Food and Bioprocess Technology
https://doi.org/10.1007/s11947-023-03311-x

RESEARCH

Screening of Aroma-Producing Performance of Anticlostridial *Lacticaseibacillus casei* Strains

Niccolò Renoldi[1] · Nadia Innocente[1] · Anna Rossi[1] · Milena Brasca[2] · Stefano Morandi[2] · Marilena Marino[1]

Received: 7 November 2023 / Accepted: 26 December 2023

Abstract
The cheesemaking industry is increasingly interested in using adjunct cultures with potential aromatic and anticlostridial activities. In this study, 34 *Lb. paracasei* and 2 *Lb. rhamnosus* strains were isolated from a semi-hard cheese and characterized for their proteolytic, esterase, and anticlostridial activity. Moreover, the strains were inoculated in a curd-based medium and the volatile compounds in the headspace of samples were evaluated by solid-phase microextraction–GC–MS analysis. Proteolytic activity was present in 30 strains, whereas only one *Lb. paracasei* strain showed esterase activity. All strains inhibited *Cl. sporogenes*, *Cl. beijerinckii*, and *Cl. butyricum*, and 18 isolates inhibited at least one *Cl. tyrobutyricum* strain. Principal component analysis and clustering analysis based on the volatilome grouped strains into three groups. One of these groups was characterized by high amounts of acids and esters and clustered with control samples inoculated with commercial starter cultures, suggesting similarity in the aroma profile. Strains belonging to this group with inhibitory effects against *Cl. tyrobutyricum* might be exploited as autochthonous adjunct cultures for the reduction of late-blowing defects in semi-hard cheeses.

Keywords Adjunct culture · Aromatic profile · Anticlostridial activity · Raw milk · Non-starter LAB

Introduction

The volatile profile is generally recognized as one of the most relevant aspects for evaluating cheese quality and its connection with the area of production. Microorganisms play an important role in developing cheese flavor through the production of proteolytic, lipolytic, and amino acid-metabolizing enzymes, which convert milk constituents into numerous volatile compounds (Afshari et al., 2020; Pogačić et al., 2016). There are several reasons to explore the potential of microorganisms in the production of aroma compounds. Firstly, new strains are always needed to modify or intensify the flavor profile of industrial cheese (Chen et al., 2012; Law, 2001). In addition, autochthonous starter cultures can also be selected to retain the unique flavor of traditional dairy products (Innocente et al., 2016, 2023). Therefore, the cheese industry is increasingly interested in using adjunct cultures for the production of cheeses with specific aromatic profiles (El Soda et al., 2000). The ability of microorganisms to generate volatile compounds is a strain-specific characteristic (Alemayehu et al., 2014; Poveda et al., 2014).

Adjunct cultures can moreover counteract microbial pathogens as well as microorganisms responsible for unwanted abnormal fermentations or able to decarboxylate amino acids and produce biogenic amines (Innocente et al., 2009; Rehaiem et al., 2012; Renes et al., 2014). To be an effective adjunct culture, the microorganisms should maintain a high cell density during the ripening process and positively contribute to the overall quality of the cheese (Irlinger et al., 2017). To ensure adaptation to the technological process and characteristics of the food matrix, adjunct cultures made by a single or a group of microbial strains can be directly isolated and selected from the same type of cheese (Gobbetti et al., 2015).

The *Lacticaseibacillus casei* group, which includes *Lb. casei*, *Lb. paracasei*, and *Lb. rhamnosus*, is of great relevance since all these species have been frequently found

✉ Nadia Innocente
nadia.innocente@uniud.it

[1] Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via Sondrio 2/A, 33100 Udine, Italy

[2] Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Celoria 2, 20133 Milan, Italy

Published online: 12 January 2024

in different semi-hard and hard cheeses at late stages of ripening, suggesting the affinity of these microorganisms to the NSLAB (non-starter lactic acid bacteria) group (Bottari et al., 2018; Gatti et al., 2014). The growth of *Lb. casei*-group species in cheese is strongly associated with several technological factors, including curd cooking conditions, physicochemical properties of the curd and cheese, the presence of other microbial species, and the ripening time (Bottari et al., 2018; Gobbetti et al., 2015). Normally, these species are part of the microflora of ripened products since they survive the technological stresses of the cheesemaking process and use alternative energy sources rather than lactose, determining strong adaptability in the cheese matrix. The *Lb. casei*-group is involved in essential biochemical activities, including the development of the characteristic volatile profile and texture of the product during cheese ripening (Carafa et al., 2019; Wang et al., 2021). For these reasons, the development of new secondary adjunct cultures for cheesemaking must consider the possible effects on the generation of aromatic compounds in the products, and not limiting the research on health functionalities (Martins et al., 2018). Many LAB (lactic acid bacteria), including *Lb. casei*-group, also exert antimicrobial activity against pathogenic and spoilage microorganisms, thus helping to increase safety and reduce waste (Mani-López et al., 2022; Morandi et al., 2019). Their use might represent a valid alternative to common antimicrobial agents, such as lysozyme, in the reduction of blowing risks related to the presence of species belonging to the *Clostridium* genus (Rodi et al., 2020). These sporeformer species are responsible for butyric fermentation which triggers undesired late-blowing defects (LBD) in semi-hard and hard cheeses during ripening (Gómez-Torres et al., 2015). Nowadays, many studies have demonstrated the anti-clostridial activity of LAB in cheese products, with a consequent control of blowing defects (Gómez-Torres et al., 2015; Rodi et al., 2020). Multiple LAB strains exploited the production of antimicrobial substances to inhibit microorganisms responsible for blowing defects with positive effects on cheese features at the same time, which makes this biological approach a promising alternative to the current strategies (Demirbaş et al., 2022). Several mechanisms involving LAB and the production of compounds of different chemical nature were proposed in the scientific literature to explain the bio-protection activity in cheese. Many strains of the *Lb. casei* group have been found to produce bacteriocins (García-Cano et al., 2019; Yang et al., 2012). In addition, LAB produce other antimicrobial substances with inhibitory effects such as organic acids (lactic and acetic acid), hydrogen peroxide, and carbonyl compounds, such as acetaldehyde, diacetyl, and acetoin (Cintas et al., 2001).

This study aimed at isolating and characterizing several indigenous strains of *Lb. casei* from a semi-hard Italian cheese to identify a suitable pool of autochthonous microorganisms with potential application as adjunct starter cultures in semi-hard cheeses. Semi-hard cheeses were chosen because they are often subject to blowing defects, which can have a significant impact on quality. Furthermore, these cheeses have a medium-long ripening period during which secondary cultures can play a very important role in developing the flavor of the product. Isolated strains have been studied for their proteolytic, esterase, and anti-clostridial activity, and for their ability to contribute to the aromatic profile of cheese.

Materials and Methods

Strain Isolation

Bacterial cultures (189) were isolated from nineteen samples of semi-hard cheeses from different artisanal and industrial dairies located in Friuli Venezia Giulia (North-East of Italy). Isolation was carried out from count plates (dilutions 10^{-5}–10^{-7}) of MMV agar, a selective medium for isolation and enumeration of *Lb. casei*-group strains, incubated at 30 °C for 48 h under anaerobic conditions (Di Lena et al., 2015). For each sample, five to ten morphologically unique colonies were randomly picked up and purified onto MRS Agar (Oxoid, Milan, I). The isolates were stored at − 80 °C in MRS broth with 30% glycerol (v/v).

Strains Identification and Typing

The DNA extraction was performed with InstaGene matrix (BioRad, Hercules, CA). DNA concentration and purity were measured by a Nanodrop™ 2000C spectrophotometer (ThermoFisher Scientific, Waltham, MA), and checked by agarose gel electrophoresis, using a 0.7% agarose gel in TBE buffer 0.5×. DNA concentration was standardized to 100 ng/µL.

PCR targeted to the *Lb. casei*-group was carried out using the primer pair LCgprpoA-F2 (5′-CACTCAARATGAAYA CYGATGA-3′) and LCgprpoA-R2 (5′-CGTGGTGAGATT GAGCCAT-3′) according to Huang et al. (2011). Each reaction mixture (20 µL) contained 1 × reaction buffer, 4 mM $MgCl_2$, 0.5 mM of dNTPs, 1 µM of each primer, 0.025 U of Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, CA), and 1 µL of bacterial DNA. The amplicons (about 360 bp) were separated in 2% agarose (Sigma Aldrich, Milan, I) in TBE buffer 0.5x.

