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Impact of dairy cultures redox potential on the growth of spoilage bacteria causing discoloration defects in soft cheeses

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ABSTRACT

Five strains belonging to the species *Pseudomonas gessardii*, *Ps. proteolytica*, *Serratia marcescens Buttiauxella* spp. were isolated and identified as the causative agents of abnormal coloration in Stracciatella and other soft cheeses. Subsequently, 95 wild and commercial lactic acid bacteria (LAB) strains were screened *in vitro* using culture-spot assay, agar well diffusion, and co-cultivation in litmus milk to evaluate both direct and deferred antagonistic activity against the spoilage stains.

Results indicated that antimicrobial activity was strain-dependent and primarily associated both with the acidification and reduction capabilities of the LAB strains. Based on these findings, four LAB mixtures, each composed of four top-performing strains, were formulated and tested during the production, ripening (7 days at 4°C), and temperature abuse storage (7 days at 10 °C) of Crescenza cheese inoculated with the spoilage strains. Among them, Mix 4, containing *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*, exhibited the highest reducing activity (E_h: 306.3 mV) and the strongest antimicrobial effect, reducing *Pseudomonas* counts by more than 2 log units. Furthermore, the application of all the LAB mixtures prevented the pigment formation under abuse storage conditions (10 °C). For the first time, evidence was provided on the contribution of the reducing activity of lactic acid bacteria in counteracting the development of spoilage bacteria in cheese and on the importance of considering this metabolic activity in the selection of LAB strains for the bio-protection of dairy products.

1. Introduction

Over the years, many natural agents from plants, animals, and microorganisms have been proposed to delay cheese spoilage. These agents are considered an efficient alternative to synthetic additives and are gaining popularity due to increasing consumer concern about food safety and increasing demand for natural ingredients. Recently Ávila, Bravo, et al. (2025) and Rossi et al. (2025) highlighted that bacteriocins and essential oils are able to control *Pseudomonas* growth in fresh cheeses. Although these compounds have been extensively studied, their use in dairy production is limited since bacteriocins are expensive to isolate and purify, and essential oils produce strong odors that make them unusable in the cheese-making process (Calo et al., 2015; Silva et al., 2018). Among the natural strategies available, the use of protective cultures appears to be the most promising method to control spoilage bacteria. These cultures are mainly composed by lactic acid bacteria (LAB) strains and their antimicrobial activity is closely related to the production of bacteriocins, H_2O_2, diacetyl, and antimicrobial peptides, as well as to their acidifying and redox capabilities (Bintsis & Papademas, 2024; Callon et al., 2016). Different strains are often used in combination to enhance their antimicrobial effectiveness, and several commercial protective mixtures are available providing a technological flexibility in terms of products and target organisms (Shi et al., 2024). When properly applied, LAB mixtures that include both starter and adjunct cultures can significantly reduce the risk of spoilage during cheese production and ripening. Some dairy products, particularly low-acid cheeses such as Crescenza and Mozzarella, remain susceptible to growth of a variety of bacterial specie products, such as Pseudomonadaceae and Enterobacteriaceae. These microorganisms can produce a range of enzymes (proteases, lipases, and lecithinases) that lead to off-flavors, unpleasant odors, and texture degradation (Martin et al., 2021). Moreover, the strains belonging to the *Pseudomonas fluorescens* group and to the *Serratia* species are frequently associated with colour defects in cheese as they are able to produce pigments ranging from yellow fluorescence and orange-brown to blue or red (Rodríguez et al., 2023; Ávila, Sánchez, et al., 2025).

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The aims of this study were to: 1) isolate, identify and characterize the spoilage strains responsible for abnormal colorations in soft cheeses; 2) screen *in vitro* both wild (n. 57) and commercially (n. 38) available adjunct cultures against five spoilage bacteria and determine their modes of action and 3) develop LAB mixtures using the most effective strains and evaluate their inhibitory activity during laboratory-scale production and ripening of Crescenza cheese.

2. Material and methods

2.1. Spoilage bacteria isolation and identification

Five spoilage bacteria were isolated from Stracciatella and soft cheeses with abnormal coloration (Fig. 1) as previously described by Decimo et al. (2014).

The purity of the isolates was checked by streaking repeatedly on Homofermentative-Heterofermentative Differential (HHD) agar (Biolife Italiana, Milan, Italy) and successively the strains were identified by the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry method (Morandi et al., 2024). The acquisition of strains mass spectrum profile was performed by Maldi Biotyper Sirius system (Bruker Daltonics, Bremen, Germany) previously calibrated using the Bruker Bacterial Test Standard (BTS, Bruker Daltonics). The assessment of the bacterial spectra quality was carried out by Flex-Analysis (version 3.4; Bruker Daltonics), while the strain identification was carried out using the MBT Compass Explorer module (Bruker Daltonics). Data were interpreted according to manufacturer interpretation, score values between 2.00 and 3.00 or 1.70 and 1.99 indicated reliable species-level and genus-level identification, respectively. Identification is considered unreliable when the scores are less than 1.7. Prior to the experiments, the strains were propagated in Brain Heart Infusion (BHI) broth (Scharlab S. L., Sentmenat, Spain) and incubated at 30 °C for 24 h.

2.2. Evaluation of pigment production

The five spoilage bacteria were characterized for their ability to produce coloured pigments. This activity was carried out as described by Chierici et al. (2016) with slight modification. Mozzarella preserving fluid (PF; water, salt, and cheese minerals) was centrifuged at 9.000 g for 30 min (Benchtop centrifuge 5425, Eppendorf, Hamburg, Germany), neutralized with sterilized NaOH 1 N and then filtered through the 0.20 μm pore size PDVF membrane filter (Minisart, Sartorius, Goettingen, Germany). After the filtration, PFs aliquots (2 mL) were inoculated with fresh cultures of spoilage bacteria (2% v/v) and carried out in 24-well microplates. The microplates were then incubated at three temperatures (4, 13 and 30 °C) and monitored daily for 10 days to evaluate the colour development. The microplates were also observed under a UV light (Enduro UV Transilluminator, Labnet, Cary, NC, USA) to detect the fluorescent pigment production.

2.3. LAB cultures and growth conditions

Ninety-five LAB cultures were considered in this study to challenge food spoilage bacteria in soft cheeses. Fifty-seven strains, isolated from raw milk cheeses (Morandi et al., 2010, 2011, 2015), belonged to the microbial collection of the Institute of Sciences of Food Production at the National Research Council of Italy (CNR-ISPA, Milan, Italy), while the other 38 strains were purchased as lyophilized cultures from various commercial suppliers (Table 1). Commercial cultures were stored according to manufacturer recommendations, and their brand name was not disclosed for proprietary purposes. Wild strains were propagated aerobically in de Man, Rogosa and Sharpe (MRS) broth (Scharlab) at 30 or 37 °C for 24 h (Table 1), while commercial freeze-dried cultures were inoculated in MRS broth, allowed to hydrate at room temperature for 30 min and then incubated aerobically at 30 or 37 °C for 24 h (Aljasir et al., 2020). Bifidobacteria were grown anaerobically (AnaeroGen, Thermo Fisher Scientific, Monza, Italy), in MRS broth at 37 °C for 48 h. All strains were refreshed twice before testing.

2.4. Screening for antimicrobial activity

2.4.1. Culture-spot assay

Culture spot assay was determined according to Wörmann et al. (2024) with minor modifications. Briefly, 5 μL of LAB fresh cultures (8 $\log_{10}$ CFU/mL) were spotted onto MRS agar plates (Sharlab) and allowed to dry in a laminar-flow hood for 15 min. Then, 8 mL of soft MRS agar (0.75 % w/v agar) was overlaid on the surface, and the plates were incubated under the same conditions described above.

