 [b]	8.3 ± 0.0 [b]	7.3 ± 0.6	4.3 ± 0.5 [a]
		cheese at 18 h	9.0 ± 0.2 [b]	8.7 ± 0.2 [b]	7.7 ± 0.5	6.7 ± 0.7 [c]
	Starter 2	milk	7.3 ± 0.1 [a]	6.9 ± 0.2 [a]	6.8 ± 0.2	7.0 ± 0.3 [b]
		curd	8.0 ± 0.1 [a]	7.7 ± 0.6 [b]	6.9 ± 0.2	5.8 ± 0.7 [a]
		cheese at 4 h	8.5 ± 0.1 [b]	8.3 ± 0.1 [b]	7.1 ± 0.2	6.0 ± 0.5 [a]
		cheese at 18 h	9.0 ± 0.3 [b]	8.8 ± 0.1 [c]	7.7 ± 0.2	7.1 ± 0.4 [b]

Starter 1: Valtellina Casera PDO autochthonous cultures; Starter 2: Silter PDO autochthonous cultures.
Means with different lowercase letters in the same column are significantly different ($P < 0.05$).

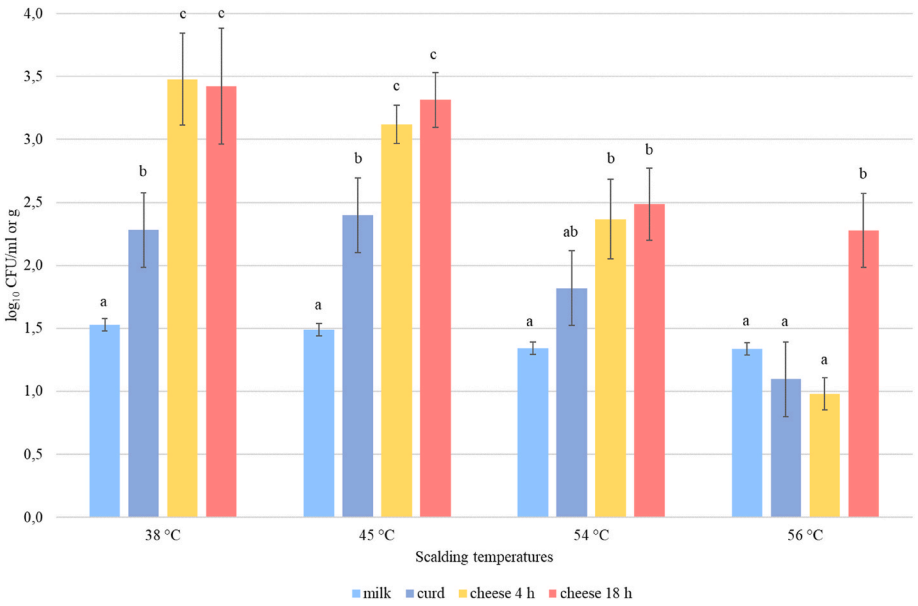
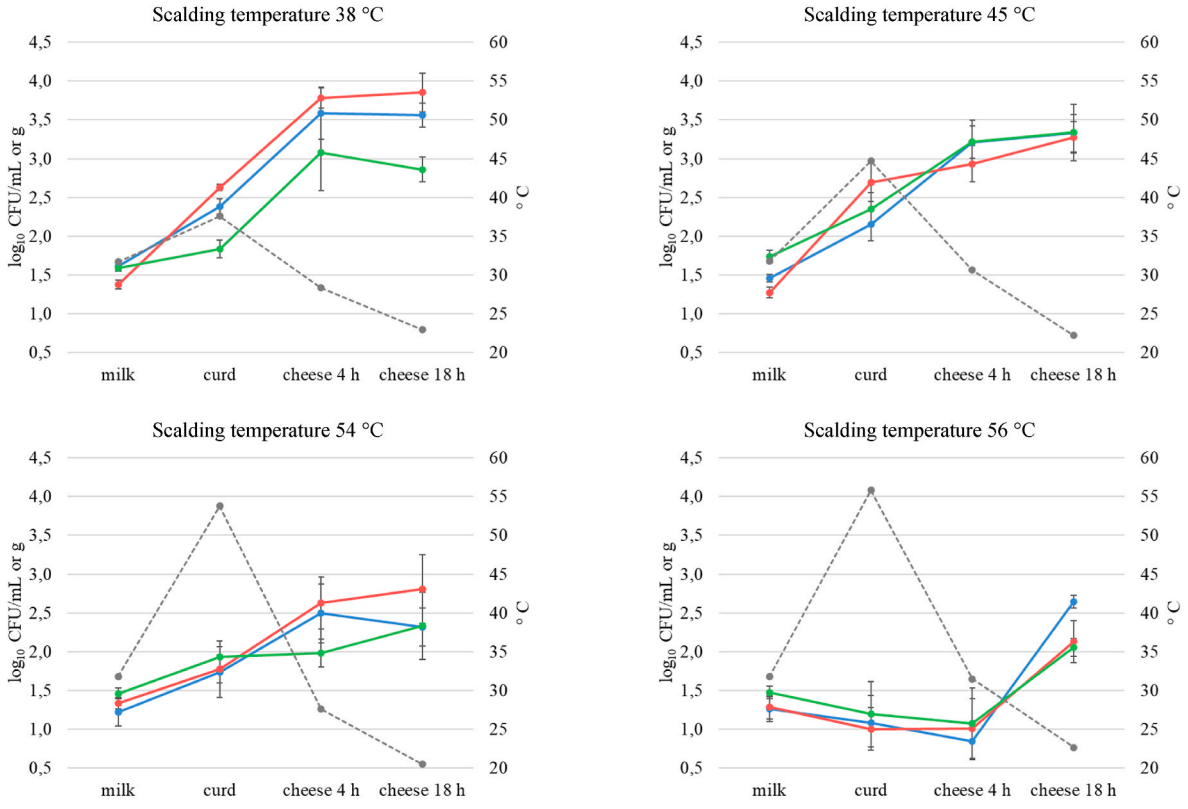