A species-specific PCR was then performed using the primer pairs Y2- casei, Y2-para, and Y2-rham for *Lb. casei*, *Lb. paracasei*, and *Lb. rhamnosus*, respectively (Ward & Timmins, 1999). Every reaction mixture (50 µL) was composed of 1 × reaction buffer, 1.5 mM MgCl2, 0.2 mM of dNTPs, 0.2 µM of each primer, 0.05 U/µL of Taq DNA

polymerase (Thermo Fisher Scientific), and 1 µL of bacterial DNA. As positive controls, *Lb. casei* DSM 20011 ^{T}, *Lb. paracasei* DSM 5622 ^{T}, and *Lb. rhamnosus* DSM 20021 ^{T} were used. The amplicons (290 bp) were separated in 2% agarose in TBE 0.5×.

The RAPD DNA fingerprinting was performed with primer M13 (Rossetti & Giraffa, 2005) and D8635 (Andrighetto et al., 2001). For primer M13, the reaction mixture (40 µL) consisted of buffer 1×, 0.2 mM of dNTPs, 0.4 µM of the primer, 3 mM of $MgCl_2$, 1.25 U of Taq DNA polymerase (Thermo Fisher Scientific), and 1 µL of bacterial DNA (Rossetti & Giraffa, 2005). For primer D8635, reactions were carried out in a 25-µL reaction mixture with buffer 1×, 0.25 mM of dNTPs, 0.8 µM of the primer, 3 mM of MgCl2, 1 U of Taq DNA polymerase (Thermo Fisher Scientific), and 1 µL of bacterial DNA (Andrighetto et al., 2002). PCR profiles were visualized after 3 h electrophoresis at 80 V with 1.5% agarose gel in TBE 0.5x. Grouping of the RAPD-PCR profiles was obtained with the Gel Compare 6.1 software package (Applied Maths, Belgium) by using the Pearson correlation similarity coefficient and the UPGMA cluster analyses.

Proteolytic and Esterase Activities

Proteolytic activity was evaluated by streaking a loopful of each isolate onto Skim Milk Agar plates (skim milk powder (Sigma) 28 g/L, casein enzymatic hydrolysate (Sigma) 5 g/L, yeast extract (Oxoid) 2.5 g/L, glucose (Carlo Erba Reagents, Milan) 1 g/L and agar (Oxoid) 15 g/L). Plates were incubated at 30 °C for 48 h under anaerobic conditions, and proteolytic activity was indicated by a clear zone surrounding the colonies (Pereira et al., 2001).

Esterase activity was evaluated by streaking each isolate onto Tributyrin agar plates (Sigma). Plates were incubated at 30 °C for 48 h. Esterase activity was indicated by the presence of a clarification halo around the culture (Morandi et al., 2013).

Anticlostridial Activity

Anticlostridial activity was determined by the standardized agar disk diffusion method using commercially available paper disks (9 mm in diameter) (Macherey–Nagel GmbH, Duren, D) (Morandi et al., 2015). Before use, strains were propagated in MRS broth at 30 °C for 24 h, while *Clostridium* strains were grown in reinforced clostridial medium (RCM; Oxoid) at 37 °C for 48 h in anaerobic conditions. Strains were spotted (20 µL) onto RCM agar plates seeded with *Clostridium* indicator-type strains, namely, *Clostridium beijerinckii* DSM 791 ^{T}, *Cl. butyricum* DSM 10702 ^{T}, and *Cl. tyrobutyricum* DSM 2637 ^{T} from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany), *Cl. sporogenes* ATCC 3584 ^{T} provided by the American Type Culture Collection (USA), and *Cl. tyrobutyricum* Coc1 and *Cl. tyrobutyricum* Coc2 from the Institute of Sciences of Food Production collection (ISPA-CNR, Milan, Italy) (Silvetti et al., 2018). Before use, lactobacilli were propagated in MRS broth (Oxoid) at 30 °C for 24 h, while *Clostridium* strains were grown in RCM broth at 37 °C for 48 h with an anaerobic incubation system (Anaerocult A, Merck Millipore, Darmstadt, Germany). The RCM agar plates were incubated anaerobically at 37 °C for 72 h. Anticlostridial activity was expressed as the difference between the diameters of the inhibition zone and the disc used (9 mm). Anticlostridial activity was considered absent (halo < 10 mm), weak (10–20 mm), moderate (21–29 mm), or strong (> 30 mm).

Growth in Curd-Based Medium

Strains having unique RAPD profiles were investigated for their ability to grow and produce volatile compounds in a curd-based medium. Samples inoculated with two commercial adjunct cultures, namely, *Lb. casei* C1a and *Lb. paracasei* C1x isolated from Lyofast CPR1 (Sacco s.r.l., Cadorago CO, I), were also assessed. The cultures were reactivated in MRS broth, then streaked onto MRS agar plates, and incubated at 30 °C for 48 h under anaerobic conditions. Cell suspensions were prepared with bacterial colonies in 1 mL of maximum recovery diluent (MRD; Oxoid). In a preliminary step, the optical density at 600 nm and viable count onto MRS agar of such suspensions were estimated, to simplify the following inoculation of medium (Pogačić et al., 2015).

A curd-based medium was prepared from a 30-day curd of semi-hard cheese, as previously described (Pogačić et al., 2015). After removing the external part, 100 g of curd was finely chopped and 200 mL of a solution containing 1.2 g/L of bacteriological peptone (Oxoid) and 1% w/v NaCl was added, then stirred for 1 h. The resultant suspension was then filtered with three layers of sterile gauze and portioned (5 mL) under stirring into 20 mL vials closed with a Teflon septum and sealed with aluminum. The vials were sterilized at 110 °C for 15 min; then, for each strain, three vials were inoculated at a final concentration of 10^7 CFU/mL and incubated at 12 °C for 30 days. After 15 and 30 days of incubation bacterial viability and pH, and volatile compounds at 30 days were evaluated. Uninoculated vials (CT0) served as control.

Evaluation of Viability

The curd-based medium was decimally diluted in MRD, and the dilutions were pour-plated onto MRS agar. Plates were incubated anaerobically at 30 °C for 48 h.

pH

pH of the inoculated curd based medium after 15 days and 30 days of incubation was measured using a pH meter Basic 20 (Crison, Barcelona, Spain) equipped with a pH electrode previously calibrated with standard solutions at pH 4.01, 7.00, and 9.21.

Analysis of Volatile Compounds

Volatile compounds were determined in duplicate by using a solid-phase microextraction (SPME) coupled to the gas chromatography-mass spectrometry (GC–MS) technique. For the extraction, an HT2800T autosampler (HTA s.r.l., Brescia, I) provided with a heater was employed. Samples were equilibrated at 60 °C for 30 min; then, a 2-cm × 50/30-µm Stableflex 24 Ga divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated SPME fiber (Supelco, Bellefonte, PA) was exposed for 30 min in the headspace for extraction. A QP2020 NX gas chromatography-mass spectrometry (GC–MS) system (Shimadzu Corporation, Kyoto, J) equipped with a DB-WAX capillary column (30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness; Agilent Technologies, CA) was used for separation and identification of volatile compounds. The fiber was desorbed in the GC injection port at 270 °C for 3 min under splitless conditions. For the analysis, the following conditions were adopted: 1 mL/min Helium flow rate; interface, source, and quadrupole temperature were 240, 200, and 150 °C, respectively. The temperature program was set initially at 50 °C for 5 min, followed by the first ramp at 10 °C/min to 230 °C, the temperature was then kept steady for 10 min, and a second ramp at 10 °C/min to 240 °C for 10 min. Scan mode with a mass range from 25 to 350 m/z was used for the analysis. Chromatographic profiles were evaluated using the GC–MS solution software ver. 4.52 (Shimadzu Corporation, Kyoto, Japan), and compounds were identified by spectra comparison using commercial standards (hexanal, 2-heptanone, 2-nonanone, 3-methylbutanoic acid, butanoic acid, and hexanoic acid) (Sigma), the NIST/EPA/NIH 20 Mass Spectral Library (John Wiley & Sons Inc., Hoboken, NJ) and Kovat's retention index (RI) from the literature (https://webbook.nist.gov/chemistry/). Data were expressed as absolute areas of the obtained peaks measured in the headspace of each curd-based medium.

