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RESEARCH

Lacticaseibacillus casei as Anti-blowing Agents: Impact on the Evolution of Ripening and Sensory Profile of Montasio Cheese

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Abstract
Recently, the *Lacticaseibacillus casei* group strains have been gaining growing interest due to their potential to be used as secondary adjunct cultures in cheese. This work aimed to test autochthonous *Lb. casei* strains as anti-blowing agents and to evaluate their impact on the evolution of the ripening and the sensory profile of Montasio cheese. The cheesemaking trial included a control production without lysozyme (C1), a control with lysozyme (C2), and four experimental productions, each containing a different pool of autochthonous *Lb. casei* strains (EX1-4). Samples were taken during ripening, and physicochemical, microbiological, and sensory analyses were carried out. *Lb. casei* counts indicate that the selected strains survived the cheesemaking and maintained their viability of about 9 log cfu g^{-1} at the end of the ripening. Only EX3 showed a significant slowdown of the proteolytic index compared to controls over time. Furthermore, from the principal component analysis, it emerged that at the end of the 120-day-long ripening, C2 and the experimental samples were characterized by similar profiles of volatile compounds. The late-blowing defect (LBD) was observed exclusively in C1, whereas it was not detected in the control sample with lysozyme or in any experimental samples. These findings not only confirmed the efficacy of lysozyme in preventing LBD but also supported the effectiveness of the selected *Lb. casei* strains as anti-blowing agents with the ability to contribute to the final volatile profile without compromising the typicality of the product.

Keywords Secondary adjunct culture · Late blowing defect · Aromatic activity · Non-starter lactic acid bacteria · Anticlostridial activity · PDO cheese

Introduction

One of the major causes of spoilage in semi-hard and hard cheeses is the late-blowing defect (LBD) caused by the outgrowth of spore-forming bacteria belonging to the *Clostridium* genus, which are initially present in the milk. This defect appears in the advanced stages of the ripening period when favorable anaerobic conditions for the germination of the endospore are established (Drouin et al., 2012). Although *Clostridium tyrobutyricum* is considered the primary cause of LBD, other contributing species include *Clostridium sporogenes*, *Clostridium beijerinckii*, and *Clostridium butyricum* (Ávila et al., 2014). These species mainly ferment lactic acid to butyric acid, acetic acid, carbon dioxide, and hydrogen. Consequently, the cheese wheel expands under the pressure of the gases, resulting in holes, cracks, and slits, accompanied by unpleasant aromas and rotten tastes because of the development of acids, thus resulting in high economic losses for producers (Sheehan, 2011). Due to the ubiquitous presence of butyric clostridia in the agricultural environment, the risk of contamination by spores can be reduced with good breeding practices, and adequate hygiene of the stable, feeding, and milking, but cannot be completely eradicated (Brändle et al., 2016). Bactofugation, microfiltration, and the addition of lysozyme or nitrate are the most used methods for preventing LBD. However, these methods are not always applicable or are prohibited for the production of certain types of cheese (Ávila et al., 2014). Among these, hen lysozyme remains the predominant and effective method to prevent the defect, but there are growing concerns about its potential allergenicity in sensitive consumers due to the considerable residual levels (250–400 mg kg^{-1}) found in

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cheese and increasing reports of allergic reactions (Bogovič Matijaši et al., 2007; Carminati et al., 2023). Furthermore, the growing demand for minimally processed and additive-free foods is pushing the dairy industry to look for alternative solutions to prevent LBD.

In recent years, there has been a growing interest in the use of lactic acid bacteria (LAB) as adjunct cultures and their antimicrobial metabolites as anti-spoilage agents. Adjunct cultures can be described as selected strains added to milk during cheesemaking for purposes other than lactic acid production (El Soda et al., 2000). Their influence on cheese ripening is a strain-specific trait, therefore to be considered suitable adjunct cultures, bacteria must not bring defects, attain and sustain high cell densities throughout the ripening period, and enhance the overall quality of the cheese (Irlinger et al., 2017). LAB can counteract undesirable microorganisms by competing for nutrients and producing various antimicrobial compounds, such as lactic acid, acetic acid, hydrogen peroxide, bacteriocins, and antifungal peptides (Rehaiem et al., 2012; Shi & Maktabdar, 2022). Inoculation of milk with LAB during the cheesemaking process has already been studied to successfully prevent LBD (Bogovič Matijaši et al., 2007; Garde et al., 2011; Gómez-Torres et al., 2014; Rilla et al., 2003). In particular, the *Lacticaseibacillus casei* group, which includes *Lb. casei*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus*, is becoming increasingly important due to its technological and antimicrobial properties (Aljewicz & Cichosz, 2017; Bertazzoni Minelli et al., 2004; Gomes da Cruz et al., 2009; Rodi et al., 2020; Rodriguez et al., 2011). These species have been identified in various semi-hard and hard cheeses during advanced stages of ripening, indicating their belonging to the non-starter lactic acid bacteria (NSLAB) group (Bottari et al., 2018; Carraro et al., 2011; Gatti et al., 2014; Marino et al., 2003). Isolating and selecting adjunct cultures from the same type of cheese they will be added to, ensures their adaptation to the cheesemaking process and the food matrix, thereby preventing any adverse impact on the technological and sensory characteristics (Gobbetti et al., 2015). This is crucial for protected designation of origin (PDO) cheeses, as they have stringent production criteria and must have features associated with the geographical environment (Innocente et al., 2013). In these cases, adding suitable adjunct microbial cultures may preserve the typical features and differentiation of PDO cheeses and protect against LBD (Centeno et al., 2022).

As a matter of fact, in a previous study, autochthonous NSLAB strains belonging to the *Lb. casei* group were directly isolated from the semi-hard PDO Montasio cheese, and tested for their potential to be used as adjunct cultures (Renoldi et al., 2024). Montasio is a semi-cooked cheese exclusively made in the North-East of Italy with raw or thermized cow's milk (Marino et al., 2008). Because of its designation and long ripening time, the secondary microflora of Montasio cheese is expected to develop the typical characteristics related to the geographical area and to counteract microorganisms responsible for undesired fermentations at the same time. In this cheese, the presence of the *Lb. casei* group becomes relevant after 60 days and remains consistently present until 150 days of ripening (Carraro et al., 2011). For these reasons, isolated strains were preliminarily tested for their anti-clostridial activity towards *Clostridium* strains and aromatic potential in a curd-based medium (Renoldi et al., 2024). Several *Lb. casei* strains showed in vitro activity against *Clostridium* spores responsible for LBD, as well as aroma profiles similar to those produced by commercial adjunct cultures for Montasio cheese. Although these autochthonous strains were already tested in a model system, the scale-up represents the next step necessary to verify the real effectiveness of these strains to be used as adjunct cultures in Montasio cheese. Therefore, the current study aimed to assess the use of selected pools of *Lb. casei* group strains as adjunct cultures in the production of Montasio cheese. To evaluate the efficiency of the added strains, physico-chemical properties, aroma profile, and sensory properties as well as the presence of LBD were monitored over time.