Next, 10 mL of soft BHI agar (0.75 % w/v) (Sharlab) was inoculated with the spoilage bacteria at 7 $\log_{10}$ CFU/mL and poured over the LAB culture-spot plates. The plates were then incubated aerobically at 30 °C for 24 h. The diameters of the culture-spots and inhibition zones surrounding each spot was measured using a callipers (Mitutoyo, Lainate, Italy) and the effective inhibition ratio (EIR) for each starter culture was calculated by applying the following equation: (inhibition diameter - spot culture diameter)/spot culture diameter. Weak, intermediate and strong inhibition were defined as a mean EIR below 0.5, between 0.5 and 1.5 and larger than 1.5, respectively (Coeuret et al., 2004). The experiment was performed in twice.

2.4.2. Agar well diffusion assay

Fresh cultures of food spoilage bacteria (8 $\log_{10}$ CFU/plate) were spread on BHI agar plates. Equidistant wells of 7 mm in diameter were punched in the plate and filled with 100 μL of LAB strains (8 $\log_{10}$ CFU/mL). Plates were incubated aerobically at 30 °C for 24 h. At the end of incubation, the diameters of the inhibition zone were measured with a

Fig. 1. Commercial Stracciatella cheeses with (A) and without (B) colour defects. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Wild and commercial LAB cultures used in this study.

Name	Genus	Species	Number of strains		Total	Growth condition
			Commercial	Wild		
BIF	*Bifidobacterium*	*brevis*	1		1	37 °C [An]
		bifidum	1		1	37 °C [An]
		lactis	1		1	37 °C [An]
CNB	*Carnobacterium*	*divergens*	1		1	30 °C
		maltaromaticum	1		1	30 °C
		spp.	1		1	30 °C
LCB	*Lacticaseibacillus*	*casei*	2	3	5	30 °C
		paracasei	6	25	31	30 °C
		rhamnosus	5	5	10	37 °C
LB	*Lactobacillus*	*acidophilus*	3		3	37 °C
LPB	*Lactiplantibacillus*	*plantarum*	10	18	28	30 °C
LAT	*Latilactobacillus*	*sakei*	2		2	30 °C
		curvatus	1		1	30 °C
LEV	*Levilactobacillus*	*brevis*	1	2	3	30 °C
LIM	*Limosilactobacillus*	*fermentum*	1	4	5	37 °C
		reuteri	1		1	30 °C
		Total	38	57	95	

[An]: anaerobic condition.

calliper and each measurement was performed in duplicate. Antimicrobial activity was estimated by subtracting the diameter of the well (7 mm) from the diameter of the zone of clearing. Based on the size of the halos, antimicrobial activity was defined as absent (no inhibition), weak (Ø < 3.0 mm), intermediate (3.0 < Ø < 6.0 mm) and strong (Ø > 6.0 mm). All the tests were performed in duplicate.

2.5. Preparation of cell-free supernatant (CFS) and neutralized CFS (NCFS)

Fresh cultures of the LAB strains with the best antimicrobial activity were centrifuged at 9.300 g for 5 min (Benchtop centrifuge 5425, Eppendorf) and supernatants were collected. Subsamples of each LAB supernatant were neutralized to pH 7.0 with 1 N NaOH to exclude the antimicrobial effects of organic acids. Both unneutralized and neutralized supernatants were then filtered through the 0.20 μm pore size PDVF membrane filter (Minisart, Sartorius) to obtain cell-free supernatants (CFS) and neutralize CFS (NCFS). The CFS and NCFS antimicrobial activity was evaluated as described below.

2.6. CFS and NCFS antimicrobial activity

The antimicrobial activity of CFS and NCFS against food spoilage bacteria was determined according to Favaro et al. (2014) with slight modifications. Ten millilitres of BHI broth was inoculated with 1% (v/v) fresh cultures of indicator strains and incubated for 3 h at 30 °C. After incubation, 100 μL of each spoilage strains were dispensed in 96-well flat bottom microtiter plate (Greiner bio-one, Cassina de Pecchi, Italy). Fifty μL of LAB CFS and NCFS were added in each well and mixed by pipette aspiration. Microplates were incubated in Infinite F200 PRO microplate reader (Tecan, Männedorf, Switzerland) at 30 °C for 24 h. Optical density (OD) measurements were carried out hourly at 595 nm. Positive (spoilage strains with the addition of 50 μL of MRS broth) and negative controls (sterile MRS and BHI broth) were included in the microplates. The influence that CFS and NCFS exert on the growth of the target microorganisms was evaluated by determining the time required to reach the inflection point of the bacterial growth curve that corresponds to the maximum growth rate (γ_{max}) before the deceleration phase, when the total number of the cells tends to stabilize (Navarro-Pérez et al., 2022). Each experiment was performed at least three times.

2.7. Inhibitory effect in co-cultivation

The efficacy of LAB cultures to inhibit the growth of spoilage bacteria was further evaluated in a co-cultivation in Litmus Milk (LM) (Biolife Italiana) (initial pH 6.5). LM was used as a model system to observe the LAB acidification and reduction activities in presence of the spoilage bacteria. This assay was performed following the temperature and time profile found in the Crescenza cheese-making process (Zhao et al., 2024). Briefly, 20 mL of LM were inoculated with a low level of spoilage bacteria (final concentration about 3 $\log_{10}$ CFU/mL) and incubated at 4 °C for 24 h to imitate the cold-storage of milk before the cheese-making process. Next, the LM samples were co-inoculated with fresh cultures of LAB strains (final concentration of 6 $\log_{10}$ CFU/mL), incubated at 38 °C for 110 min (curdling and cutting) and then shifted to room temperature (about 22 °C) for 3 h (moulding). Later, the LM samples were place at 13 °C for 1 h (salting) and finally stored at 3-5 °C in a cooled incubator (VELP Scientifica, Usmate Velate, Italy) for 7 days (ripening). LM containing spoilage bacteria without the addition of LAB

cultures were used as control samples. During the assay, bacterial enumeration was performed at different time points: 1) after inoculation of spoilage bacteria prior to cold-storage, 2) after the inoculation with LAB cultures, 3) after 24 h and 4) 7 days in the cooled incubator. At each time point, one mL of LMs was serially diluted in quarter-strength Ringer's solution (Scharlab) and inoculated in *Pseudomonas* agar (Biolife Italiana) with PP *Pseudomonas* supplement (Biolife Italiana), Petrifilm Enterobacteriaceae Count Plate (Neogen, Lansing, MI, USA) and Petrifilm Lactic Acid Bacteria Count Plates (Neogen) for the determination of *Pseudomonas* spp., Enterobacteriaceae and LAB content. *Pseudomonas* agar plates and Petrifilm Lactic Acid Bacteria Count Plates were incubated at 30 °C for 48 h, while the Petrifilm Enterobacteriaceae Count Plate were kept at 37 °C for 24 h. The antimicrobial activity of LAB cultures was expressed as inhibitory power (IP) defined as the difference between the spoilage bacteria counts in control samples (expressed in $\log_{10}$ CFU/mL) and the load of spoilage bacteria in samples inoculated with LAB strain (Callon et al., 2016). IP values were determined after 24 and 7 days of incubation at 4 ± 1 °C. All the tests were performed in duplicate.