Statistical Analysis

Statistical analyses were carried out using the Origin Pro 9 software (OriginLab, Northampton, MA). Statistical differences between the mean values of control and inoculated samples ($p < 0.05$) were assessed using a *t*-test. Correlation analysis, precisely principal component analysis (PCA), and heatmap with hierarchical clustering analysis on volatile compounds were performed using pre-processed data, in the specific log10[*x*]-transformed and scaled data, while Euclidean distances for continuous variables and Ward's minimum variance method were set for clustering.

Results and Discussion

Strain Characterization

From nineteen samples of Montasio cheese, 189 microbial strains were isolated. A total of 149 cultures were identified as members of the *Lb. casei* group. Since the isolates came from count plates with a very high dilution of the cheese sample (10^{-5}–10^{-7}), this confirms that *Lb. casei* represents a predominant NSLAB group in the cheese considered in this study (Marino et al., 2003). Amplification profiles of the 149 *Lb. casei*-group strains were investigated by RAPD-PCR. Thirty-six unique RAPD profiles (Fig. 1) were found, highlighting their unique patterns. Strains were identified as *Lb. paracasei* (34 strains) and *Lb. rhamnosus* (2), which fully reflects the diffusion of the *Lb. casei* group in different semi-hard cheeses. Indeed, very recently in an extensive metagenomic investigation on the microbiota of 45 different types of Italian PDO raw milk cheeses, *Lb. casei* was detected in only 4 out of 128 samples, while *Lb. paracasei* and *Lb. rhamnosus* in 68 and 27, respectively (Fontana et al., 2023). Thus, the selected 36 strains were qualitatively evaluated for their proteolytic, esterase, and anti-clostridial activity. Thus, these 36 strains were qualitatively evaluated for their proteolytic, esterase, and anti-clostridial activity. In cheesemaking, microbial proteolytic and lipolytic/esterolytic activities are required since they contribute to the formation of textural and sensory characteristics of cheeses (McSweeney, 2004). Thirty strains isolated in this study, along with the two strains, C1a and C1x, coming from commercial adjunct cultures, were positive for proteolytic activity on milk proteins, whereas esterase activity was present in one *Lb. paracasei* strain (Table 1). *Lb. paracasei* and *Lb. rhamnosus* strains with proteolytic activity have already been isolated from cheese in previous studies (Bonomo & Salzano, 2013; García-Cano et al., 2019). During cheese ripening, when most of the residual lactose has been already metabolized by starter bacteria, peptides and amino acids constitute the main energy source for NSLAB and *Lb. casei*-group bacteria. Generally, strains having proteolytic activity exhibit cell membrane proteinases (CEP), plural peptide transporters, such as Opp transporters, and intracellular peptidases which degrade proteins generating amino acids and peptides, which are important precursors of chemical compounds involved in the development of cheese sensory attributes during ripening (Christensen et al., 2023;

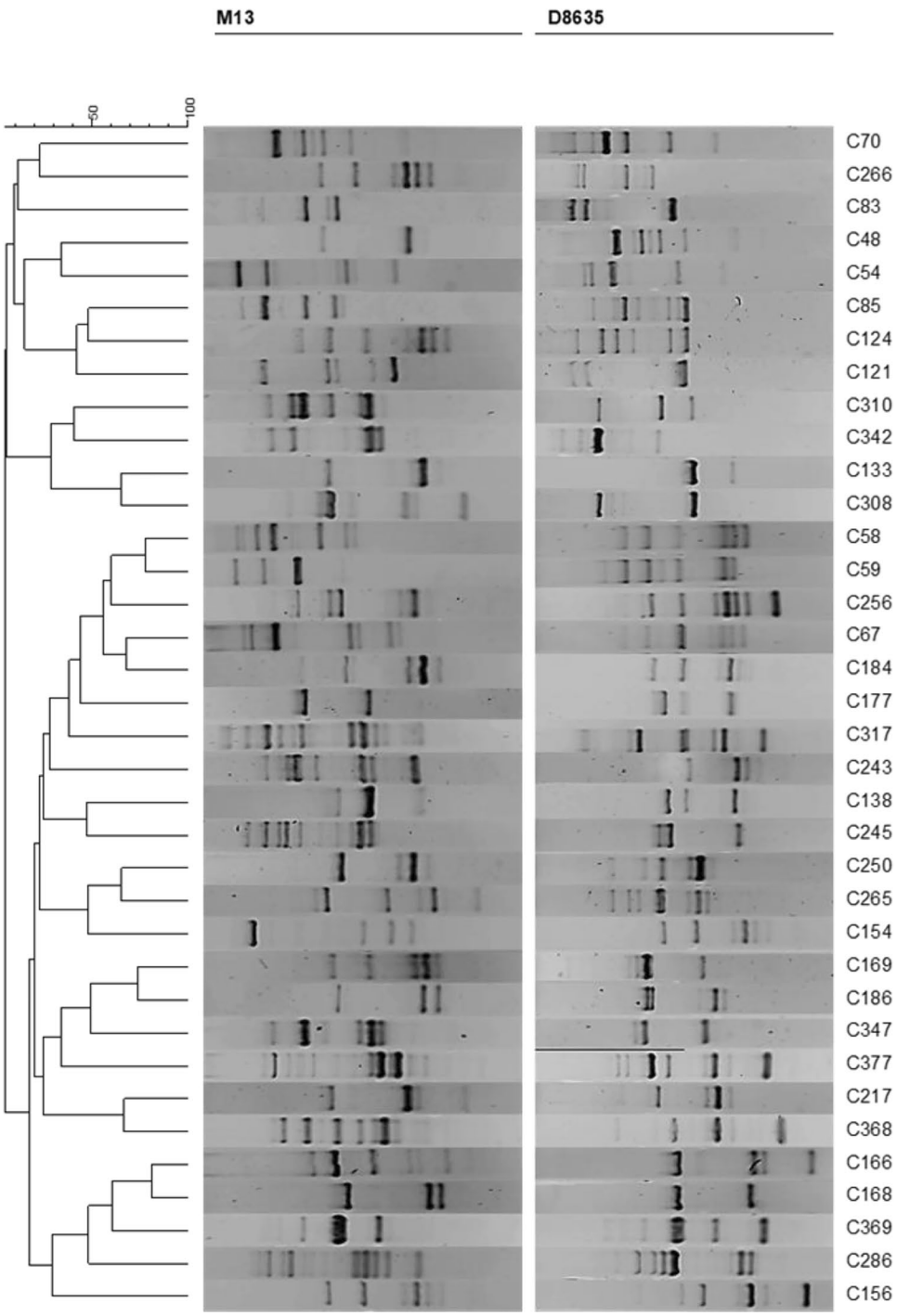


Fig. 1 Dendrogram derived from the combined RAPD-PCR profiles of isolates

McSweeney, 2004; Novak et al., 2022). In the ripening of some cheeses, an important contribution is also provided by lipolysis, which causes the release of free fatty acids, monoglycerides, and diglycerides, essential for flavor and aroma development. As for strains isolated in this study, only C217 showed to be esterolytic on a solid medium. Lipolytic and esterolytic activities are less widespread than proteolytic within the *Lb. casei* group and have been rarely reported in the literature (Bonomo & Salzano, 2013; Meng et al., 2018). Although lipolysis plays a relevant role in the ripening of a limited number of cheeses, LAB esterases contribute to the synthesis of esters from glycerides and alcohols (Chen et al., 2021). It has been hypothesized that an esterase from *Lb. casei* could synthesize ethyl esters as a_w decreased during cheese ripening, suggesting a possible impact on cheese flavor development (Fenster et al., 2003; Mukdsi et al., 2018).