Materials and Methods

Bacterial Strains

Eight strains belonging to the species *Lb. paracasei* (named C70, C121, C138, C177, C184, C245, and C308) and *Lb. rhamnosus* (C154) were used in this study. The strains were previously isolated from Montasio cheese, and characterized for their anticlostridial, proteolytic, and esterase activity, as well as for their ability to produce volatiles in a curd-based medium (Renoldi et al., 2024). Based on their ability to contribute to volatilome and to inhibit clostridial spores, strains were combined into four pools (I-IV) as shown in Table 1. Strains were maintained at − 80 °C in De Man-Rogosa-Sharpe agar (MRS) broth (Oxoid, Milan, Italy) with 30% glycerol (w/v) until use. After reactivation in MRS broth, each strain was inoculated at 1% (v/v) into 50 mL of MRS broth incubated overnight at 30 °C. Cells were then recovered by centrifugation (10.000 × g for 5 min at 4 °C) and washed three times with Maximum Recovery Diluent (MRD) (Oxoid, Milan, Italy). The microbial biomasses were pooled, resuspended in sterile UHT milk (200 mL with a concentration of about 9 log cfu mL^{-1}), and used for cheesemaking.

Table 1 Pools of *Lb. casei* group strains inoculated in each experimental cheesemaking trial

Pool	Strain	Species	Main activity[a]
I	C70	*Lb. paracasei*	Anticlostridial
	C308	*Lb. paracasei*	Aromatic
	C177	*Lb. paracasei*	Anticlostridial
	C121	*Lb. paracasei*	Anticlostridial
II	C70	*Lb. paracasei*	Anticlostridial
	C308	*Lb. paracasei*	Aromatic
	C184	*Lb. paracasei*	Anticlostridial
	C138	*Lb. paracasei*	Anticlostridial
III	C70	*Lb. paracasei*	Anticlostridial
	C308	*Lb. paracasei*	Aromatic
	C177	*Lb. paracasei*	Anticlostridial
	C154	*Lb. rhamnosus*	Aromatic
IV	C70	*Lb. paracasei*	Anticlostridial
	C308	*Lb. paracasei*	Aromatic
	C184	*Lb. paracasei*	Anticlostridial
	C245	*Lb. paracasei*	Anticlostridial

[a]Source, Renoldi et al. (2024)

Cheese Manufacture

The following cheesemaking trials were carried out on the same day using the same batch of milk: control production without lysozyme (C1), control production with lysozyme (250 mg kg^{-1}) (C2), and four different experimental productions (EX1, EX2, EX3, EX4) where the *Lb. casei* pools I – IV (Table 1) were added. All cheesemaking processes were carried out following the traditional production method outlined by Innocente et al. (2013) with few modifications. After thermization at 64.5 °C for 15 s, milk was processed in a 200-L Milk&Cheese vat (FDstore s.r.l., Modena) using a thermophilic commercial starter culture (Aniferm Montasio NMB, ALCE s.r.l, Novara, Italy). In the experimental production, the *Lb. casei* pool was added in the vat at a final concentration of about 6 log cfu mL^{-1}. The curd was transferred into circular molds and pressed for 2 h at 300 kPa. The cheese was then salted in brine (NaCl 16–18% w/w) for 24 h at 11 °C, then ripened at 8 °C for 120 days at a relative humidity of 86%. Milk, and cheeses at different stages of ripening were analyzed for microbiological and physicochemical purposes.

Microbiological Analysis

Milk and cheese samples at 10, 30, 60, 90, and 120 days of ripening were decimally diluted in MRD and dilutions spread onto Maltose-MRS-Vancomycin (MMV) agar plates (Di Lena et al., 2015) for *Lb. casei* enumeration. Plates were incubated at 30 °C for 48 h under microaerophilic conditions.

Detection of butyric clostridial spores in milk was performed with the Most Probable Number (MPN) technique. 10, 1, and 0.1 mL of milk were inoculated in a 3 × 3 dilution series in RCM-lactate medium and treated at 85 °C for 15 min, cooled, and incubated at 37 °C for 7 days under anaerobic conditions (Andrighetto et al., 2023). Results were expressed in MPN L^{-1} following tables of Mcbride (2003).

Analysis of Volatile Compounds

The volatile fraction of cheese samples at 10, 30, 60, 90, and 120 days of ripening was analyzed using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC–MS) as previously described by Renoldi et al. (2024). Briefly, a 2 cm × 50/30 µm Stableflex 24Ga divinylbenzene/carboxene/poly-dimethylsiloxane (DVB/CAR/PDMS) coated SPME fiber (Supelco, Bellefonte, Pennsylvania) was exposed to the headspace for extraction after equilibration at 60 °C for 30 min. A QP2020 NX GC–MS system (Shimadzu Corporation, Kyoto, Japan) with a DB-WAX capillary column (30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness; Agilent Technologies, Santa Clara, California) was used to separate and identify volatile compounds. For the analysis, scan mode with a mass range of 25 to 350 m/z was employed. The GC–MS software version 4.52 (Shimadzu Corporation) was utilized to evaluate the chromatographic profiles. The compounds were identified through spectra comparison, utilizing the NIST/EPA/NIH 20 Mass Spectral Library (John Wiley & Sons Inc., Hoboken, New Jersey) and Kovat's retention index (RI) from the literature (https://webbook.nist.gov/chemistry/). The data were reported as absolute areas of peaks recorded in the sample's headspace.

Quantification (mg kg^{-1}) was performed for butyric acid as the main product of butyric fermentation. For this purpose, the butyric acid standard (Sigma Aldrich, Milan, Italy) was used at different concentrations for external calibration to obtain the calibration curve ($R^2 = 0.99$).

Acquisition of Cheese Images

Images of cheese samples at 120 days of ripening were acquired using a digital camera (EOS 550D, Canon, Milan, Italy) equipped with an image acquisition cabinet (Immagini & Computer, Bareggio, Italy).

Chemical and Physicochemical Analyses

Samples were obtained by manually removing the rind and the underrind part, 1 cm from the crust, and then ground

with a mixer (1000 W La Moulinette mixer; Moulinex, Écully, France).