2.8. LAB cultures redox potential (E_h)

A multi-channel pH-E_h-meter (Acidification Monitoring System and Analyser AMSA, Star Ecotronics, Milan, Italy) equipped with redox electrodes (In Lab Redox, Mettler-Toledo; Milan, Italy) was used to evaluate the reduction activity of the adjunct cultures. LAB strains were inoculated at a level of 2% in UHT milk (Parmalat S.p.a., Collecchio, Italy) as previously reported by Brasca et al. (2007). Two replicates for each bacterial strain were used to estimate the reduction activity, determining the minimum value of E_h ($E_{h\ min}$) at the temperature and time profile found in the Crescenza cheese-making process (see section 2.7). E_h values were reported as oxidation-reduction potential (ORP), uncorrected for pH and without reference to the standard hydrogen electrode (Caldeo & McSweeney, 2012; Bulat & Topcu, 2019). Before each analysis, the accuracy of E_h electrodes were checked as described by Caldeo and McSweeney (2012).

2.9. Cheese manufacturing

2.9.1. Preparation of spoilage bacteria and LAB inoculum

One cocktail containing the five spoilage microorganisms was prepared according to Aljasir et al. (2020) and Wörmann et al. (2024) with slight modification. The strains were grown separately in of BHI broth (Sharlab) overnight at 30 °C. Two subsequent transfers in BHI broth were made prior to use (final concentration 8 - 9 $\log_{10}$ CFU/mL). Equal volumes (1 mL) of fresh cultures of each spoilage strain were combined to produce the cocktail. After centrifugation at 5.000*g* for 10 min (Benchtop centrifuge 5425, Eppendorf), the supernatant was discarded, and the pellet was washed twice in buffered peptone water (BPW) (Sharlab). The resulting pellet was resuspended in BPW and serially diluted in UHT milk to obtain the target concentrations described hereafter for each experiment (3 $\log_{10}$ CFU/mL).

Four mixtures composed by four LAB strains were used in the experimental cheese-making process. These strains were chosen considering their antimicrobial activity determined in the previously assays (Trevisiol et al., 2025). LAB strains were grown separately in MRS broth incubated overnight at 30 °C (final concentration 8 - 9 $\log_{10}$ CFU/mL). After reactivation, cells were recovered by centrifugation (5.000 g for 10 min), washed three time with Ringer solution, resuspended in UHT milk and used for cheesemaking.

2.9.2. Crescenza cheese-making process

Crescenza cheese was made at the laboratory scale as previously reported by Zhao et al. (2024) with slight modifications. Cheeses were obtained in Schott bottle containing 200 mL of fresh pasteurized homogenized whole milk (Latteria Soresina, Soresina, Italy). The Schott bottles were placed in a water bath in which the temperature was controlled by a thermostat (Julabo Corio C, Seelbach, Germany). A volume of spoilage bacteria, reaching 3 $\log_{10}$ CFU/mL in milk, and 200 μL of calcium chloride (35%) (Sigma-Aldrich, St. Louis, MO USA) were added to each bottle. The milk was then heated to 38 °C and inoculated with a commercial starter culture (ST021, *Streptococcus thermophilus*, Sacco S.r.L, Cadorago, Italy) according to the manufacturer's instruction, then LAB cultures were added individually (7 $\log_{10}$ CFU/mL) and the milk was left for 1 h at 38 °C. Subsequently, 50 μL of calf rennet (Naturen Extra 220, CHR Hansen, Hørsholm, Denmark) was added and the curd obtained was cut into grains sized ca. 1.5 to 2.0 cm. After 45 min a second cut was performed (0.5 to 1.0 cm size) and successively, the whey was drained. Then, the curd was transferred into cheese molds and placed at room temperature (about 22 °C), for 3 h. Fresh cheeses were then salted by immersion in NaCl brine (15% NaCl) at 13 °C for 1 h. Finally, the samples were ripened at 4 ± 1 °C for 7 days and subsequently stored at 10 ± 1 °C for 7 days in order to simulate the temperature conditions most commonly found in the European domestic refrigerators (Bonanno et al., 2024). To assess the color development due to the spoilage bacteria growth, the cheeses were visually observed daily during the ripening period. In this study six cheeses were produced at the same time, three without the addition of the adjunct cultures (controls) and three inoculated with the pool of LAB strains.

2.9.3. Temperature, pH and redox measurements

During the cheese-making process, temperature, pH and E_h were automatically recorded at 10-min interval by a multi-channel pH-E_h-meter (Acidification Monitoring System and Analyzer) equipped with combined pH and redox electrodes (In Lab Power 51343110 and In Lab Redox, Mettler-Toledo; Milan, Italy). Before each analysis, pH electrodes were calibrated using two standard solutions (pH 4.0 and 7.0; Scharlab) while the accuracy of E_h electrodes was checked as described by Caldeo and McSweeney (2012). E_h values were reported as ORP. E_h evolution was followed up to the cheese salting, since, as shown by several authors, this parameter remains almost constant throughout the ripening period (Abraham et al., 2013; Bulat & Topcu, 2019), while pH measurements were also performed at three time points during the cheese storage (after after 24 h and 7 days at 4 ± 1 °C and after 7 days at 10 ± 1 °C).

2.9.4. Microbiological analysis

Samples were collected for bacterial enumeration at the time of LAB inoculation and in three time points: after the addition of the adjunct cultures and during the ripening period (after 24 h and 7 days at 4 ± 1 °C and after 7 days at 10 ± 1 °C).

For microbiological analyses, milk (1 mL) and cheese (5 g) samples were homogenized (Stomacher BagMixer, Interscience, St. Nom, France) for 2 min in quarter-strength Ringer's solution (9 mL) and in 2% (w/v) sterile K_2HPO_4 (45 mL) (Sigma-Aldrich) buffer solution, respectively. Samples were then serially diluted in quarter-strength Ringer's solution and inoculated in *Pseudomonas* agar with PP *Pseudomonas* supplement, in Petrifilm Enterobacteriaceae Count Plate and in Petrifilm Lactic Acid Bacteria Count Plates. All the plates were incubated as described in section 2.7. The LAB strain's inhibitory power (IP) was calculated according to Callon et al. (2016).

2.10. Statistical analysis

All data related to the antimicrobial activity, acidifying and redox capabilities, were presented as means ±standard deviations (SD). Significant differences ($P < 0.05$) among the data were calculated by one-way ANOVA using Minitab ver. 14.13 software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Identification of spoilage bacteria and pigment production

Five spoilage bacteria that lead to cheese discoloration were isolated from Stracciatella and soft cheeses. The strains were identified by MALDI-TOF MS analysis, and were found to belong to the species *Pseudomonas gessardii* (STL2 and SLP3), *Ps. proteolytica* (SLP5) and *Serratia marcescens* (PS92). The STL1 strain was identified at the genus level as *Buttiauxella* spp. (score: 1.85) (Table 2). The pigment production of these bacteria was evaluated at three different temperatures; 4, 13 and 30 °C (Table 2). All *Pseudomonas* strains produced a yellow-green pigment that appeared fluorescent under UV light. In the two *Ps. gessardii* strains this activity was detected under all conditions tested, while in *Ps. proteolytica* STL5 it was only evident at 13 and 30 °C. These results are consistent with those reported by Ávila, Sánchez, et al. (2025) and Chierici et al. (2016), who demonstrated that strains belonging to the *Pseudomonas fluorescens* complex are involved in the fluorescent yellow or green discoloration of fresh cheeses, and that this phenomenon is strictly strain-dependent and influenced by the incubation temperature.