Isolated strains were also tested for their antimicrobial ability against six different butyric acid-producing

Table 1 Proteolytic, esterase, and anticlostridial activities of selected and commercial strains

Strain	Species*	Proteolytic activity	Lipolytic activity	*Cl. sporogenes* ATCC 3584	*Cl. beijerinckii* DSM 791	*Cl. butyricum* DSM 10702	*Cl. tyrobutyricum* DSM 2637	*Cl. tyrobutyricum* Coc1	*Cl. tyrobutyricum* Coc2
C48	LP	+	-	Weak	Moderate	Moderate	-	Weak	Weak
C54	LP	+	-	Weak	Moderate	Moderate	-	Moderate	Weak
C58	LP	+	-	Weak	Strong	Moderate	-	Weak	Weak
C59	LP	+	-	Weak	Strong	Moderate	-	Moderate	Weak
C67	LP	+	-	Weak	Strong	Moderate	-	Weak	Weak
C70	LP	-	-	Weak	Strong	Moderate	Weak	Moderate	Moderate
C83	LP	+	-	Weak	Moderate	Moderate	-	-	-
C85	LP	-	-	Weak	Strong	Weak	-	-	-
C121	LP	+	-	Weak	Strong	Moderate	-	Weak	Moderate
C124	LP	+	-	Weak	Strong	Moderate	-	Weak	-
C133	LP	-	-	Weak	Moderate	Moderate	-	Weak	-
C138	LP	+	-	Weak	Strong	Moderate	-	Weak	Weak
C154	LR	+	-	Weak	Moderate	Moderate	-	-	-
C156	LP	+	-	Weak	Moderate	Moderate	-	Moderate	Moderate
C166	LP	+	-	Weak	Strong	Moderate	-	Moderate	Moderate
C168	LP	+	-	Weak	Moderate	Moderate	-	Moderate	Moderate
C169	LP	+	-	Weak	Moderate	Weak	-	Weak	Weak
C177	LP	+	-	Weak	Moderate	Moderate	Weak	Weak	Weak
C184	LP	+	-	Weak	Strong	Moderate	Weak	Weak	Weak
C186	LP	+	-	Weak	Strong	Moderate	-	Moderate	Moderate
C217	LP	-	+	Weak	Strong	Moderate	-	-	Weak
C243	LP	+	-	Weak	Weak	Weak	-	-	-
C245	LP	-	-	Weak	Moderate	Moderate	-	-	-
C250	LP	+	-	Weak	Weak	Moderate	-	-	-
C256	LP	+	-	Weak	Moderate	Moderate	-	-	-
C265	LR	+	-	Weak	Moderate	Weak	-	-	-
C266	LP	+	-	Weak	Weak	Weak	-	-	-
C286	LP	+	-	Weak	Weak	Weak	-	-	-
C308	LP	+	-	Weak	Weak	Weak	-	-	-
C310	LP	-	-	Weak	Weak	Moderate	-	-	-
C317	LP	+	-	Weak	Weak	Moderate	-	-	-
C342	LP	+	-	Weak	Moderate	Moderate	-	-	-
C347	LP	+	-	Weak	Weak	Weak	-	-	-
C368	LP	+	-	Weak	Moderate	Moderate	-	-	-
C369	LP	+	-	Weak	Weak	Weak	-	-	-
C377	LP	+	-	Weak	Strong	Moderate	-	-	-
C1a	LP	+	-	nd	nd	nd	nd	nd	nd
C1x	LP	+	-	nd	nd	nd	nd	nd	nd

*Species: LP = *L. paracasei*; LR = *L. rhamnosus*. Anticlostridial activity: weak (halo between 10 and 20 mm); moderate (halo between 21 and 29 mm); strong (halo larger than 30 mm). nd: not determined

clostridia (Table 1). All strains weakly inhibited *Cl. sporogenes* ATCC 3584 ^{T}, while weak to strong anticlostridial activities were found against *Cl. beijerinckii* DSM 791 ^{T} and *Cl. butyricum* DSM 10702 ^{T}. Among butyric acid-producing clostridia, *Cl. tyrobutyricum* is the main strain responsible for LBD in semi-hard and hard cheese during ripening, significantly causing food waste and economic losses (Christiansen et al., 2010). For this reason, the activity of the isolated strains against *Cl. tyrobutyricum* was tested against three different strains. Eighteen isolates showed to weakly or moderately inhibit at least one *Cl. tyrobutyricum* strain, and three *Lb. paracasei* strains, (C70, C177, and C184) were active against all *Cl. tyrobutyricum* strains. The anticlostridial activity,

albeit moderate, of the strains of this study along with their ability to adapt to the stressful environment of the cheese during ripening make them particularly attractive for the development of an adjunct culture.

Growth in Curd-Based Medium

After qualitative characterization, the selected strains, together with the two commercial adjunct cultures (C1a and C1x) were incubated for 30 days at 12 °C in a curd-based medium to evaluate the ability to grow and contribute to a volatile profile in a simulated cheese environment.

All strains proliferated after 15 days of incubation (Table 2), due to their ability to gain energy from residual lactose and/or the nitrogen fraction (amino acids and peptides) present in the curd-based medium. Similar results have been already reported for the *Lb. casei*-group (Pogačić et al., 2015, 2016). The initial pH of the curd-based medium was 5.77. As expected, all strains showed acidifying ability, with a pH of the medium after 15 days ranging from 4.25 to 4.71 (Table 2). As for 30 days of incubation, results highlighted the different abilities of strains to proliferate due to different microbial metabolisms and adaptive capacities. The bacterial counts of most strains, including the commercial ones, remained similar to 15 days, probably having already reached the stationary phase of growth. After 30 days only slight variations in pH were observed. Only four strains (C217, C286, C369, and C1x) showed a decrease in pH values at 30 days of incubation. During cheese ripening, sensory defects might be formed from intense acidification of the matrix. As a matter of fact, *Lb. paracasei* and *Lb. rhamnosus* are not responsible for cheese acidification because despite belonging to the NSLAB microflora they are distinctly characterized by slow to moderate acidification activities (Meng et al., 2018). Eight strains showed a decreased viability after 30 days, which might be due to autolysis phenomena. Autolysis is a general property of LAB, present in the *Lb. casei* group, and plays a fundamental role in cheese; indeed, it has been shown that the intracellular enzymes released by lysis are mainly involved in flavor formation (Bancalari et al., 2017). Moreover, it has to be highlighted that those microorganisms may persist in a viable but not culturable (VBNC) state during the cheese ripening, while remaining metabolically active. Thus, conventional culture-dependent methods may not effectively reveal their presence (Ruggirello et al., 2014). The VBNC state in *Lb. casei* strain grown under cheese-like conditions could be linked to a physiological response to carbohydrate starvation, a condition characteristic of post-fermentation cheese (Hussain et al., 2009).

Table 2 Microbial growth in curd-based medium of isolated strains with unique profile and controls, and pH values of the medium after 15 days and 30 days of incubation at 12 °C