Determination of pH

The pH of curd just after the extraction from the vat, and of cheeses after 10, 30, 60, 90, and 120 days of ripening was determined using a pH meter (Basic 20, Crison, Barcelona, Spain) calibrated with standard solutions at pH 4, 7, and 9. Measurements were carried out by immersing the electrode in a dispersion of water and cheese prepared by suspending 5 g of the sample in 10 mL of distilled water at room temperature (Innocente & Biasutti, 2013). The mixture was then homogenized at 7000 rpm for 1 min using an Ultra Turrax T18 homogenizer (IKA, Staufen im Breisgau, Germany).

Determination of the Moisture

Moisture of cheese samples at 10, 30, 60, 90, and 120 days of ripening was assessed gravimetrically by using 2 g of sample. The weight difference of the sample before and after overnight drying in a Vuotomatic 50 vacuum oven at 75 °C (Bicasa, Milan, Italy) was measured (Innocente et al., 2007).

Determination of the Proteolytic Index

The percentage ratio between water-soluble nitrogen and total nitrogen was used as the proteolytic index (PI), as previously described by Masotti et al. (2010). The determination of total nitrogen of cheese samples at 10, 30, 60, 90, and 120 days of ripening was performed following the Kjeldahl method (IDF, 2011) on 0.5 g of cheese. For the determination of water-soluble nitrogen, 4 g of cheese was smashed in a mortar with distilled water at 40 °C until the formation of a smooth dispersion, diluted to 100 mL using distilled water at room temperature, and analyzed using the Kjeldahl method.

Sensory Analysis

A panel of ten judges was trained for a quantitative descriptive sensory test on 120-day-ripened cheeses following the method proposed by Bérodier et al. (1997). Basic tastes (spicy, salty, acid, bitter), structural and mechanical characteristics (elasticity, hardness, deformability, friability, adhesiveness), and the intensity of aroma and odor were measured using a seven-point scale. To create a comprehensive and representative odor and aroma profile of each cheese sample, the descriptors detected by 50% of the judges were considered key attributes.

Samples were prepared by removing the external part (1.5 cm from the crust) of the cheese wheels and obtaining sticks 1.5 cm thick, 1.5 cm wide, and 5 cm high. Samples were placed in Petri dishes randomly coded with a three-digit number and stored at 15–16 °C until tasting. To conduct the tests the "SmartSensory Box2, internet of sense" version 2.2.44 software (Smart Sensory Box, Sassari, Italy) was used. The study complied with the principles established by the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine (protocol n. 0019200).

Statistical Analysis

Data are expressed as mean ± standard deviation of at least three measurements carried out on three different curds from the same cheesemaking process. Statistical analysis of the data was performed using the R program, version 4.1.0 (The R Foundation for Statistical Computing, Vienna, Austria). The difference between means was calculated using ANOVA and post hoc Tukey's test ($p < 0.05$). Origin Pro 9 (OriginLab, Northampton, England) was used to perform principal component analysis (PCA) and a heatmap with hierarchical clustering analysis on volatile compounds based on Ward's variance method and a Euclidean distance metric. It was carried out with pre-processed, scaled, and transformed log10[x] data.

Results and Discussion

Microbiological Analyses

From the MPN analysis, it emerged that the number of clostridial spores in the thermised milk used for cheesemaking was 800 spores L^{-1}. From the literature data, threshold spore concentrations associated with this defect in cheese widely range from 5 to 1000 spores L^{-1} of milk (Ingham et al., 1998; Klijn et al., 1995). This large variability may be attributable to several factors, such as the clostridial strains involved and the processing conditions applied during cheese production and ripening (Burtscher et al., 2020).

The *Lb. casei* group viable counts in milk and during ripening are shown in Table 2. In both control samples, *Lb. casei* strains were already present in the milk used for cheesemaking, confirming that this group is part of the milk microbiota of Montasio cheese (Marino et al., 2003). As expected, during the transition from milk to curd, *Lb. casei* counts increase, also because of the concentration effect from whey removal. The magnitude of the increase in microbial loads in the first 10 days was slightly different in the samples, as this was the result not only of the metabolic activity of the microflora of the acidifying starter and the raw milk, but also of the presence of lysozyme (in C2) and the metabolism carried out by the different pools of *Lb. casei* added in the experimental samples C3-C6. The

Table 2 Log counts (expressed as cfu mL^{-1} and cfu g^{-1} for milk and cheese, respectively) of *Lacticaseibacillus casei* group strains during ripening

Ripening time (d)	Milk	10	30	60	90	120
C1	2.17 ± 0.13^{Bb}	5.34 ± 0.10^{Bb}	8.26 ± 0.24^{Aa}	8.51 ± 0.42^{Aa}	8.88 ± 0.61^{Aa}	8.97 ± 0.33^{Aa}
C2	2.20 ± 0.06^{Bb}	4.45 ± 0.14^{Bb}	8.32 ± 0.34^{Aa}	8.37 ± 0.34^{Aa}	8.74 ± 0.41^{Aa}	9.66 ± 0.23^{Aa}
EX1	6.34 ± 0.12^{Ab}	8.27 ± 0.22^{Aa}	8.96 ± 0.27^{Aa}	9.05 ± 0.27^{Aa}	9.35 ± 0.35^{Aa}	9.81 ± 0.64^{Aa}
EX2	5.92 ± 0.17^{Ab}	8.71 ± 0.34^{Aa}	8.90 ± 0.19^{Aa}	9.20 ± 0.19^{Aa}	9.26 ± 0.52^{Aa}	9.46 ± 0.35^{Aa}
EX3	5.76 ± 0.23^{Ab}	7.94 ± 0.26^{Aab}	8.65 ± 0.11^{Aa}	8.85 ± 0.11^{Aa}	9.28 ± 0.44^{Aa}	9.38 ± 0.28^{Aa}
EX4	6.17 ± 0.31^{Ab}	8.22 ± 0.09^{Aa}	8.68 ± 0.18^{Aa}	9.06 ± 0.55^{Aa}	9.41 ± 0.59^{Aa}	9.36 ± 0.44^{Aa}

Different capital letters mean statistically different values within the same column ($p < 0.05$). Different lowercase letters mean statistically different values within the same row ($p < 0.05$)

concentration of *Lb. casei* gradually increased reaching approximately 8–9 log cfu g^{-1} in experimental productions after 10 days of ripening, whereas, in the control productions, these values were reached after 30 days of ripening (Table 2). After 60 days of ripening the *Lb. casei* counts reached their maximum and remained constant for up to 120 days in all samples. These results confirm the ability of *Lb. casei* strains to grow and dominate the microflora in semi-hard cheeses during the ripening phase, as previously observed by Christiansen et al. (2010). Although microbial counts were similar between control and experimental samples after 30 days of ripening (Table 2), it is important to highlight that the strains present in the experimental samples were specifically selected for their demonstrated anti-clostridial ability and their ability to produce volatile compounds similar to those made by commercial adjunct cultures for Montasio cheese (Renoldi et al., 2024).