Although defects caused by *Pseudomonas* in fresh dairy products are well documented, the mechanisms of pigment production remain poorly understood. Aromatic amino acids released from milk proteins via the proteolytic activity of *Pseudomonas* enzymes can serve as precursors for the biosynthesis of pigments such as indigoidine, leading to visible discoloration (del Olmo et al., 2018). Some pigments also appear to possess antimicrobial activity and are regulated by quorum sensing, with their production likely stimulated at high bacterial densities, providing a competitive advantage to the producing strains (Angell et al., 2006). *Pseudomonas* spp. produce siderophores such as pyoverdines, which facilitate iron uptake. Once bound to iron, pyoverdines act as signaling molecules, inducing genes involved in toxin production and modulating protease and lipase activities, further contributing to the biochemical changes associated with spoilage and discoloration (Quintieri, Caputo, Brasca, & Fanelli, 2021).

Considering the strains belonging to the Enterobacteriaceae family, *Buttiauxella* STL1 caused orange pigmentation only at low temperatures (4 and 13 °C after 7 and 3 days, respectively), while the intensity of red pigment produced by *Se. marcescens* PS92 increased with the rising temperature and the intense red colour developed after only 1 day at 30 °C These findings were in line with those reported by Rodríguez et al. (2023), who showed that the optimum temperature for prodigiosin production was 32 °C. Prodigiosin is a red pigment that is synthesized by *Se. marcescens* strains, which are responsible for the discolouration in Cabrales cheese (Rodríguez et al., 2023).

To date, limited data are available on the presence of *Buttiauxella* in dairy products, and no correlation had been observed until now between this microorganism and colour defects in cheese. This is the first time that a strain of this genus has been linked to an orange colour defect in food, specifically in Stracciatella cheese (Fig. 1).

Table 2
Spoilage strains isolated from Stracciatella and soft cheese, and their pigment production. In the square brackets are reported the days required to observe colour development at different incubation temperatures (4, 13 and 30 °C).

Strains	Species (Source)	MALDI-TOF	Colour development		
		Score	4 °C	13 °C	30 °C
STL2	*Ps. gessardii* (Stracciatella cheese)	2.05	[3 days]	[1 day]	[1 day]
SLP3	*Ps. gessardii* (Soft cheese)	2.13	[6 days]	[1 day]	[1 day]
SLP5	*Ps. proteolytica* (Soft cheese)	2.13	Colourless	[1 day]	[6 days]
STL1	*Butiauxella* spp. (Stracciatella cheese)	1.85	[7 days]	[3 days]	Colourless
PS92	*Se. marcescens* (Soft cheese)	2.36	Colourless	[3 days]	[1 day]

Isolates with MALDI-TOF score values between 2.00 and 3.00 were identified at the species level, while those with scores ranging from 1.70 to 1.99 were identified at the genus level.

[a] Pigment production detected under UV light.

3.2. Antimicrobial activity of LAB cultures

The second aim of this study was to evaluate the antimicrobial activity of LAB cultures against spoilage bacteria. For this purpose, 95 different strains belonging to 8 genera (*Bifidobacterium* spp., *Carnobacterium* spp., *Lacticaseibacillus* spp., *Lactobacillus* spp., *Lactiplantibacillus* spp., *Latilactobacillus* spp., *Levilactobacillus* spp. and *Limosilactobacillus*) were tested against *Pseudomonas* spp. and Enterobacteriaceae isolated from Stracciatella and soft cheeses. Two different tests were used to identify the most active strains: deferred antagonism using the culture-spot method and direct antagonism using the agar well diffusion technique. The results obtained using the culture-spot method showed high EIR values, suggesting that most LAB cultures were effective against the target bacteria (Table 3). The highest mean EIR values were observed in strains belonging to the genera *Latilactobacillus* (3.6 ± 0.7) and *Lacticaseibacillus* (3.4 ± 0.6) (data not shown). These data agreed with those of Bassi et al. (2020) who reported that the strains belonging to *Lat. sakei* and *Lac. rhamnosus* species were the most effective against Gram-negative spoilage bacteria isolated from fresh cheese.

A different scenario emerged when the antimicrobial activity was assessed by agar well diffusion method. As shown in Table 3, in the direct antagonism test, LAB cultures were generally ineffective in controlling the growth of spoilage bacteria. In fact, only one target strain (*Ps. proteolytica* SLP5) was strongly inhibited by three strains belonging to *Lactiplantibacillus plantarum* species. In addition, only two cultures exhibited an appreciable antimicrobial activity against *Se. marcescens* PS92 (Table 3). The genus *Lactiplantibacillus*, particularly the *Lpb. plantarum* species, proved to be the most effective LAB group in inhibiting spoilage bacteria. As recently reported by Shi et al. (2024), strains belonging to this species produce a broad spectrum of antimicrobial agents that can hamper the growth of undesirable bacterial species, making it the most promising natural competitor of the microbiota responsible for food spoilage.

As described above, the two methods used to evaluate the antimicrobial activity of LAB strains did not show similar results. These discrepancies can be attributed to the different approaches (deferred and direct antagonism) that characterize the two assays considered. In the culture-spot method (deferred antagonism), LAB cultures may produce different metabolites (organic acids, hydrogen peroxide and bacteriocins) that diffuse into the medium before the growth of the indicator strains, thus hampering their development. This phenomenon does not occur in the agar well diffusion assay (direct antagonism) since the LAB strains and target bacteria grow simultaneously (Gensler et al., 2020).

Considering the results of antimicrobial activity, 10 strains (3 wild

Table 3
Number of LAB strains that showed antimicrobial activity against target spoilage bacteria by culture-spot and agar well diffusion assays. In the brackets are reported the total number of strains considered for each genus considered.

Target strains	Antimicrobial activity	Antimicrobial activity								
		BIF	CNB	LCB	LB	LPB	LAT	LEV	LIM	Total
		(3)	(3)	(46)	(3)	(28)	(3)	(3)	(6)	(95)
Culture spot assay (EIR)										
Ps. gessardii	2+	-	-	-	-	-	-	-	-	-
STL2	3+	3	3	46	3	28	3	3	6	95
Ps. gessardii	2+	-	-	-	-	-	-	-	-	-
SLP3	3+	3	3	46	3	28	3	3	6	95
Ps. gessardii	2+	-	-	-	-	-	-	-	-	-
SLP5	3+	3	3	46	3	28	3	3	6	95
Buttiauxella spp.	2+	-	1	-	-	-	-	-	-	1
STL1	3+	3	2	46	3	28	3	3	6	94
Se. marcescens	2+	-	-	-	-	-	-	-	-	-
PS92	3+	3	3	46	3	28	3	3	6	95
Agar well diffusion assay (mm)										
Ps. gessardii	2+	1	1	12	1	14	-	1	-	30
STL2	3+	-	-	-	-	-	-	-	-	-
Ps. gessardii	2+	-	2	10	-	11	-	-	-	23
SLP3	3+	-	-	-	-	-	-	-	-	-
Ps. proteolytica	2+	-	2	29	-	19	-	-	1	51
SLP5	3+	-	-	-	-	3	-	-	-	3
Buttiauxella spp.	2+	-	1	-	-	11	-	-	-	12
STL1	3+	-	-	-	-	-	-	-	-	-
Se. marcescens	2+	-	-	1	-	1	-	-	-	2
PS92	3+	-	-	-	-	-	-	-	-	-

EIR: Effective inhibition ratio.
BIF:*Bifidobacterium* spp., CNB: *Carnobacterium* spp., LCB: *Lacticaseibacillus* spp., LB: *Lactobacillus* spp., LPB: *Lactiplantibacillus* spp., LAT: *Latilactobacillus* spp., LEV: *Levilactobacillus* spp., LIM: *Limosilactobacillus* spp.
Culture-spot assay: 2+: intermediate inhibition (0.5 < EIR <1.5); 3+: strong inhibition (EIR >1.5).
Agar well diffusion assay: 2+: intermediate inhibition (inhibition zone between 3.0 and 6.0 mm); 3+ strong inhibition (inhibition zone >6.0 mm).

and 7 from commercial cultures) were selected for further analyses (Table 4). *Lpb. plantarum* LPB68 and *Lac. paracasei* LAC16 were chosen for their antimicrobial activity against *Se. marcescens* PS92, while *Carnobacterium* spp. CNB1, *Lpb. plantarum* LPB48, LPB61, LPB68, LPB84, LPB233, LPB281 and LPBA as well as *Lac. paracasei* LAC02 strains were selected because they proved to be the most active strains against *Pseudomonas* spp. and *Buttiauxella* spp. In addition to our tests, CNB1 and LPB48 strains were also selected for their documented antimicrobial activity against Gram-negative bacteria in dairy products (Bassi et al., 2020; Spanu et al., 2017).