Fig. 3. *E. coli* STEC counts during the laboratory cheese-making process conducted with different scalding temperatures. The figure shows mean values ± standard deviation. Bars with different letters differ significantly ($P < 0.05$).



Blue line: S11 strain; Red line: S13 strain; Green line: S19 strain; Dotted line: Scalding temperatures

Fig. 4. *E. coli* STEC strain development during the laboratory cheese-making process conducted with different scalding temperatures (38, 45, 56 and 58 °C). The data were expressed as means ± standard deviation.

and the pH values (from 6.7 to 5.0) achieved during the production of uncooked and semi-cooked cheeses are insufficient to hamper *E. coli* development, thus creating a favourable condition for the STEC growth.

With a curd cooking at 54 °C for 15 min, the *E. coli* STEC development was lower than those obtained under the previously tested conditions, in fact its concentration did never exceed 3 logs (Fig. 3). In this state, the growth of *E. coli* S19 was affected by the process temperature, since its content increased only when the curd was cooled after the whey draining (Fig. 4). Our findings contrast with those reported by Miszczycha et al. (2013) that in an experimental cheese-making process

did not observ *E. coli* growth curd samples cooked at 54 °C for 35 min. These dissimilarities are probably related to the different time of curd-cooking applied (15 *vs* 35 min) and by the fact that, as described by other authors, the *E. coli* STEC heat resistance is biotype-dependent since the heat shock response varies from strain to strain (Peng, Schafroth, Jakob, Stephan, & Hummerjohann, 2013; Peng, Tasara, Hummerjohann, & Stephan, 2011).

A different scenario was observed increasing the cooking temperature by 2 °C (56 °C for 15 min). In this situation the *E. coli* load did not increase in the first 4 h, rising about 1.3 log with the cheese storage at ambient temperature (Figs. 3 and 4). Considering that the time and temperature profiles applied in this laboratory cheese-making model reflects the situation of cheese peripheral zones (Schwendimann et al., 2020), these results agreed with Ercolini, Fusco, Blaiotta, Sarghini & Coppola. (2005), who showed as in Grana Padano PDO cheese (curd cooking 55 °C × 20 min), *E. coli* STEC are defeated by the high temperature in the central part of the wheel, but they may survive, thanks to the favourable thermal conditions, in the external part.