Strain	Growth (Log CFU/mL)		pH	
	15 d	30 d	15 d	30 d
C48	7.93 ± 0.09	7.97 ± 0.05	4.33 ± 0.02	4.32 ± 0.02
C54	8.52 ± 0.02	8.59 ± 0.01	4.33 ± 0.02b	4.57 ± 0.04a
C58	8.70 ± 0.01	8.68 ± 0.01	4.26 ± 0.01	4.27 ± 0.01
C59	8.50 ± 0.18	8.42 ± 0.02	4.27 ± 0.01	4.27 ± 0.01
C67	8.26 ± 0.37	8.11 ± 0.01	4.25 ± 0.01	4.26 ± 0.01
C70	8.01 ± 0.23a	7.20 ± 0.02b	4.40 ± 0.20	4.24 ± 0.05
C83	8.52 ± 0.18	8.29 ± 0.20	4.29 ± 0.04	4.28 ± 0.01
C85	8.61 ± 0.05	8.68 ± 0.16	4.44 ± 0.11	4.41 ± 0.16
C121	8.39 ± 0.08	8.56 ± 0.01	4.64 ± 0.01	4.61 ± 0.15
C124	8.72 ± 0.03	8.73 ± 0.02	4.57 ± 0.01	4.60 ± 0.01
C133	8.32 ± 0.00	8.41 ± 0.05	4.46 ± 0.01	4.44 ± 0.02
C138	8.83 ± 0.14	7.68 ± 0.97	4.56 ± 0.02	4.45 ± 0.23
C154	8.21 ± 0.02	8.43 ± 0.18	4.42 ± 0.02	4.34 ± 0.06
C156	8.37 ± 0.00	8.16 ± 0.15	4.29 ± 0.02	4.28 ± 0.01
C166	8.80 ± 0.29	8.29 ± 0.02	4.28 ± 0.03	4.31 ± 0.01
C168	8.70 ± 0.01a	6.60 ± 0.06b	4.29 ± 0.01	4.27 ± 0.03
C169	8.42 ± 0.26	7.93 ± 0.68	4.47 ± 0.21	4.30 ± 0.04
C177	8.01 ± 0.02a	7.79 ± 0.02b	4.51 ± 0.15	4.61 ± 0.01
C184	8.65 ± 0.00	8.72 ± 0.24	4.69 ± 0.03	4.55 ± 0.17
C186	8.38 ± 0.08	7.85 ± 0.52	4.51 ± 0.22	4.49 ± 0.12
C217	8.61 ± 0.37a	7.13 ± 0.12b	4.71 ± 0.01a	4.31 ± 0.02b
C243	8.54 ± 0.02a	6.98 ± 0.10b	4.62 ± 0.10	4.57 ± 0.03
C245	8.40 ± 0.00	8.40 ± 0.02	4.32 ± 0.09	4.34 ± 0.03
C250	8.58 ± 0.06a	7.71 ± 0.04b	4.28 ± 0.01	4.26 ± 0.01
C256	8.31 ± 0.23	8.72 ± 0.06	4.71 ± 0.01	4.69 ± 0.02
C265	8.55 ± 0.10a	7.20 ± 0.23b	4.63 ± 0.01	4.61 ± 0.01
C266	8.48 ± 0.19	8.38 ± 0.03	4.44 ± 0.20	4.61 ± 0.05
C286	8.06 ± 0.03	8.17 ± 0.02	4.54 ± 0.02a	4.28 ± 0.04b
C308	8.40 ± 0.67	8.13 ± 0.22	4.68 ± 0.04	4.66 ± 0.04
C310	8.40 ± 0.02a	8.09 ± 0.03b	4.43 ± 0.04	4.48 ± 0.01
C317	8.13 ± 0.50	7.15 ± 0.16	4.51 ± 0.26	4.50 ± 0.24
C342	8.62 ± 0.08	8.69 ± 0.12	4.44 ± 0.09	4.45 ± 0.03
C347	8.15 ± 0.25	7.99 ± 0.04	4.47 ± 0.12	4.48 ± 0.15
C368	8.28 ± 0.04	8.00 ± 0.18	4.44 ± 0.17	4.45 ± 0.16
C369	8.54 ± 0.75	8.51 ± 0.02	4.57 ± 0.03a	4.28 ± 0.01b
C377	8.44 ± 0.20	7.93 ± 0.22	4.40 ± 0.19	4.39 ± 0.20
C1a	8.84 ± 0.10	8.85 ± 0.19	4.44 ± 0.01	4.46 ± 0.02
C1x	8.64 ± 0.08	8.77 ± 0.10	4.61 ± 0.02a	4.40 ± 0.03b

In the same row, different letters indicate means statistically different ($p < 0.05$)

Volatile Compound Production

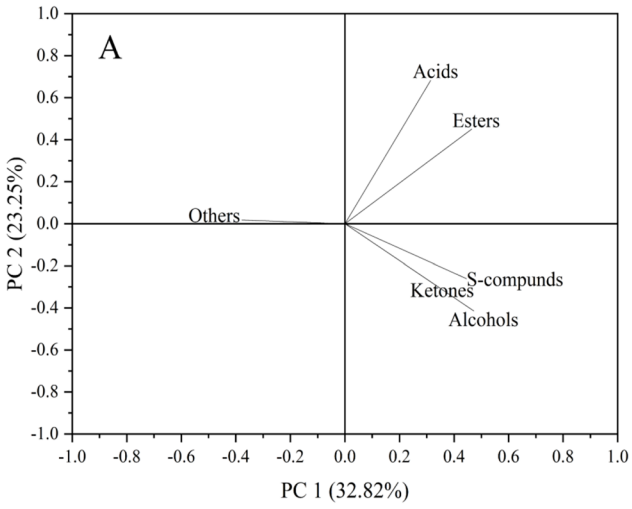
A total of 55 volatile compounds were identified in the headspace of vials containing curd-based medium inoculated with *L. casei-group* strains (Table S1) at 30 days of

incubation. In the specific, 11 ketones, 3 esters, 18 alcohols, 14 acids, 3 sulfur compounds, 3 aldehydes, 2 furans, and 1 lactone grouped as "others" were detected.

Principal component analysis (PCA) was carried out to highlight the differences in the production of volatile compounds among selected strains at 30 days of incubation in the curd-based medium (Fig. 2). In addition, a clustering analysis based on the chemical classes of volatile compounds was performed and the results were shown on heatmaps (Fig. 3). The first two components of PCA described 56.1% of the total variance (Fig. 2). PC1 accounted for 32.8% of the variability, while PC2 accounted for 23.2%. The first component was positively associated with all volatile compound classes, except for "others." PC2 was instead positively associated with acids and esters, and negatively correlated with sulfur compounds, ketones, and alcohols (Fig. 2A). The uninoculated curd-based medium (CT0, control medium at 0 days) was in the left negative quadrant, far from the other samples, indicating that the production of flavors was different from those of all strains at 30 days of incubation (Fig. 2B). CT0 contained significant amounts of aldehydes, such as hexanal and benzaldehyde, ethanol, acetoin, and acetic acid (Fig. 3B). These volatiles either come from raw milk or can also derive from the metabolic activity of starter lactic acid bacteria (SLAB) inoculated to the formation of the curd that was used to prepare the cheese-based medium (Pogačić et al., 2016). Ethanol, acetoin, and acetic acid are principally produced from lactose metabolism, while aldehydes are normally generated from the catabolism of amino acids (McSweeney & Sousa, 2000).

Hierarchical clustering separated strains into three distinct groups (Fig. 3A). Group I contained strains C54 (a proteolytic strain), C85, and C245 (not proteolytic), which were characterized by a high production of ethanol (Fig. 3B). Ethanol can originate from the lactate metabolism via acetaldehyde dehydrogenase activity, which many LAB including *Lb. paracasei* activate under limiting nutritional conditions, or from the fermentation of pentoses (e.g., ribose) released by SLAB lysis. Moreover, ethanol can be produced within the catabolism of amino acids, which produces acetaldehyde that can be further reduced to ethanol by alcohol dehydrogenase (McSweeney & Sousa, 2000). A significant increase in the relative percentage of ethanol in a cheese medium fermented by specific *Lb. casei* strains has been already reported, which may suggest a specific strain dependency in ethanol production (Sgarbi et al., 2013). In addition, group I was also characterized by a remarkable formation of dimethyl disulfide and ketones, in particular, diacetyl, acetoin, and 2-heptanone, which are relevant flavor contributors in several cheese varieties (Smit et al., 2005). Dimethyl disulfide originates from the catabolism of methionine, while alkan-2-ones are commonly produced by the β-oxidation of saturated fatty acids, and diacetyl results from lactose and citrate metabolism (McSweeney, 2004).

Group II was characterized by a low to moderate ability to produce volatiles, with the only class "others" as representative of this group (Fig. 2B). Indeed, samples in this group are located in the same quadrant as the non-inoculated control sample (CT0) (Fig. 2B). Benzaldehyde and 2-furan-methanol were the principal compounds, arising probably from the proteolytic activity and following amino acids degradation and phenylalanine catabolism (McSweeney, 2004). Recently,



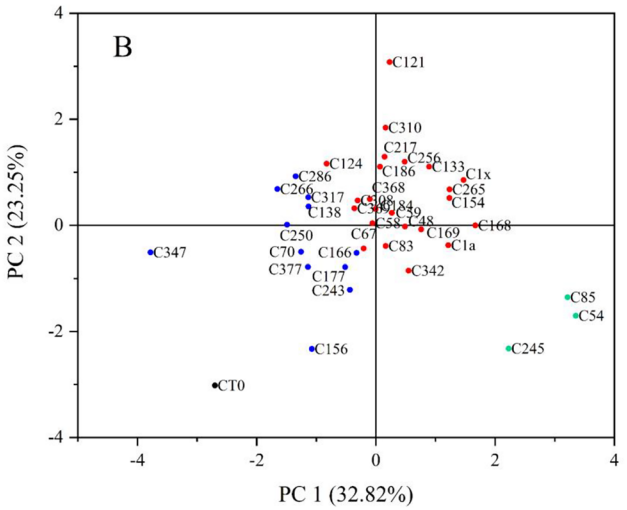


Fig. 2 PCA loading (**A**) and score (**B**) plots for classes of volatile compounds generated in curd-based medium inoculated with *L. casei*-group strains and incubated for 30 days at 12 °C. Based on the cluster analysis, strains were grouped as follows: green dots, group I; blue dots, group II; red dots, group III; black dot, uninoculated sample