LBD Presence in Cheese Samples

To monitor the occurrence of the late blowing defect in both control and experimental cheese samples, the concentration of butyric acid (mg kg^{-1}) was measured throughout the ripening process (Fig. 1). A progressive increase in butyric acid levels was observed in all samples over time. However, after 60 days of ripening, the butyric acid content of the lysozyme-free control sample (C1) was significantly higher ($p < 0.05$) than other samples. The experimental samples (EX) and the lysozyme-treated control samples (C2) showed negligible differences over time. After 120 days of ripening, these samples displayed small, numerous, and uniformly distributed holes, typical of Montasio PDO cheese. These characteristic holes can be attributed to facultative or obligate heterofermentative bacteria (lactobacilli and *Leuconostoc*), but can also derive from the formation of γ-butyric acid from the bacterial metabolization of the glutamic acid naturally present in the cheese (Fig. 2B, C) (Innocente & Corradini,

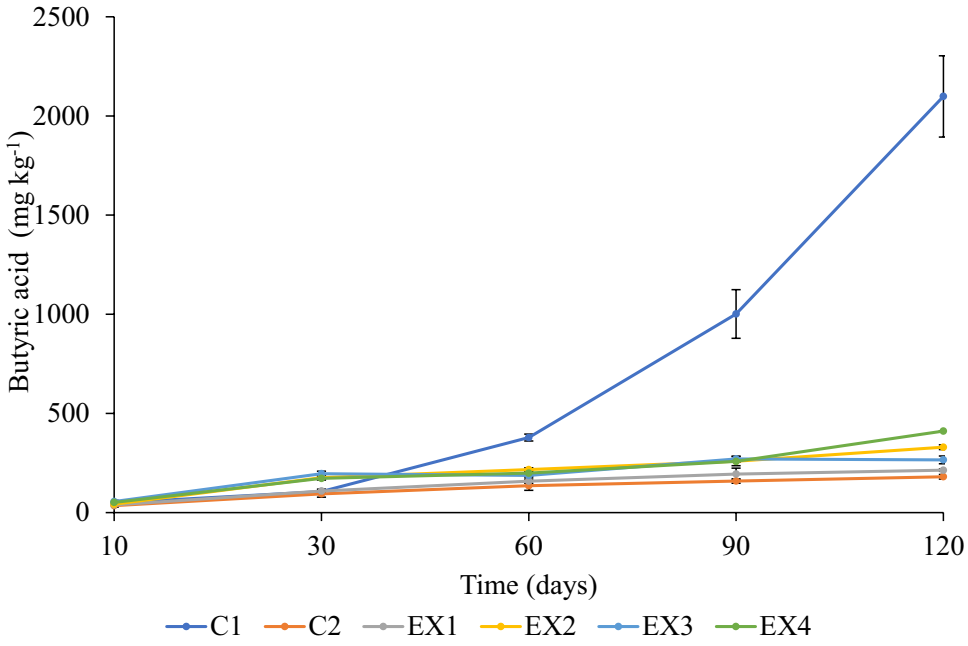


Fig. 1 Evolution of butyric acid (mg kg^{-1}) present in the headspace of cheese samples (mean ± SD, $n = 3$)

Fig. 2 Pictures of the cheeses at 120 days of ripening. **A** Control sample without lysozyme (C1). **B** Control sample with lysozyme (C2). **C** Experimental sample with the inoculum of *Lb. casei* (EX1)

1998; Innocente et al., 2002; Maifreni et al., 2002). The absence of visible signs of LBD in these samples suggests that the concentration of butyric acid may have been too low to be linked to the defect. The lack of the defect in sample C2 was likely due to the presence of lysozyme, which is known to have anticlostridial activity (Carminati et al., 2023). In the case of experimental samples, it is potentially attributable to the antimicrobial effect of the intentionally inoculated *Lb. casei* strains, which phenotypically demonstrated the ability to inhibit clostridia (Renoldi et al., 2024). The anticlostridial activity may be attributed to the production of several molecules, including organic acids (mainly lactic and acetic acid), bacteriocins, hydrogen peroxide, and carbonyl compounds (acetoin, diacetyl, and acetaldehyde) (García-Cano et al., 2019; Özogul & Hamed, 2018). It cannot be excluded that the *Lb. casei* strains present in sample C1 from the beginning (2.17 log cfu $mL^{-1} \pm 0.13$) may have shown antimicrobial activity against clostridia; however, their limited load in milk determined different growth kinetics compared to the experimental samples, where the initial *Lb. casei* load was approximately 6 log cfu mL^{-1}. Therefore, the inoculated strains' faster multiplication and more effective antimicrobial activity may have caused the absence of the defect. Conversely, in the control sample produced without lysozyme (C1) the LBD was notably present, and at 90 and 120 days of ripening was visible to the naked eye with several holes and cracks typical of this defect (Fig. 2A). These results suggest that the *Lb. casei* pools, previously selected for their anticlostridial properties, were as effective as lysozyme in preventing LBD.

Physicochemical Analysis

In addition to the microbiological analyses, the impact of the *Lb. casei* pools on the physicochemical characteristics of the cheese was also evaluated. Moisture evolution as a function of ripening time is shown in Table 3.

During the ripening period, moisture content progressively decreased both in control and experimental samples, due to the dehydration process that characterizes this phase. The data indicate that dehydration progresses similarly across the various samples, with all cheeses exhibiting comparable moisture values starting from 60 days of ripening.

Similar considerations can be made for the pH values of cheeses at different ripening times, with no significant differences between samples. The pH of the curd just after the extraction from the vat was very similar between all the samples ranging from 6.35 ± 0.01 to 6.48 ± 0.01. This underlines that the presence of the strain pools did not interfere with the acidifying activity of the starter. At the end of the ripening process, the pH of the cheeses decreased to a range of 5.15 ± 0.01 to 5.49 ± 0.01 (data not shown).