The content of *E. coli* STEC in whey samples is shown in Table 4. A difference in pathogen concentration between whey and curd was observed and was attributed to both the bacterial cells' entrapment during the transition from milk to curd and to *E. coli* growth (Bellio et al., 2017; Otero et al., 2022). In particular, a difference of approximately 1 log is due to the cell concentration within curd formation, while the additional increase is attributed to the *E. coli* development (Peng, Hoffmann, et al., 2013). In the present study, we used two different autochthonous starter cultures for cheeses production and no differences in the *E. coli* development were observed, confirming that the pH values achieved in the Alpine cheeses before the salting (about pH: 5.0) were not able to hamper the STEC strains growth (Bellio et al., 2017).

Our findings showed that raw milk cheese safety is strictly related to their production technology. Starting from milk spiked with low STEC level (1.4 ± 0.2 $\log_{10}$ CFU/mL), uncooked cheeses could be a potential threat to human health, since *E. coli* STEC grew reaching high concentrations (about 3.5 $\log_{10}$ CFU/g) during the early stages of their manufacture. Moreover, the short ripening period (about 15–30 days) and the slow acidification of these products, make these types of cheese a suitable substrate for STEC growth (Otero et al., 2022).

Also, the semi-cooked cheese technology allowed the STEC development in fact, with a scalding temperature of 45 °C, before salting the load of *E. coli* strains exceeded 3 logs. Considering our findings and the length of the ripening period of these cheeses (usually more than 70 days), starting from raw milk with low STEC level the safety of this product should be ensured. As reported by many authors, during the ripening of semi-cooked cheeses an approximate reduction of ≥1 $\log_{10}$ CFU/g of STEC per month occurs (Miszczycha et al., 2013; Bellio et al., 2017).

The manufacturing process of cooked cheese hampered the *E. coli* STEC growth, confirming the effectiveness of the hurdles imposed during the cheese-making, such as, scalding temperatures ranging from 54 to 56 °C. In addition, the long ripening periods (more than 60 days) are known to reduce the pathogen content at levels that are known to avoid the illness (Peng, Schafroth, et al., 2013).

Our data highlight that, excluding the cooked ripened cheese, dairy products made from raw milk even with a very low *E. coli* content (<50 CFU/mL) may represent a potential risk for consumers, especially young children and elderly people. Therefore, dairy farms producing raw milk cheeses should apply strict control measures to prevent and monitor the milk and cheese contamination. In particular, the monitoring and the control of raw milk contamination at farm level is a crucial point to improve the safety of dairy products. Considering that the raw milk cheeses constitute an important part of the Alpine region's economy and tradition, understanding the behaviour of *E. coli* STEC dairy products is crucial to preserve the human health and the value of cheese products. Further research and practical applications are needed i) to reduce the presence of *E. coli* in raw milk, ii) to investigate the different behaviour of *E. coli* STEC biotypes and iii) to develop effective technological procedures (scalding temperatures and ripening parameters) to prevent and eliminate this microorganism from cheese production, without neglecting the need for continuous monitoring to improve the safety of these products.

Table 4
E. coli STEC content in curd and whey samples at different scalding temperatures (38, 45, 56 and 58 °C). The data were expressed as means ± standard deviation.

Starter	Scalding temperatures	*E. coli* STEC ($\log_{10}$ CFU/g)	
		Curd	Whey
Starter 1	38 °C	2.3 ± 0.3 [b]	0.8 ± 0.9 [a]
	45 °C	2.3 ± 0.4 [b]	0.7 ± 0.6 [a]
	54 °C	1.9 ± 0.2 [b]	0.2 ± 0.3 [a]
	56 °C	1.1 ± 0.4 [a]	0.6 ± 0.6 [a]
Starter 2	38 °C	2.3 ± 0.3 [b]	0.4 ± 0.7 [a]
	45 °C	2.4 ± 0.3 [b]	0.7 ± 0.7 [a]
	54 °C	1.7 ± 0.3 [b]	0.2 ± 0.3 [a]
	56 °C	1.0 ± 0.3 [a]	0.1 ± 0.1 [a]

Starter 1: Valtellina Casera PDO autochthonous cultures; Starter 2: Silter PDO autochthonous cultures.
Means with different lowercase letters in the same column are significantly different ($P < 0.05$).

CRediT authorship contribution statement

Stefano Morandi: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Tiziana Silvetti:** Writing – review & editing, Investigation. **Francesca Bonazza:** Writing – review & editing. **Milena Brasca:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.117029.

Data availability

Data will be made available on request.

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