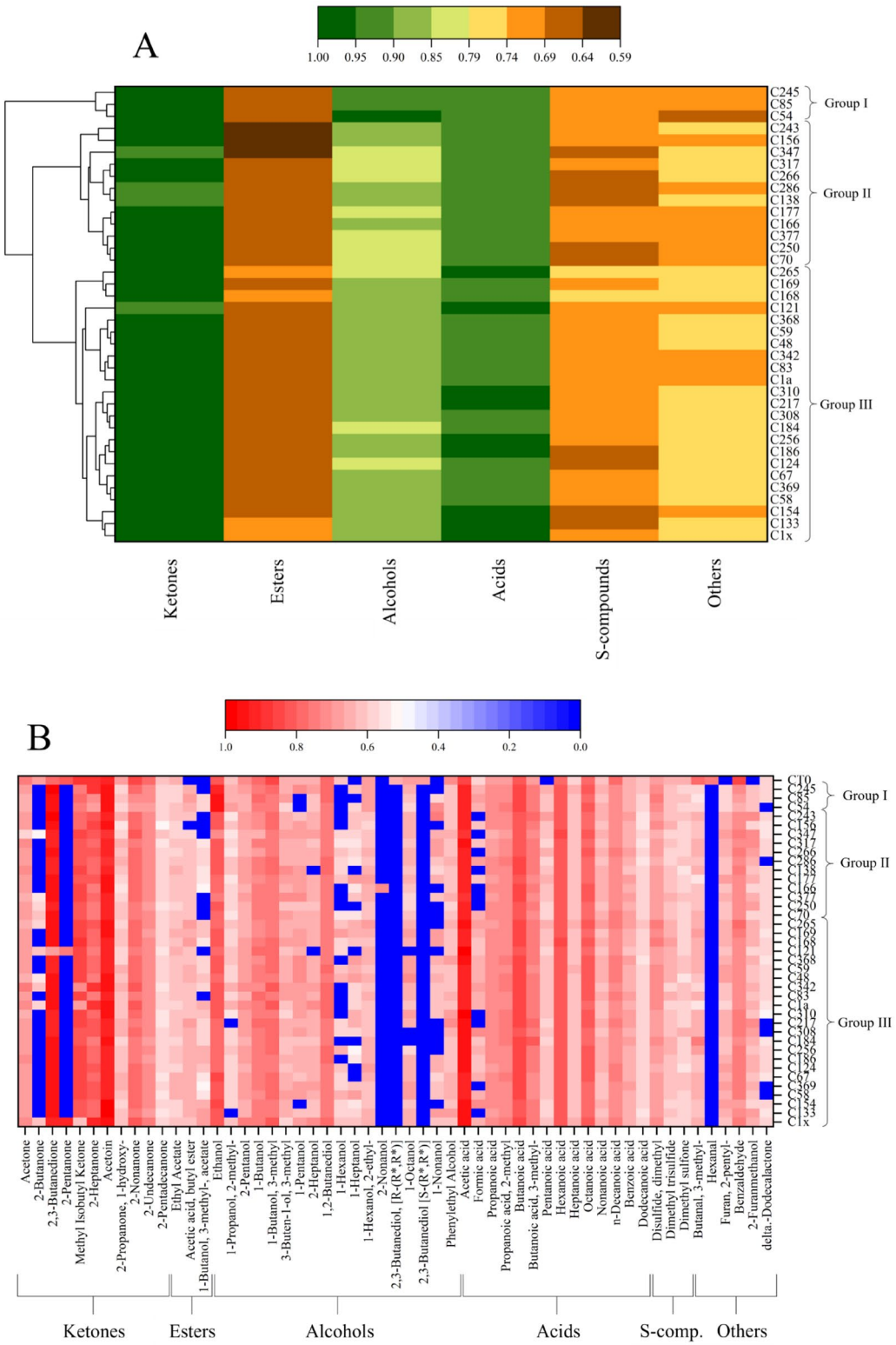


Fig. 3 Hierarchical clustering analysis **A** based on classes of volatiles and heatmap **B** showing the distribution of chemical compounds produced in curd-based medium incubated for 30 days at 12 °C

benzaldehyde has been detected in curd-based media after inoculation and growth of *Lb. casei* group strains (Picon et al., 2019).

Group III clustered 21 isolated *Lb. casei*-group strains and commercial strains C1a and C1x, highlighting that 58% of the strains isolated in this study were similar to commercial controls in terms of volatile production at 30 days of incubation. Acids, esters, and ketones were the predominant classes representative of this group of strains (Figs. 2B and 3A). Acetic, butanoic, hexanoic, and octanoic acids were detected as prevalent fatty acids (FAs). In a medium like the one used in this study, short- and medium-chain FAs can either derive from the catabolism of amino acids or by oxidation of ketones, esters, and aldehydes. A significant amount of these FAs was previously detected in semi-hard cheeses at the late stages of ripening (Innocente et al., 2013), where *Lb. casei* group strains are dominant (Innocente & Biasutti, 2013; Marino et al., 2003). In cheese, short and medium-chain FAs are key odorant components due to their low perception thresholds (Curioni & Bosset, 2002). Butyl acetate was the most abundant ester, as the result of esterification reactions between acetic acid and 1-butanol. Regarding ketones, once more diacetyl, acetoin, methyl isobutyl ketone, and 2-heptanone showed the highest absolute area (Fig. 3B). Although C217 was the only strain showing esterolytic activity on agar medium, no significant differences in the volatiles' profile were detected between C217 and the other strains belonging to group III at 30 days, suggesting that esterolytic activity may not be a relevant selection criterion for strains for semi-hard cheeses. Both *Lb. rhamnosus* strains (C154 and C265) were in group III and showed a high production of acetoin and diacetyl (Fig. 3B), as previously reported by several authors (Pogačić et al., 2016; Sgarbi et al., 2013). From previous findings, in semi-hard cheeses after 60 days of ripening similar volatile compounds to that produced by group III strains were detected (Innocente & Biasutti, 2013; Innocente et al., 2013; Thomsen et al., 2012). This suggests that most of the investigated *Lb. casei* strains could be used as adjunct cultures to generate an appropriate aroma profile and to prevent blowing defects in these cheeses.

Conclusion

Strains of *Lacticaseibacillus casei*-group are part of NSLAB in semi-hard cheeses and can therefore produce aromatic compounds during ripening and possibly protect the product from undesirable fermentations. Results obtained in this work showed that all *Lb. casei*-group strains isolated from semi-hard cheeses inhibited at least one of the *Clostridium* strains responsible of LBD, confirming the potentiality of use strains belonging to the *Lb. casei-group* as bio-protective cultures. The volatilome of strains in group III was characterized by high amounts of acids and esters and were clustered with commercial control strains suggesting similarity in the aroma profile. Thus, such strains might be taken into consideration to create a suitable pool of autochthonous microorganisms with potential application as bio-protective cultures to reduce late-blowing defects in semi-hard cheeses. Further studies on cheese products should be carried out to test the efficiency of combinations of these strains in terms of inhibition of clostridia and improvement of sensory properties of cheese.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11947-023-03311-x.

Author Contribution Niccolò Renoldi: data curation; formal chemical-physical analysis; investigation; methodology; writing—original draft; writing—review and editing. Nadia Innocente: conceptualization, project administration; resources; supervision; writing—review and editing. Anna Rossi: data curation; formal microbiological analysis; investigation; methodology; writing—review and editing. Milena Brasca: formal microbiological analysis; methodology; writing—review. Stefano Morandi: formal microbiological analysis; methodology; writing—review. Marilena Marino: conceptualization, project administration; resources; supervision; writing—review and editing.

Funding Open access funding provided by Università degli Studi di Udine within the CRUI-CARE Agreement.

Data Availability Data will be made available on request.

Declarations

Competing Interest The authors declare no competing interests.