To investigate the nature of the LAB antimicrobial activity, the influence of CFSs and NCFSs on the growth spoilage bacteria was determined. This assay was performed evaluating the time needed to reach the inflection point of the indicator strains growth curve, which corresponds to their maximum growth rate (Navarro-Pérez et al., 2022). As reported in Table 5, it is possible to note that all the CFS exerted bactericidal effect against the spoilage bacteria; in fact, after 24 h of incubation, no growth of the target strains was observed. In contrast, after pH neutralization, almost all the NCFSs lost their antimicrobial capabilities. These findings confirm that the acid produced by the LAB cultures acts as an important hurdle to the spoilage bacteria growth in culture media. The antimicrobial effect of organic acids produced by LAB strains is well documented (Mani-López et al., 2022). These compounds, usually lactic and acetic acids but also tartaric, citric, malic, oxalic, and succinic acid, can determine an environmental pH reduction that hampers the growth of spoilage bacteria. Moreover, these molecules can penetrate the cytoplasmic membrane of target microorganisms and cause an intracellular acidification and successively the collapse of the transmembrane proton motive force (Mani-López et al., 2022). The antimicrobial activity of organic acids is not only related to pH

Table 4
Antimicrobial activity assessed by agar well diffusion assay and values of redox potential (Eh_{min}) in UHT milk of selected LAB strains.

Species	Source	Strains	Antimicrobial activity					$E_{h\ min}$
			STL2	SLP3	SLP5	STL1	PS92	(mV)
Carnobacterium spp.	Commercial	CNB1	2+	1+	2+	1+	-	−240.8
Lpb. plantarum	Commercial	LPB48	1+	1+	2+	1+	-	−326.7
	Wild	LPB61	2+	2+	3+	2+	-	−340.5
	Commercial	LPB68	2+	1+	2+	2+	2+	−303.6
	Wild	LPB84	2+	2+	3+	2+	-	−328.9
	Wild	LPB233	2+	2+	2+	2+	1+	−237.0
	Commercial	LPB281	2+	2+	3+	2+	1+	−328.1
	Commercial	LPBA	2+	1+	2+	2+	-	−309.4
Lac. paracasei	Commercial	LAC02	2+	1+	2+	2+	-	−337.8
	Commercial	LAC16	1+	1+	2+	1+	2+	−340.0

Antimicrobial activity: : no activity; 1+: low; 2+: intermediate; 3+: strong.
STL2: *Ps. gessardii*; SLP3: *Ps. gessardii*; SLP5: *Ps. proteolytica*; STL1: *Buttiauxella* spp.; PS92: *Se. marcescens*.

Table 5
Effect of neutralized cell-free supernatants (NCFS) and cell-free supernatants (CFS) on the time (h) required to reach the maximum growth rate of spoilage bacteria.

Sample	Species	Strains	Maximum growth rate (h)				
			STL2	SLP3	SLP5	STL1	PS92
Control +			12	6	13	7	8
CFS	*Carnobacterium* spp.	CNB1	>24	>24	>24	>24	>24
	Lpb. plantarum	LPB48	>24	>24	>24	>24	>24
		LPB61	>24	>24	>24	>24	>24
		LPB68	>24	>24	>24	>24	>24
		LPB84	>24	>24	>24	>24	>24
		LPB233	>24	>24	>24	>24	>24
		LPB281	>24	>24	>24	>24	>24
		LPBA	>24	>24	>24	>24	>24
	Lac. paracasei	LAC02	>24	>24	>24	>24	>24
		LAC16	>24	>24	>24	>24	>24
NCFS	*Carnobacterium* spp.	CNB1	12	6	13	7	8
	Lpb. plantarum	LPB48	12	6	13	7	8
		LPB61	11	6	16	7	8
		LPB68	13	7	11	7	7
		LPB84	13	6	13	7	8
		LPB233	13	6	13	7	8
		LPB281	12	6	13	7	7
		LPBA	12	6	13	7	8
	Lac. paracasei	LAC02	12	6	12	7	8
		LAC16	12	6	13	7	8

Control +: indicator strain with the addition of 50 μL of MRS broth.
STL2 and SLP3: *Ps. gessardii*; SLP5: *Ps. proteolytica*; STL1: *Buttiauxella* spp.; PS92: *Se. marcescens*.

reduction, but also to other mechanisms; for instance, lactate can permeabilize the outer membrane of Gram-negative bacteria, causing structural alterations in the phospholipid component and consequently the cell death (Alakomi et al., 2000). Acetic acid exerts strong membrane-disruptive effects in *Escherichia coli* strains, leading to cytoplasmic membrane destabilization, dissipation of the proton motive force and release of cellular constituents. Citric acid increases periplasmic permeability, whereas malic acid interferes with membrane-associated proteins, further compromising the integrity of Gram-negative bacterial cells (Zhang et al., 2023; Yoon et a., 2024). In addition to the acid production, the antimicrobial activity of LAB strains could be related to their reduction capability, in fact, at the temperatures and times found in the Crescenza cheese-making process all the selected LAB showed Eh_{min} values lower than – 200 mV (Table 4). As reported by Brasca et al. (2007), LAB species commonly used in cheese production differ widely in their ability to lower the redox potential during fermentation, and *Lpb. plantarum* and *Lac. paracasei* belong to the most reducing species among those used in dairy chain.

3.3. Inhibitory activity of LAB cultures against spoilage bacteria in litmus milk (LM)

The inhibitory effect of the LAB cultures on the growth of spoilage bacteria was further evaluated in LM following the parameters of the Crescenza cheese-making process. LM was previously inoculated with spoilage bacteria (3 $\log_{10}$ CFU/mL), and after 24 h of incubation at 4 °C, LAB strains were added at a concentration of 7 $\log_{10}$ CFU/mL. The use of LM allows the evaluation of the reduction activity of LAB cultures, as this metabolic process induces characteristic color changes in the medium, from purple to white. Considering the control samples (without the addition of LAB strains), the colour of LM inoculated with *Buttiauxella* and *Se. marcescens* changed from purple to red, while no colour changes were observed in the samples spiked with *Ps. gessardii* and *Ps. proteolytica* strains. With the addition of the adjunct cultures, the colour of LM samples changed from purple to white within the first 6 h of incubation, highlighting that the spoilage bacteria did not have a negative effect on the growth and the redox activity of LAB strains. Changes in redox potential are of great importance in the dairy sector, since the use of reducing LAB strains during the cheese-making process hampers the growth of aerobic bacteria (*Pseudomonas* and *Bacillus* spp.) in cheese-matrix (Beresford et al., 2001). In the control samples, without the addition of the LAB cultures, the content of *Ps. gessardii* SLP3 constantly increased achieving values of 5.5 ± 0.1 $\log_{10}$ CFU/mL after 7 days of incubation, while in the same conditions, the level of *Ps. gessardii* STL2 and *Ps. proteolytica* SLP5 raised about 1 log reaching values of 3.7 ± 0.3 and 3.8 ± 0.1 $\log_{10}$ CFU/mL. *Buttiauxella* STL1 grew during the first 24 h (achieving 3.8 ± 0.1 $\log_{10}$ CFU/mL) and its level remained constant for 7 days, while *Se. marcescens* did not grow under the conditions tested, but its cells were alive and viable throughout the experiment (2.9 ± 0.2 $\log_{10}$ CFU/mL). Considering the LAB cultures inoculated in LM samples, their content increased for all strains with an average value of 1.5 $\log_{10}$ CFU/mL (from 7.5 ± 0.3 to 9.0 ± 0.2). Overall, LAB counts in co-culture did not differ significantly from those in uninoculated controls (data not shown).