Table 3 Evolution of the moisture (%) of the cheese sample during the ripening period (mean ± SD, $n = 3$)

Ripening time (d)	0	10	30	60	90	120
C1	44.34 ± 0.08^{Ba}	42.49 ± 0.15^{Bb}	40.44 ± 0.21^{Cc}	39.48 ± 0.06^{Ad}	37.40 ± 0.08^{Be}	36.06 ± 0.09^{Cf}
C2	45.82 ± 0.13^{Aa}	43.13 ± 0.07^{Ab}	42.18 ± 0.04^{Ac}	40.52 ± 0.10^{Ad}	38.80 ± 0.15^{Ae}	37.21 ± 0.39^{Af}
EX1	45.82 ± 0.16^{Aa}	43.04 ± 0.02^{ABb}	41.24 ± 0.06^{Bc}	39.65 ± 0.30^{Ad}	37.40 ± 0.23^{Be}	36.52 ± 0.42^{Bf}
EX2	44.49 ± 0.15^{Ba}	42.88 ± 0.10^{ABb}	41.09 ± 0.25^{Bc}	40.08 ± 0.02^{Ad}	38.84 ± 0.11^{Ae}	37.47 ± 0.25^{Af}
EX3	43.03 ± 0.07^{Ca}	41.06 ± 0.47^{Cb}	40.48 ± 0.11^{Cbc}	39.92 ± 0.08^{Ac}	38.51 ± 0.43^{Ad}	37.21 ± 0.02^{ABe}
EX4	42.54 ± 0.02^{Da}	40.96 ± 0.12^{Cb}	39.71 ± 0.05^{Dc}	39.23 ± 0.04^{Ad}	38.21 ± 0.05^{Ae}	36.27 ± 0.22^{BCf}

Different capital letters mean statistically different values within the same column ($p < 0.05$). Different lowercase letters mean statistically different values within the same row ($p < 0.05$)

The proteolytic index (PI) was also monitored over time and results are reported in Table 4. In all samples, PI gradually increased over time due to the action of proteolytic activities that resulted in the steady release of water-soluble small and medium-sized peptides. The formation of water-soluble nitrogen compounds during the ripening phase is a key index of the proteolytic activity of semi-hard cheeses. It reflects the extent and rate of casein hydrolysis, by the action of rennet and the microbial and non-microbial proteases present at the beginning of the ripening process (Tarakci & Kucukoner, 2006). For both control and experimental samples, the PI values ranged from 7–9% after 10 days to 11–15% at 90 days of storage. At each ripening time, sample EX3 showed PI values significantly lower than those of the control sample produced with the addition of lysozyme (C2). At 120 days of ripening, the control sample produced without lysozyme (C1) showed the highest PI value compared to the other samples at the same ripening time. The observed disparity could be attributed to the germination and proliferation of undesired spoilage microorganisms in sample C1, such as those belonging to the proteolytic species *Clostridium sporogenes* (Brändle et al., 2016; Carminati et al., 2024). On the other hand, experimental samples (EX) and sample C2 showed similar PI values also at the end of ripening, probably because the undesired microorganisms were inactivated by the addition of selected *Lb. casei* and lysozyme. This variability might be attributable to the fact that the ability to release proteolytic enzymes during the ripening period is strongly influenced by the species and strains of *Lb. casei* considered (Stefanovic et al., 2018). Moreover, it is noteworthy that the viability of *Lb. casei* was uniform across all samples from 30 days onward. This consistency likely explains the similar PI values observed among the different samples (Table 2). This similarity in PI values can represent a positive outcome for their employment in cheeses with a PDO label, as typicality is the key feature of these products.

The volatile profile is widely regarded as one of the most crucial factors in determining cheese quality (Pogačić et al., 2016). For this reason, previously isolated strains were used, selected not only for their anticlostridial activity but also for their ability to produce volatiles in a model system where they were inoculated individually (Renoldi et al., 2024). The type of volatile compounds detected in cheese was very similar to those made by the individual strains. However, in cheese, the selected strains were added as a pool, and the resulting volatiles were produced not only by the added bacteria but also by the interaction of starter bacteria, the enzymatic activities of native milk enzymes, rennet enzymes, and accompanying lipases (Smit et al., 2005). Forty-six volatile compounds contributing to the volatile profile of Montasio cheese were identified using HS-SPME-GC/MS (Table S1). Volatiles include 13 acids, 17 alcohols, 2 aldehydes, 4 esters, 7 ketones, and 3 sulfur compounds.

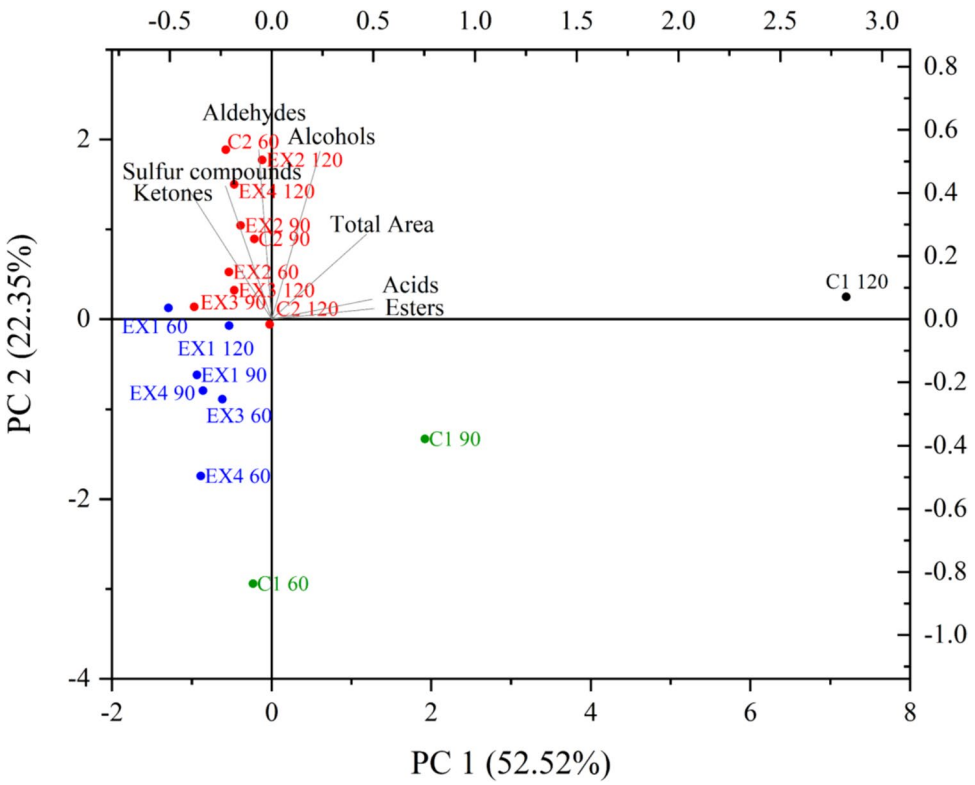
The principal component analysis (PCA) was used to highlight the differences in volatile chemical production among samples taken at 60, 90, and 120 days of ripening (Fig. 3). A clustering analysis based on the chemical classes of volatile compounds was performed, resulting in cheese samples being grouped into four main clusters (Fig. 4).