References

Afshari, R., Pillidge, C. J., Dias, D. A., Osborn, A. M., & Gill, H. (2020). Cheesomics: The future pathway to understanding cheese flavour and quality. *Critical Reviews in Food Science and Nutrition, 60*(1), 33–47. https://doi.org/10.1080/10408398.2018.1512471

Alemayehu, D., Hannon, J. A., McAuliffe, O., & Ross, R. P. (2014). Characterization of plant-derived lactococci on the basis of their volatile compounds profile when grown in milk. *International Journal of Food Microbiology, 172*, 57–61. https://doi.org/10.1016/j.ijfoodmicro.2013.11.024

Andrighetto, C., Borney, F., Barmaz, A., Stefanon, B., & Lombardi, A. (2002). Genetic diversity of *Streptococcus thermophilus* strains

isolated from Italian traditional cheeses. *International Dairy Journal, 12*(2–3), 141–144. https://doi.org/10.1016/S0958-6946(01)00134-0

Andrighetto, C., Knijff, E., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., et al. (2001). Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. *Journal of Dairy Research, 68*(2), 303–316. https://doi.org/10.1017/S0022029901004800

Bancalari, E., Savo Sardaro, M. L., Levante, A., Marseglia, A., Caligiani, A., Lazzi, C., et al. (2017). An integrated strategy to discover Lactobacillus casei group strains for their potential use as aromatic starters. *Food Research International, 100*, 682–690. https://doi.org/10.1016/j.foodres.2017.07.066

Bonomo, M. G., & Salzano, G. (2013). Genotypic and technological diversity of *Leuconostoc mesenteroides* and *Lactobacillus paracasei* subsp. *paracasei* strains for use as adjunct starter cultures in Pecorino di Filiano cheese. *International Journal of Dairy Technology*, *66*(3), 402–409. https://doi.org/10.1111/1471-0307.12040

Bottari, B., Levante, A., Neviani, E., & Gatti, M. (2018). How the fewest become the greatest. L. casei's impacton long ripened cheeses. *Frontiers in Microbiology, 9*, 1–6. https://doi.org/10.3389/fmicb.2018.02866

Carafa, I., Stocco, G., Franceschi, P., Summer, A., Tuohy, K. M., Bittante, G., & Franciosi, E. (2019). Evaluation of autochthonous lactic acid bacteria as starter and non-starter cultures for the production of Traditional Mountain cheese. *Food Research International, 115*, 209–218. https://doi.org/10.1016/j.foodres.2018.08.069

Chen, C., Huang, K., Yu, H., & Tian, H. (2021). The diversity of microbial communities in Chinese milk fan and their effects on volatile organic compound profiles. *Journal of Dairy Science, 104*, 2581–2593. https://doi.org/10.3168/jds.2020-19053

Chen, L. S, Cui, J., Ding, Q. B., Ma, Y., Chen, L. J., Dong, J. Y., et al. (2012). The effect of yeast species from raw milk in China on proteolysis and aroma compound formation in Camembert-type cheese. *Food and Bioprocess Technology, 5*, 2548–2556. https://doi.org/10.1007/s11947-011-0589-4

Christensen, L. F., Høie, M. H., Bang-Berthelsen, C. H., Marcatili, P., & Hansen, E. B. (2023). Comparative structure analysis of the multi-domain, cell envelope proteases of lactic acid bacteria. *Microorganisms, 11*(9), 2256. https://doi.org/10.3390/microorganisms11092256

Christiansen, P., Vogensen, F. K., Nielsen, E. W., & ArdÖ, Y. (2010). Potential of anticlostridial *Lactobacillus* isolated from cheese to prevent blowing defects in semihard cheese. *International Journal of Dairy Technology, 63*(4), 544–551. https://doi.org/10.1111/j.1471-0307.2010.00626.x

Cintas, L. M., Casaus, M. P., Herranz, C., Nes, I. F., & Hernández, P. E. (2001). Review: Bacteriocins of lactic acid bacteria. *Food Science and Technology International, 7*(4), 281–305. https://doi.org/10.1106/R8DE-P6HU-CLXP-5RYT

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal, 12*(12), 959–984. https://doi.org/10.1016/S0958-6946(02)00124-3

Demirbaş, F., Dertli, E., & Arıcı, M. (2022). Prevalence of Clostridium spp., in Kashar cheese and efficiency of Lactiplantibacillus plantarum and Lactococcus lactis subsp. lactis mix as a biocontrol agents for Clostridium spp. *Food Bioscience*, *46*. https://doi.org/10.1016/j.fbio.2022.101581

Di Lena, M., Quero, G. M., Santovito, E., Verran, J., De Angelis, M., & Fusco, V. (2015). A selective medium for isolation and accurate enumeration of *Lactobacillus casei*-group members in probiotic milks and dairy products. *International Dairy Journal, 47*, 27–36. https://doi.org/10.1016/j.idairyj.2015.01.018

El Soda, M., Madkor, S. A., & Tong, P. S. (2000). Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science, 83*(4), 609–619. https://doi.org/10.3168/jds.s0022-0302(00)74920-4

Fenster, K. M., Parkin, K. L., & Steele, J. L. (2003). Intracellular esterase from *Lactobacillus casei* LILA: Nucleotide sequencing, purification, and characterization. *Journal of Dairy Science, 86*(4), 1118–1129. https://doi.org/10.3168/jds.S0022-0302(03)73694-7

Fontana, F., Longhi, G., Alessandri, G., Lugli, G. A., Mancabelli, L., Tarracchini, C., et al. (2023). Multifactorial microvariability of the Italian raw milk cheese microbiota and implication for current regulatory scheme. *mSystems*, *8*(1), 1–16. https://doi.org/10.1128/msystems.01068-22

García-Cano, I., Rocha-Mendoza, D., Ortega-Anaya, J., Wang, K., Kosmerl, E., & Jiménez-Flores, R. (2019). Lactic acid bacteria isolated from dairy products as potential producers of lipolytic, proteolytic and antibacterial proteins. *Applied Microbiology and Biotechnology, 103*(13), 5243–5257. https://doi.org/10.1007/s00253-019-09844-6

Gatti, M., Bottari, B., Lazzi, C., Neviani, E., & Mucchetti, G. (2014). Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. *Journal of Dairy Science, 97*(2), 573–591. https://doi.org/10.3168/jds.2013-7187

Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L., & Fox, P. F. (2015). Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends in Food Science and Technology, 45*(2), 167–178. https://doi.org/10.1016/j.tifs.2015.07.016

Gómez-Torres, N., Garde, S., Peirotén, Á., & Ávila, M. (2015). Impact of Clostridium spp. on cheese characteristics: Microbiology, color, formation of volatile compounds and off-flavors. *Food Control, 56*, 186–194. https://doi.org/10.1016/j.foodcont.2015.03.025

Huang, C. H., Chang, M. T., Huang, M. C., & Lee, F. L. (2011). Application of the SNaPshot minisequencing assay to species identification in the *Lactobacillus casei* group. *Molecular and Cellular Probes, 25*(4), 153–157. https://doi.org/10.1016/j.mcp.2011.03.002

Hussain, M. A., Rouch, D. A., & Britz, M. L. (2009). Biochemistry of non-starter lactic acid bacteria isolate *Lactobacillus casei* GCRL163: Production of metabolites by stationary-phase cultures. *International Dairy Journal, 19*(1), 12–21. https://doi.org/10.1016/j.idairyj.2008.07.004

Innocente, N., Renoldi, N., Moret, E., Maifreni, M., & Marino, M. (2023). Volatilome of brine-related microorganisms in a curd-based medium. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2022-23051

Innocente, N., & Biasutti, M. (2013). Automatic milking systems in the protected designation of origin Montasio cheese production chain: Effects on milk and cheese quality. *Journal of Dairy Science, 96*(2), 740–751. https://doi.org/10.3168/jds.2012-5512

Innocente, N., Biasutti, M., Rita, F., Brichese, R., Comi, G., & Iacumin, L. (2016). Effect of indigenous *Lactobacillus rhamnosus* isolated from bovine milk on microbiological characteristics and aromatic profile of traditional yogurt. *LWT - Food Science and Technology, 66*, 158–164. https://doi.org/10.1016/j.lwt.2015.10.031

Innocente, N., Marino, M., Marchesini, G., & Biasutti, M. (2009). Presence of biogenic amines in a traditional salted Italian cheese. *International Journal of Dairy Technology, 62*(2), 154–160. https://doi.org/10.1111/j.1471-0307.2009.00479.x

Innocente, N., Munari, M., & Biasutti, M. (2013). Characterization by solid-phase microextraction-gas chromatography of the volatile profile of protected designation of origin Montasio cheese during ripening. *Journal of Dairy Science, 96*, 26–32. https://doi.org/10.3168/jds.2012-5689