As shown in Table 6, *Lpb. plantarum* strains LPB61, LPB84, LPB233 and LPB281 exhibited strong antimicrobial activity against *Ps. gessardii* SLP3 (IP > 3.0) while all tested LAB strains were able to inhibit the growth of *Ps. proteolytica* SLP5 (IP ≥ 2.0). In contrast, the development of *Buttiauxella* STL1 and *Ps. gessardii* STL2 was strongly hindered only by the *Lpb. plantarum* LPB233 (IP: 2.5). None of the strains that inhibited the growth of *Se. marcescens* on agar plates (*Lpb. plantarum* LPB68 and *Lac. paracasei* LAC16) were able to hamper its growth in LM samples (IP: 0.0). In these conditions *Lpb. plantarum* LPB233 was found to be the most effective against the growth of spoilage bacteria. This strain showed the same activity of the other *Lpb. plantarum* (see Tables 4 and 5) in plate assay, highlighting that the antimicrobial power is strictly related to the environmental parameters, such as temperature, pH and grown medium (Wörmann et al., 2024). It is also worth considering that, the simultaneous presence of different bacterial species could lead to changes in the LAB metabolic pathways that can positively or negatively affect their activity against undesirable microorganisms (Canon, Nidelet, Guédon, Thierry, & Gagnaire, 2020).

Table 6
Inhibitory power (IP) of LAB cultures against spoilage bacteria in Litmus Milk. This assay was performed following the temperature and time profile found in the Crescenza cheese-making process. The data reported represent the IP of LAB at the end of the ripening period (7 days at 4 °C).

Species	Strains	Inhibitory power (IP)			
		STL2	SLP3	SLP5	STL1
Carnobacterium spp.	CNB1	0.0	0.3 ± 0.1	2.1 ± 0.1	0.3 ± 0.2
Lpb. plantarum	LPB48	0.2 ± 0.1	0.1 ± 0.1	2.1 ± 0.3	0.3 ± 0.1
	LPB61	0.6 ± 0.1	3.6 ± 0.3	2.1 ± 0.2	0.5 ± 0.3
	LPB68	0.8 ± 0.1	0.2 ± 0.1	2.0 ± 0.2	0.9 ± 0.2
	LPB84	0.5 ± 0.1	3.6 ± 0.3	2.3 ± 0.2	0.3 ± 0.1
	LPB233	2.5 ± 0.1	3.5 ± 0.2	2.0 ± 0.1	2.5 ± 0.3
	LPB281	0.3 ± 0.1	3.6 ± 0.3	2.3 ± 0.2	1.0 ± 0.1
	LPBA	0.4 ± 0.2	0.3 ± 0.1	2.0 ± 0.2	0.3 ± 0.1
Lac. paracasei	LAC02	0.3 ± 0.1	0.4 ± 0.2	2.1 ± 0.1	0.8 ± 0.2
	LAC16	0.2 ± 0.1	0.1 ± 0.1	2.0 ± 0.1	0.3 ± 0.1

IP: difference between the mean values of spoilage bacteria content in control samples and their load (mean values) in samples inoculated with LAB strains (both expressed in $\log_{10}$ CFU/mL).
STL2: *Ps. gessardii*; SLP3: *Ps. gessardii*; SLP5: *Ps. proteolytica*; STL1: *Buttiauxella* spp. Values are given as mean ± SD.

3.4. Inhibitory activity of adjunct culture mixtures against spoilage bacteria in Crescenza cheeses

Based on the antimicrobial and redox potential activity of the LAB strains, nine were selected to formulate four distinct LAB mixtures for use in the experimental cheese-making processes. Mixture 1 (Mix 1) was composed of four *Lpb. plantarum* strains (LPB48, LPB68, LPB84 and LPB233) while Mixture 2 (Mix 2) contained one *Carnobacterium* spp. (CNB1) and three *Lpb. plantarum* (LPB48, LPB233 and LPB281). Mixtures 3 and 4 (Mix 3 and 4) were obtained combining *Lpb. plantarum* and *Lac. paracasei* strains, precisely, Mix 3 contained LPB48, LPB61, LPB233 and LAC02 strains, whereas Mix 4 was composed of LPB48, LPB68, LPB233 and LAC16 strains. The content of LAB added during the cheesemaking and ripening process was determined using Petrifilm Lactic Acid Bacteria Count Plates. This system allowed us to enumerate only the LAB cultures, as it does not detect the presence of *St. thermophilus* ST021 used in this study (Neogen Corporation, 2024). All mixtures showed a similar trend: they were inoculated approximately at the same level (Mix 1: 7.9 ± 0.3 $\log_{10}$ CFU/mL; Mix 2: 7.6 ± 0.2 $\log_{10}$ CFU/mL; Mix 3: 7.5 ± 0.3 $\log_{10}$ CFU/mL and Mix 4: 7.6 ± 0.6 $\log_{10}$ CFU/mL), reached their maximum within the 7 days of storage at 4 °C (increasing their content by approximately 1.0-1.3 log) and then remained constant until the end of the experiments. This observation was consistent with Spanu et al. (2017) and Milesi et al. (2008) who showed as the adjunct lactobacilli content (*Carnobacterium* spp. and *Lpb. plantarum*) in soft cheeses gradually grew up to 10^8 CFU/g and did not decrease during the storage period.

No significant differences (P > 0.05) were found in the acidification evolution during the cheese-making process among control and experimental cheeses, except for those produced with the Mix 2 that showed a large drop in pH during the cooking step (T1) (Table 7). In the cheeses obtained by the addition of Mix 4, the acidification kinetics was slightly slowed down, but not significantly. During the ripening period the pH values recorded were similar in all cheese samples (from 5.3 to 5.5) both after 7 days at 4 °C (T4) than after 7 days at 10 °C (T5).

Otherwise, significant differences in the evolution of redox potential were observed among the experimental cheese-making processes (Table 7). In control cheeses, from the addition of starter culture (T0) to the salting stage (T2), E_h showed values constantly ranging from +107.9 to +120.9 mV. The redox potential of these samples did not reach a negative value because the strain used as starter (*St. thermophilus*) belong to a bacterial species known to have good acidifying power but poor reducing capacity (Morandi et al., 2016). In cheeses obtained with the addition of Mix 1 the redox potential decreased slowly without reaching negative values, while in the other samples manufactured with Mix 2, Mix 3 and Mix 4 the E_h dropped after the cooking step (T1), achieving values significantly different from those of the control samples (Table 7). It is important to highlight that the mixtures containing *Lpb. plantarum* and *Lac. paracasei* strains showed particularly strong reducing activity, reaching E_h values of −197.3 mV (Mix 3) and −306.3 mV (Mix 4) at T5. The reducing activity of LAB strains is related to their metabolic activity, in particular the use of oxidising molecules as electron

Table 7
pH and Eh evolution during the Crescenza cheese-making process obtained with *St. thermophilus* ST021 (CTRL) and *St. thermophilus* ST021 combined with the adjunct culture mixtures.