The two PCA axes accounted for 74.87% of the total variability, with PC1 and PC2 describing 52.52% and 22.35% of the variability, respectively (Fig. 3). PC1 was positively associated with total area, esters, alcohols, and acids, while it was negatively associated with ketones, aldehydes, and sulfur compounds. PC2 was positively associated with the abundance of all the volatile classes that characterize cheese, including sulfur compounds, acids, esters, alcohols, ketones, and aldehydes. All control samples produced with the addition of lysozyme (C2), all the EX2 samples, EX3 at 90 and 120 days, and EX4 at 120 days were positioned in the upper quadrants and grouped in cluster I (red) (Figs. 3 and 4). The remaining experimental samples fall into the left negative quadrant and grouped all together in cluster II (blue). Control samples produced without the addition of lysozyme (C1) at 60 and 90 days were grouped in cluster III (green), while only sample C1 at 120 days of ripening was located far from the other samples in the PCA biplot and grouped alone in cluster IV (black) (Figs. 3 and 4).

Table 4 Evolution of the proteolytic index (%) of cheeses during the ripening period (mean ± SD, $n = 3$)

Ripening time (d)	10	30	60	90	120
C1	7.60 ± 0.23^{Bd}	10.70 ± 0.11^{ABc}	10.86 ± 0.12^{Cc}	15.69 ± 0.59^{Ab}	21.73 ± 0.66^{Aa}
C2	8.27 ± 0.18^{Ac}	11.69 ± 0.13^{Ab}	12.75 ± 0.17^{Ab}	15.26 ± 1.10^{Aa}	15.87 ± 0.86^{BCa}
EX1	9.05 ± 0.06^{Ad}	10.90 ± 0.60^{Ac}	12.98 ± 0.23^{Ab}	15.82 ± 1.04^{Aa}	16.70 ± 0.63^{Ba}
EX2	8.20 ± 0.71^{ABc}	9.06 ± 0.18^{Bc}	12.50 ± 0.07^{ABb}	14.28 ± 0.89^{Ba}	14.12 ± 0.62^{Ca}
EX3	7.71 ± 0.12^{Bc}	9.78 ± 0.83^{Bb}	10.78 ± 0.13^{Cb}	11.74 ± 0.95^{Cab}	13.40 ± 0.76^{Da}
EX4	7.45 ± 0.17^{Bd}	10.24 ± 1.18^{ABc}	12.11 ± 0.44^{Bb}	12.14 ± 0.20^{BCb}	15.23 ± 0.13^{BCa}

Different capital letters mean statistically different values within the same column ($p < 0.05$). Different lowercase letters mean statistically different values within the same row ($p < 0.05$)



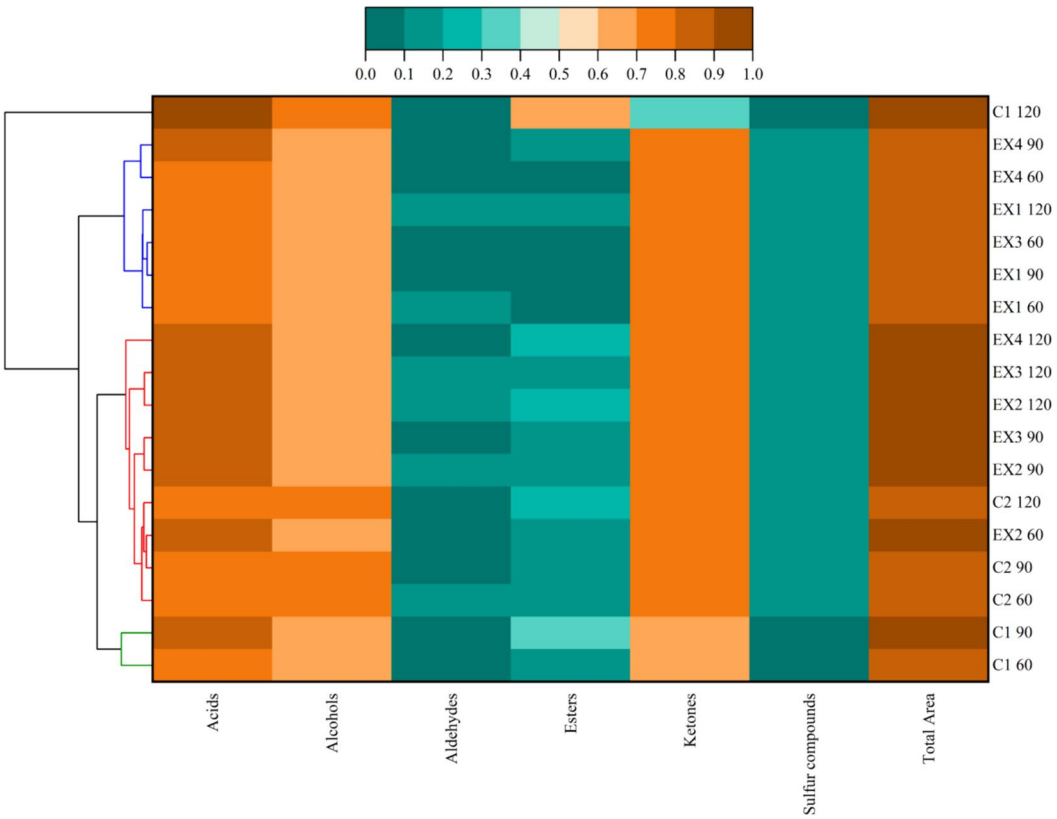


Fig. 3 PCA loading and score plots for classes of volatile compounds generated in the cheeses during the ripening period. Based on the cluster analysis, cheese samples were grouped as follows: red dots, group I; blue dots, group II; green dots, group III; black dots, group IV

Fig. 4 Hierarchical clustering analysis based on classes of volatiles and heatmap. Cheese samples were grouped as follows: red line, group I; blue line, group II; green line, group III, black line, group IV

Principal component analysis and hierarchical clustering analysis revealed distinct profiles among the groups (Figs. 3 and 4). Cluster I was characterized by a high total area of volatile compounds, predominantly acids, alcohols, and ketones, with lesser amounts of aldehydes. Cluster II shares similar compounds with Cluster I but with lower percentages of acids and alcohols, a lower total area, and a slightly higher percentage of esters than Cluster I. On the other hand, Cluster III showed a slightly lower percentage of ketones and a higher percentage of sulfur compounds compared to Cluster I and II. Cluster IV was characterized by the highest total area and the highest values of acids and esters. Comparing samples at 120 days of ripening (Fig. 5), C1 (Cluster IV) differs from all other samples by the higher percentages of butanoic acid, pentanoic acid, propanoic acid, and 1-butanol, compounds associated with the occurrence of LBD. In contrast, the control sample produced with lysozyme (C2) and the experimental samples (EX), excluding EX1, exhibited comparable volatile profiles. Therefore, despite EX3 revealing a lower proteolytic index compared to the other samples, as shown in Table 4, its aromatic profile remains similar to the other samples of the cluster. This observation highlights the efficacy of the selected strains in producing aromas commonly associated with Montasio cheese produced via conventional commercial adjunct cultures. The fact that EX1 belongs to a different cluster than the other experimental samples at the end of the ripening period, distinguished by a lower percentage of ethyl acetate, 1-butanol 3-methyl-acetate, 1-hexanol, and pentanoic acid could be attributed to the presence of the strain *Lb. paracasei* C121. This strain, which is absent in all other samples, may have a reduced capacity to produce the desired volatile compounds.