Irlinger, F., Helinck, S., & Jany, J. L. (2017). Secondary and adjunct cultures. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.), *Cheese, chemistry, physics and microbiology* (Fourth Edi., pp. 273–300). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-417012-4.00011-9

Law, B. A. (2001). Controlled and accelerated cheese ripening: The research base for new technologies. *International Dairy Journal, 11*, 383–398. https://doi.org/10.1016/S0958-6946(01)00067-X

Mani-López, E., Arrioja-Bretón, D., & López-Malo, A. (2022). The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria in vitro and foods. *Comprehensive Reviews in Food Science and Food Safety, 21*, 604–641. https://doi.org/10.1111/1541-4337.12872

Marino, M., Maifreni, M., & Rondinini, G. (2003). Microbiological characterization of artisanal Montasio cheese: Analysis of its indigenous lactic acid bacteria. *FEMS Microbiology Letters, 229*(1), 133–140. https://doi.org/10.1016/S0378-1097(03)00816-4

Martins, I. B. A., Deliza, R., dos Santos, K. M. O., Walter, E. H. M., Martins, J. M., & Rosenthal, A. (2018). Viability of probiotics in goat cheese during storage and under simulated gastrointestinal conditions. *Food and Bioprocess Technology, 11*, 853–863. https://doi.org/10.1007/s11947-018-2060-2

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*(2), 127–144. https://doi.org/10.1016/B978-0-12-417012-4.00018-1

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait, 80*(3), 293–324. https://doi.org/10.1051/lait:2000127

Meng, Z., Zhang, L., Xin, L., Lin, K., Yi, H. X., & Han, X. (2018). Technological characterization of *Lactobacillus* in semihard artisanal goat cheeses from different Mediterranean areas for potential use as nonstarter lactic acid bacteria. *Journal of Dairy Science, 101*(4), 2887–2896. https://doi.org/10.3168/jds.2017-14003

Morandi, S., Cremonesi, P., Silvetti, T., & Brasca, M. (2013). Technological characterisation, antibiotic susceptibility and antimicrobial activity of wild-type Leuconostoc strains isolated from north Italian traditional cheeses. *Journal of Dairy Research, 80*(4), 457–466. https://doi.org/10.1017/S0022029913000447

Morandi, S., Silvetti, T., Battelli, G., & Brasca, M. (2019). Can lactic acid bacteria be an efficient tool for controlling Listeria monocytogenes contamination on cheese surface? The case of Gorgonzola cheese. *Food Control, 96*, 499–507. https://doi.org/10.1016/j.foodcont.2018.10.012

Morandi, S., Silvetti, T., Miranda Lopez, J. M., & Brasca, M. (2015). Antimicrobial activity, antibiotic resistance and the safety of lactic acid bacteria in raw milk valtellina casera cheese. *Journal of Food Safety, 35*(2), 193–205. https://doi.org/10.1111/jfs.12171

Mukdsi, M. C. A., Maillard, M.-B., Medina, R. B., & Thierry, A. (2018). Ethyl butanoate is synthesised both by alcoholysis and esterification by dairy lactobacilli and propionibacteria. *LWT - Food Science and Technology, 89*, 38–43. https://doi.org/10.1016/j.lwt.2017.10.012

Novak, J., Leboš Pavunc, A., Butorac, K., Banić, M., Čuljak, N., Rak, H., ... & Kos, B. (2022). Caseinolytic proteases of *Lactobacillus* and *Lactococcus* strains isolated from fermented dairy products. *Mljekarstvo*, *72*(1), 11–21. https://doi.org/10.15567/mljekarstvo.2022.0102

Pereira, C. I., Crespo, M. T. B., & Romäo, M. V. S. (2001). Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and *L. homohiochii*. *International Journal of Food Microbiology*, *68*(3), 211–216. https://doi.org/10.1016/S0168-1605(01)00534-7

Picon, A., López-Pérez, O., Torres, E., Garde, S., & Nuñez, M. (2019). Contribution of autochthonous lactic acid bacteria to the typical flavour of raw goat milk cheeses. *International Journal of Food Microbiology, 299*, 8–22. https://doi.org/10.1016/j.ijfoodmicro.2019.03.011

Pogačić, T., Maillard, M. B., Leclerc, A., Hervé, C., Chuat, V., Valence, F., & Thierry, A. (2016). *Lactobacillus* and *Leuconostoc* volatilomes in cheese conditions. *Applied Microbiology and Biotechnology, 100*(5), 2335–2346. https://doi.org/10.1007/s00253-015-7227-4

Pogačić, T., Maillard, M. B., Leclerc, A., Hervé, C., Chuat, V., Yee, A. L., et al. (2015). A methodological approach to screen diverse cheese-related bacteria for their ability to produce aroma compounds. *Food Microbiology, 46*, 145–153. https://doi.org/10.1016/j.fm.2014.07.018

Poveda, J. M., Nieto-Arribas, P., Seseña, S., Chicón, R., Castro, L., Palop, L., & Cabezas, L. (2014). Volatile composition and improvement of the aroma of industrial Manchego cheese by using *Lactobacillus paracasei* subsp. *paracasei* as adjunct and other autochthonous strains as starters. *European Food Research and Technology*, *238*(3), 485–494. https://doi.org/10.1007/s00217-013-2127-2

Rehaiem, A., Martínez, B., Manai, M., & Rodríguez, A. (2012). Technological performance of the Enterocin A producer *Enterococcus faecium* MMRA as a protective adjunct culture to enhance hygienic and sensory attributes of traditional fermented milk "Rayeb." *Food and Bioprocess Technology, 5*, 2140–2150. https://doi.org/10.1007/s11947-010-0501-7

Renes, E., Diezhandino, I., Fernández, D., Ferrazza, R. E., Tornadijo, M. E., & Fresno, J. M. (2014). Effect of autochthonous starter cultures on the biogenic amine content of ewe's milk cheese throughout ripening. *Food Microbiology, 44*, 271–277. https://doi.org/10.1016/j.fm.2014.06.001

Rodi, J. O., Ramos, M. J. G., Gadea, P. D., & Reginensi, S. M. (2020). Screening of anti-clostridial lactic acid bacteria strains isolated from uruguayan dairy farms. *Journal of Microbiology, Biotechnology and Food Sciences*, *9*(6), 1170–1175. https://doi.org/10.15414/JMBFS.2020.9.6.1170-1175

Rossetti, L., & Giraffa, G. (2005). Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *Journal of Microbiological Methods, 63*(2), 135–144. https://doi.org/10.1016/j.mimet.2005.03.001

Ruggirello, M., Dolci, P., & Cocolin, L. (2014). Detection and viability of *Lactococcus lactis* throughout cheese ripening. *PLoS ONE, 9*(12), e114280. https://doi.org/10.1371/journal.pone.0114280

Sgarbi, E., Lazzi, C., Tabanelli, G., Gatti, M., Neviani, E., & Gardini, F. (2013). Nonstarter lactic acid bacteria volatilomes produced using cheese components. *Journal of Dairy Science, 96*(7), 4223–4234. https://doi.org/10.3168/jds.2012-6472

Silvetti, T., Morandi, S., & Brasca, M. (2018). Growth factors affecting gas production and reduction potential of vegetative cell and spore inocula of dairy-related Clostridium species. *LWT - Food Science and Technology, 92*, 32–39. https://doi.org/10.1016/j.lwt.2018.02.014

Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*, 591–610. https://doi.org/10.1016/j.femsre.2005.04.002

Thomsen, M., Martin, C., Mercier, F., Tournayre, P., Berdagué, J. L., Thomas-Danguin, T., & Guichard, E. (2012). Investigating semihard cheese aroma: Relationship between sensory profiles and gas chromatography-olfactometry data. *International Dairy Journal, 26*(1), 41–49. https://doi.org/10.1016/j.idairyj.2012.04.009

Wang, Y., Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., et al. (2021). Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. *Frontiers in Bioengineering and Biotechnology, 9*, 1–19. https://doi.org/10.3389/fbioe.2021.612285

Ward, L. J. H., & Timmins, M. J. (1999). Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. *Letters in Applied Microbiology, 29*(2), 90–92. https://doi.org/10.1046/j.1365-2672.1999.00586.x

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express, 2*(1), 1–12. https://doi.org/10.1186/2191-0855-2-48