	Time	Samples				
	points	CTRL	Mix 1	Mix 2	Mix 3	Mix 4
pH	T0	6,6 ± 0,1	6,6 ± 0,1	6,6 ± 0,2	6,6 ± 0,1	6,6 ± 0,2
	T1	6,4 ± 0,1	6,1 ± 0,1	5,7 ± 0,2^{A}	6,1 ± 0,2	6,4 ± 0,1
	T2	5,5 ± 0,2	5,4 ± 0,1	5,2 ± 0,1	5,3 ± 0,1	5,7 ± 0,1
	T3	5,4 ± 0,1	5,4 ± 0,1	5,2 ± 0,1	5,3 ± 0,1	5,7 ± 0,1
	T4	5,3 ± 0,1	5,3 ± 0,1	5,3 ± 0,1	5,5 ± 0,1	5,5 ± 0,1
	T5	5,4 ± 0,1	5,3 ± 0,1	5,3 ± 0,1	5,5 ± 0,1	5,3 ± 0,1
E_h	T0	107,9 ± 56,1	105,5 ± 56,1	102,9 ± 56,1	103,7 ± 56,1	105,8 ± 56,1
(mV)	T1	110,7 ± 21,1	108,7 ± 12,1	104,8 ± 17,1	101,3 ± 7,2	106,9 ± 24,7
	T2	120,9 ± 18,7^{A}	63,0 ± 8,4^{B}	−104,5 ± 18,0^{C}	−197,3 ± 25,9^{D}	−306,3 ± 13,7^{E}

Values are given as mean ± SD.
Values followed by capital letters are different from the CTRL samples at the same time point ($P < 0.05$).
T0: Milk after the LAB mixtures addition; T1: cooking; T2: salting; T3: cheese after 24 h at 4 °C; T4: cheese after 7 days at 4 °C; T5: cheese after 7 days at 10 °C.
Mix 1: *Lpb. plantarum* LPB48, LPB68, LPB84, LPB233.
Mix 2: *Carnobacterium* spp. CNB1 and *Lpb. plantarum* LPB48, LPB233, LPB281.
Mix 3: *Lpb. plantarum* LPB48, LPB61, LPB233 and *Lac. paracasei* LCP02.
Mix 4: *Lpb. plantarum* LPB48, LPB84, LPB233 and *Lac. paracasei* LCP16.

acceptors (NADH oxidase) and the production of reducing compounds such as trioses and acetaldehyde (Caldeo & McSweeney, 2012; Roussel et al., 2022). As reported by many authors (Aljasir et al., 2020; Cachon, 2023; Gensler et al., 2020), during milk fermentation, the simultaneous presence of several LAB strains (starter and adjunct cultures) could negatively influence the activities of the same strains by reducing their performance (bacteriocin production, acidifying and reducing activity, etc.). Recently, Li et al. (2025) demonstrated that certain molecules, defined as quorum sensing inhibitors and produced by various bacteria (including LAB), can alter the normal redox activity of LAB cultures. It is probably for this reason that, mixtures composed of LAB strains with a good reduction capability (Mix 1 and 2) showed a lower reducing power than the individual strains.

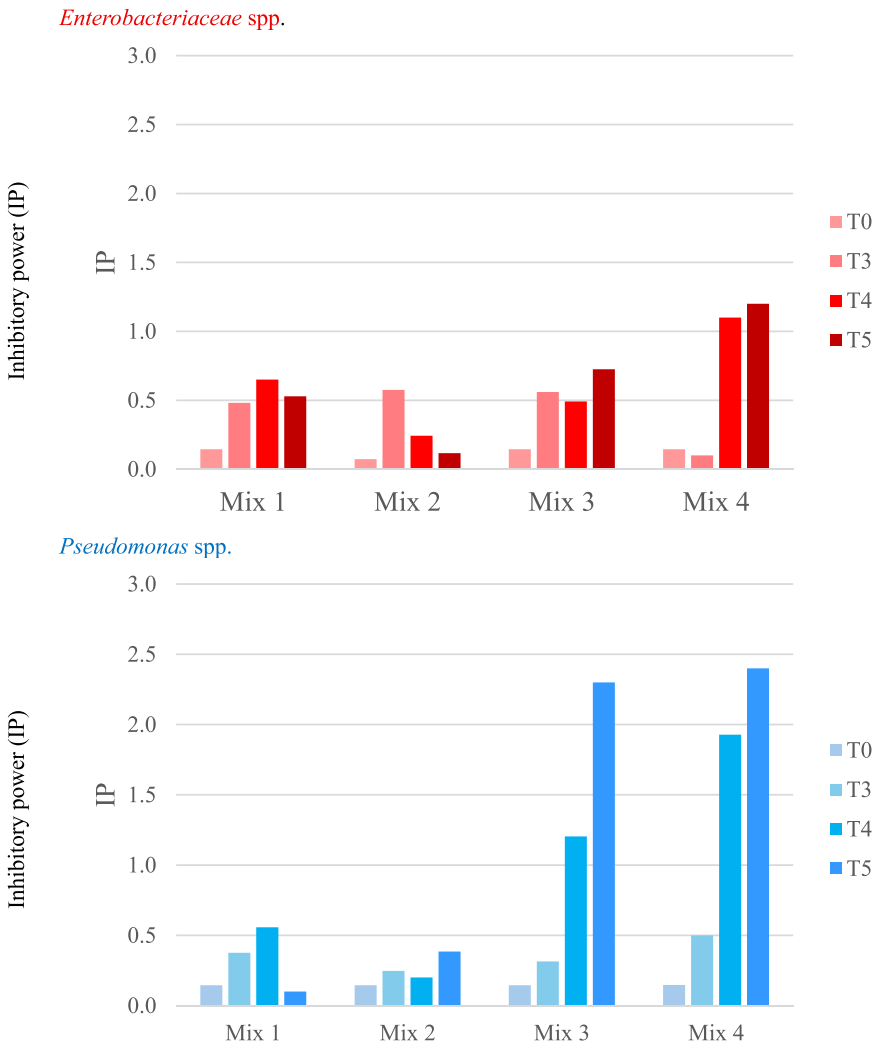
The effect of the LAB mixtures on the spoilage bacteria development was evaluated applying the cultivation-dependent approach. At the start of the trials, the average content of Enterobacteriaceae and *Pseudomonas* spp. in all samples was 2.8 ± 0.2 and 2.9 ± 0.3 $\log_{10}$ CFU/mL, respectively. In control cheeses, during the cheese-making process and ripening periods, these values increased achieving 4.3 ± 0.6 and 4.4 ± 0.8 $\log_{10}$ CFU/g.