Sensory Profile

Sensory analysis was performed on samples at 120 days of ripening since the inoculation of secondary adjunct cultures into the PDO cheese should not influence the

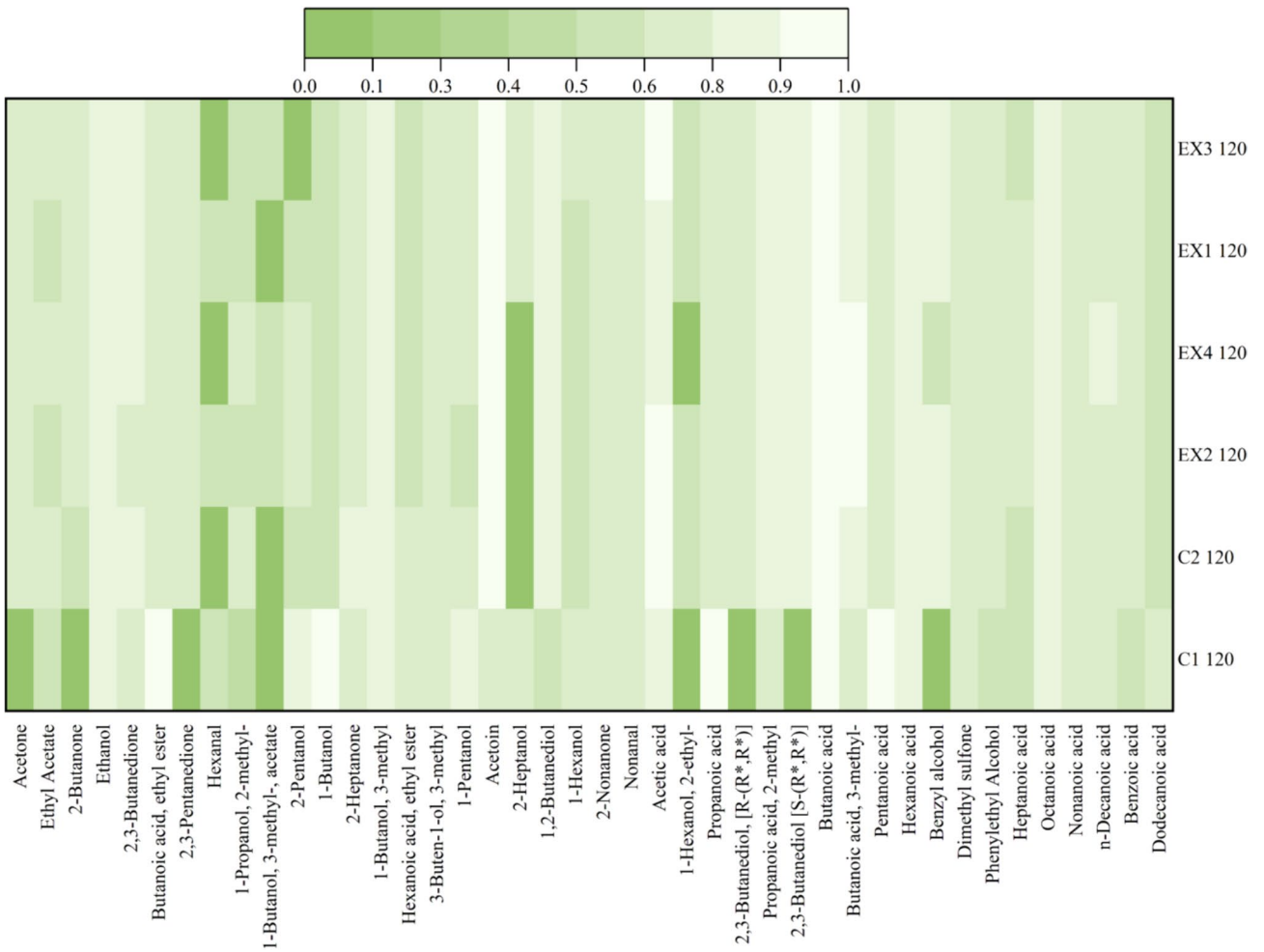


Fig. 5 Hierarchical clustering analysis showing the distribution of volatile compounds in the cheese samples at the end of the 120-day ripening period

expected sensorial characteristics of the products, as those represent the most important factor in the choice of food products by consumers (Karimi et al., 2012).

Data obtained from the sensory analysis regarding basic tastes, structural and mechanical characteristics, and aroma and odor intensity are shown in Table 5. Most of the sensory analysis descriptors can correlate with the physicochemical properties (Srisawas & Jindal, 2007).

Regarding basic tastes, no significant differences were observed among the samples in terms of spiciness and acidity. The similarity in perceived acidity can be attributed to all samples having comparable pH values at the end of the ripening period. Minimal differences in saltiness were noted between samples C1 and EX2, with C1 exhibiting the lowest salty perception and EX2 the highest. Significant differences in bitterness were observed between samples C2 and EX1 compared to sample C1, which exhibited higher bitterness levels. This can be explained by the higher percentages of butyric acid (Fig. 4), pentanoic acid, propanoic acid, and 1-butanol (Fig. 3), compounds all associated with LBD. However, these differences do not appear to be attributable to the addition of the cheese strains, as there is no clear distinction between control and experimental samples. Concerning structural descriptors, samples demonstrate similar characteristics in terms of deformability, friability, and adhesiveness. However, slight variations can be seen in elasticity and hardness between samples C2 and EX1, with C2 exhibiting the higher elasticity and EX1 showing the highest hardness value, probably due to its lower moisture content compared to C2 (Table 3). In the other samples, there does not seem to be any correlation between physicochemical properties (pH, moisture content, and PI) and texture parameters. Aroma and odor intensity descriptors are comparable across all samples. In addition to the descriptors mentioned above, odor and aroma descriptors of the 120-day-old samples were also identified by the trained panel (Table 6).