As shown in Fig. 2, the level of Enterobacteriaceae in cheeses produced with Mix 1, 2, and 3 was comparable to that observed in the control samples ($0.1 < IP < 0.7$). A different trend was detected in cheeses obtained with Mix 4, where a reduction of approximately 1 log was recorded after the ripening stage at 4 °C (IP: 1.1) and the subsequent storage at 10 °C (IP: 1.2) (Fig. 2). A similar reduction in Enterobacteriaceae content was achieved by Bassi et al. (2020) using protective cultures containing *C. maltoromaticum*, *Lac. rhamnosus*, and *Lat. sakei* in the production of fresh cheeses. This activity was related to the production of organic acids (formate and acetate) and the synthesis of bacteriocins. The ability of the LAB species to inhibit the growth of Enterobacteriaceae in dairy products is documented (Rangel-Ortega et al., 2023); however, most studies have focused on *E. coli* and *Salmonella* spp., while data on *Se. marcescens* and *Buttiauxella* spp. are not currently available. Considering the *Pseudomonas* spp. content, Mix 1 and Mix 2 were unable to effectively inhibit the growth of these bacteria throughout the cheese-making process, showing IP values ranging between 0.1 and 0.6. In contrast, during the ripening period of Crescenza cheese (4 °C for 7 days), *Pseudomonas* growth was limited by Mix 3 (IP: 1.2), while Mix 4 reduced the level of spoilage bacteria by approximately 2 log (IP: 1.9) (Fig. 2). At 10 °C, the temperature most frequently found in the European domestic refrigerators (Bonanno et al., 2024), Mix 3 and 4 displayed the strongest antimicrobial activity, reducing the *Pseudomonas* counts by more than 2 log (IP: 2.3 and 2.4) (Fig. 2). These results are consistent with previous studies reporting that the use of protective cultures containing various LAB strains can effectively inhibit *Pseudomonas* growth during the storage of soft cheeses (Bassi et al., 2020; Spanu et al., 2017). Interestingly, Mix 4, which had the greatest reducing capability (E_h: 306,3 mV), was the most effective mixture against spoilage bacteria. This observation supports the hypothesis that LAB strains characterised by high reducing activity are able to influence the microbial diversity in dairy products, for example by excluding the development of obligate aerobic bacteria such as *Pseudomonas* (Brasca et al., 2007; Cachon, 2023). Moreover, our results confirm the hypothesis reported by Callon et al. (2016), which suggested that LAB antimicrobial activity was related not only to the production of bacteriocins and/or organic acids, but also to their reducing ability. During ripening at 4 °C, no abnormal surface discoloration was observed in any of the samples. However, at the end of storage at 10 °C, a pale pink discoloration appeared on the surface of the control cheeses (Fig. 3). This observation is consistent with Ruan et al. (2024), who showed that *Se. marcescens* strains did not produce prodigiosin at 4 °C, whereas, a small amount of the pigment was synthesized at 10 °C. These findings suggest that LAB cultures can inhibit pigment production by spoilage bacteria even when their growth is not suppressed. This effect could be attributed to quorum sensing inhibitors produced by LAB strains, such as *Lpb. plantarum*, which downregulate genes involved in pigment synthesis (Prazdnova et al., 2022).

Protective cultures are microorganisms used to control the growth of undesirable bacteria in fermented foods without altering sensorial properties and must not pose health risks. In the European Union, LAB must hold Qualified Presumption of Safety (QPS) status granted by European Food Safety Authority (EFSA), providing a standardized preassessment of microbial safety and in the United States, protective cultures may be used if they comply with Generally Recognized as Safe (GRAS) requirements established by the U.S. Food and Drug Administration (FDA). Like QPS, GRAS status indicates safety based on scientific evidence or a documented history of safe use (Garín-Murguialday et al., 2026). Once QPS or GRAS status has been recognized, protective cultures are not subject to defined maximum usage limits; however, if their viable cells remain in the finished cheese, they must be listed on the label, distinguishing them from starter cultures driving primary fermentation (Fernandes et al., 2025).

The use of these cultures can generate significant economic benefits for the dairy sector, as it could extend product shelf life up to 20–50%, reducing waste-related losses by 5–15% (estimated at € 200–500 million annually across the EU dairy sector), and decreasing costs associated with the use of chemical preservatives. For this reason, the use of protective cultures is expected to grow, with an annual growth rate ranging from 4.3% to 23.3% by the end of the decade (Fischer & Titgemeyer, 2023). Currently, dairy products represent the primary field of application for these bacteria, representing for 57.9% of commercially available cultures. They are followed by sausages and other meat products (23.7%), milk analogues (9.2%), vegan products (3.9%), and fish and seafood products (2.6%) (Fischer & Titgemeyer, 2023).

Several dairy products containing strains of *Lpb. plantarum* and *Lac. paracasei* are commercially available (e.g., Actimel yogurt contains *Lac. paracasei*; Nonno Nanni probiotic stracchino contains *Lactobacillus acidophilus* and *Lac. casei*). These products are primarily formulated to



IP: difference between the mean values of spoilage bacteria content in control samples and their load (mean values) in samples inoculated with the mixtures of adjunct cultures (both expressed in $\log_{10}$ CFU/mL).
T0: Milk after the LAB mixtures addition; T3: cheese after 24 h at 4 °C; T4: cheese after 7 days at 4 °C; T5: cheese after 7 days at 10 °C.
Mix 1: *Lpb. plantarum* LPB48, LPB68, LPB84, LPB233.
Mix 2: *Carnobacterium* spp. CNB1 and *Lpb. plantarum* LPB48, LPB233, LPB281.
Mix 3: *Lpb. plantarum* LPB48, LPB61, LPB233 and *Lac. paracasei* LCP02.
Mix 4: *Lpb. plantarum* LPB48, LPB84, LPB233 and *Lac. paracasei* LCP16.

Fig. 2. Inhibitory power (IP) of LAB mixtures against spoilage Crescenza cheese samples.

provide health benefits, such as supporting the immune system and maintaining intestinal microbiota balance. However, they may have some limitations, including higher prices compared with non-probiotic equivalents, specific storage requirements to preserve probiotic viability until consumption, and labeling restrictions to comply with health claim regulations. While these commercial products are designed for human health, the protective cultures used in this study were set up to improve product quality and shelf life. The application of these protective cultures may involve some drawbacks, such as additional production costs and the need for proper application to ensure effectiveness. On the other hand, they offer several advantages, including replacing synthetic chemical preservatives, reducing food waste by extending product shelf life, and, in some cases, enhancing the organoleptic characteristics of the product, as recently described by Bonazza et al. (2025).

4. Conclusion

Cheese producers are always looking for new strategies to ensure the quality and safety of their products while extending their shelf life. For this reason, new technological approaches are necessary to reduce the content of undesired microorganisms throughout the dairy chain. In this study, five spoilage bacteria responsible for cheese discoloration were isolated from Stracciatella and other soft cheeses. The strains were identified by MALDI-TOF MS analysis and assigned to *Ps. gessardii* (2 strains), *Ps. proteolytica*, *Se. marcescens* and *Buttiauxella* spp. Wild and commercial LAB strains were first screened *in vitro* and then evaluated for their ability to inhibit these spoilage bacteria during Crescenza cheese production and storage. Our results demonstrated that certain LAB mixtures were able to inhibit the growth of *Pseudomonas* strains at concentrations comparable to those typically found in natural contamination. This inhibitory activity was both species- and strain-dependent

Fig. 3. Development of a pale pink coloration on the surface of the control cheeses after 7 days of storage at 10 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and was associated with the technological properties of the LAB strains, in particular showing a strong correlation with their reducing power. The LAB mixture exhibiting the highest reducing activity showed the strongest anti-spoilage effect. These findings emphasize the importance of redox potential as a key parameter for selecting LAB cultures, as controlling reduction kinetics during cheese manufacture can improve both the quality and safety of dairy products.

CRediT authorship contribution statement

S. Morandi: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **T. Silvetti:** Writing – review & editing, Formal analysis. **F. Bonazza:** Writing – review & editing, Formal analysis. **M. Brasca:** Writing – review & editing, Supervision, Funding acquisition, 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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Data availability

Data will be made available on request.

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