In all samples, descriptors associated with the "lactic" family, recalling aromas of milk, fresh curd, butter, and yogurt, were consistently detected for both odor and aroma assessments. Specifically, prominent odor descriptors encompassed "fresh lactic" (describing scents similar to milk, curd, cream, and butter), "cooked lactic" (characterizing aromas reminiscent of melted butter and boiled milk), and "acid lactic" (evoking sour notes akin to curd, yogurt, and sour milk). Likewise, key aroma descriptors included "fresh lactic" and "acid lactic". The only sample to which descriptors other than those belonging to the "lactic" family were associated is the control sample produced without the addition of lysozyme (C1), in which the "butyric" descriptor was detected both in the aroma and in the odor. The fact that the panel judges detected the presence of butyric acid only in this sample supports the hypothesis that butyric acid was

Table 5 Intensity of descriptors in cheese at 120 days of ripening

	Elasticity	Hardness	Deformability	Friability	Adhesiveness	Spicy	Salty	Acid	Bitter	Aroma intensity	Odor intensity
C1	3.88 ± 1.13^{ab}	3.75 ± 0.71^{ab}	3.63 ± 1.19^{a}	3.25 ± 1.39^{a}	3.75 ± 1.39^{a}	1.50 ± 0.53^{a}	3.13 ± 0.64^{b}	3.13 ± 1.13^{a}	2.38 ± 1.30^{a}	4.38 ± 1.06^{a}	4.75 ± 1.04^{a}
C2	4.50 ± 1.60^{a}	2.75 ± 0.71^{b}	4.25 ± 0.71^{a}	2.50 ± 1.07^{a}	3.25 ± 0.89^{a}	1.63 ± 1.77^{a}	4.63 ± 1.69^{ab}	3.75 ± 1.28^{a}	1.00 ± 0.00^{b}	4.25 ± 1.04^{a}	3.63 ± 0.52^{a}
EX1	2.50 ± 1.20^{b}	4.25 ± 1.04^{a}	$3.6\,3 \pm 1.30^{a}$	3.50 ± 1.31^{a}	2.38 ± 0.74^{a}	1.00 ± 0.00^{a}	4.75 ± 1.39^{ab}	3.75 ± 1.49^{a}	1.13 ± 0.35^{b}	4.00 ± 1.12^{a}	4.11 ± 0.99^{a}
EX2	3.00 ± 0.82^{ab}	3.86 ± 1.21^{ab}	3.14 ± 1.07^{a}	3.43 ± 0.53^{a}	3.29 ± 0.76^{a}	1.14 ± 0.38^{a}	5.43 ± 0.98^{a}	2.86 ± 1.21^{a}	1.71 ± 0.95^{ab}	4.29 ± 1.25^{a}	3.43 ± 0.98^{a}
EX3	4.25 ± 1.28^{ab}	3.25 ± 1.04^{ab}	3.63 ± 0.74^{a}	3.36 ± 0.74^{a}	3.63 ± 1.19^{a}	1.25 ± 0.46^{a}	4.25 ± 1.04^{ab}	2.50 ± 1.41^{a}	1.38 ± 0.74^{ab}	4.13 ± 1.13^{a}	3.50 ± 0.76^{a}
EX4	4.00 ± 1.00^{ab}	3.56 ± 1.13^{ab}	3.44 ± 1.33^{a}	3.22 ± 0.83^{a}	3.78 ± 0.83^{a}	1.22 ± 0.44^{a}	4.11 ± 1.27^{ab}	2.67 ± 1.00^{a}	1.33 ± 0.50^{ab}	4.44 ± 1.42^{a}	4.44 ± 1.33^{a}

Different capital in letters in columns mean statistically different values ($p < 0.05$)

Different lowercase letters in lines mean statistically different values ($p < 0.05$)

Table 6 Odor and aroma descriptors of the samples at 120 days of ripening

Sample	Odor		Aroma	
	Family	Subfamily	Family	Subfamily
C1	Lactic; other	Cooked; butyric	Lactic; other	Cooked; butyric
C2	Lactic	Fresh	Lactic	Acid
EX1	Lactic	Fresh; cooked	Lactic	Fresh; acid
EX2	Lactic	Fresh	Lactic	n.d
EX3	Lactic	Fresh; cooked	Lactic	Fresh; acid
EX4	Lactic	Fresh; acid	Lactic	Fresh; acid

n.d. no descriptor was detected by at least 50% of the judges

present in sufficient quantities to cause the onset of the LBD exclusively in sample C1.

Conclusions

In this work, the impact of selected *Lb. casei* strains on the biological and technological properties of PDO Montasio cheese was studied. The inoculated strains survived the cheesemaking process and were able to maintain a viability of almost 9 log cfu g^{-1} at the end of the ripening period. The physicochemical properties related to cheese ripening (moisture, pH, proteolytic index) of the experimental cheeses were very similar to those shown by the control cheese produced with the addition of lysozyme. Moreover, data reveal that the selected strains of *Lb. casei* performed comparably to lysozyme as an anti-blowing agent. This conclusion arises from the absence of the LBD in the control sample with lysozyme and the experimental samples inoculated with the *Lb. casei* strains. Conversely, only the sample was produced without the addition of lysozyme or *Lb. casei* exhibited the defect after 90 days of ripening.

The sensory profile of the experimental cheeses also showed a great similarity with those of the control cheese produced with the addition of lysozyme, confirming that that the inoculated strains were able to produce aromas similar to those produced by commercial starters for Montasio cheese. Isolating autochthonous strains with aromatic and anticlostridial activities can therefore be considered as a valid strategy in the case of PDO-labeled products to make the label clean by removing the lysozyme. In addition, the implementation of these adjunct cultures could be pivotal in mitigating the widespread issue of food waste and the economic losses associated with this defect. The results of this study demonstrate that it is possible to produce cheeses free from late-blowing defects by using selected strains of *Lactobacillus casei*, achieving an effect similar to that obtained with the addition of lysozyme. Therefore, the use of these strains could help achieve a cleaner label by avoiding the use of lysozyme.

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Author Contribution F.T.: data curation; formal analysis; writing—original draft. N.R.: data curation; formal analysis; investigation; methodology; writing—review and editing. A.R.: data curation; formal analysis; investigation; methodology; writing—review and editing. G.D.F.: formal analysis; investigation. M.M.: conceptualization, project administration; resources; supervision; writing—review and editing. N.I.: conceptualization, project administration; resources; supervision; writing—review, and editing.

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Data Availability Data will be made available on request.

Declarations

Competing Interests The authors declare no competing interests.

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