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Nonstarter lactic acid bacteria volatilomes produced using cheese components

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ABSTRACT

In long-ripened cheese, flavor formation occurs during ripening. The metabolism of lactic acid bacteria (LAB) leads to the production of different compounds that contribute to the flavor of cheese. The contribution of LAB to the formation of cheese flavor has previously been studied. However, the specific nonstarter LAB (NSLAB) metabolic reactions in ripened cheese that lead to the formation of flavor compounds remain unclear. In ripened cheese, the nutrient sources available include small peptides or amino acids, citrate, lactate, free fatty acids, and starter LAB cell lysis products. Thus, the aim of this study was to evaluate the ability of NSLAB to produce volatile flavor compounds by using an in vitro system that used only the nutrients available in ripened cheese as the energy source. Moreover, the potential contribution of the NSLAB volatilome on total cheese flavor is discussed. For this purpose, the production of volatile compounds on cheese-based medium (CBM) and on starter LAB lysed cell medium (LCM) by 2 *Lactobacillus casei* and 2 *Lactobacillus rhamnosus* strains, previously isolated from ripened Parmigiano Reggiano cheese, was investigated. The generated volatile compounds were analyzed with head-space gas chromatography mass spectrometry. Overall, ketones, aldehydes, alcohols, and acids were the most abundant compounds produced. Differences in volatilome production were found between NSLAB grown in LCM and CBM. The catabolic metabolism of amino acids and fatty acids were required for NSLAB growth on LCM. Conversely, pyruvate metabolism was the main catabolic pathway that supported growth of NSLAB in CBM. This study can be considered a first step toward a better understanding of how microbiota involved in the long ripening of cheese may contribute to the development of cheese flavor.

Key words: volatilome, nonstarter lactic acid bacteria, cheese flavor compound

INTRODUCTION

Flavor perception is very important in the distinction of fermented dairy products and for the determination of their quality (Olson, 1990). The combination of a large number of sapid volatile and nonvolatile compounds, in the correct ratios and concentrations, leads to the formation of cheese flavor (McSweeney and Sousa, 2000). The flavors of different cheeses have already been characterized, such as Cheddar (Zehentbauer and Reineccius, 2002), Camembert (Kubickovà and Grosh, 1997), Gorgonzola (Moio et al., 2000), Grana Padano (Moio and Addeo, 1998), Parmigiano Reggiano (Langford et al., 2012), Pecorino Crotonese (Randazzo et al., 2010), and Pecorino Romano and Fiore Sardo (Di Cagno et al., 2003). To date, at least 600 volatile compounds have been detected and identified in cheese (Maarse and Visscher, 1989).

Cheese is a biochemically dynamic product, and during its ripening significant microbial and biochemical changes occur. Freshly made curds of several types of cheese have bland and similar flavors. However, in relation to cheese, the production of flavor compounds produced during ripening can vary in terms of the time of production and the environmental conditions in which they are produced (McSweeney and Sousa, 2000). Thus, the formation of flavors is a rather slow process that involves several chemical and biochemical reactions that occur during aging (Smit et al., 2005). This complex process involves 3 major metabolic pathways: (1) metabolism of lactate and citrate, (2) liberation of FFA and their subsequent metabolism, (3) degradation of the casein matrix of the curd to a range of peptides, which is followed by degradation to free AA, and ultimately involves the catabolism of free AA (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001). Metabolically volatile compounds derived from amino acids were studied (Tavaria et al., 2002; Fernández and Zuniga, 2006), and the ability of lactic acid bacteria (**LAB**) to generate flavor compounds through AA catabolism was observed (Yvon and Rijnen, 2001). Both lipolysis and the pathways of FFA catabolism that directly affect cheese flavor have been reported (Collins et al.,

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2003). Additionally, the ability of LAB to use lactate and citrate for the production of flavor compounds has been confirmed (McSweeney, 2004).

Research in this area has mainly focused on single species, such as *Lactococcus lactis* (which is added as a starter for Cheddar, Gouda, and other cheese varieties; Broadbent and Steele, 2005), *Lactobacillus delbrueckii* (and the subspecies *bulgaricus*), and *Streptococcus thermophilus* (Helinck et al., 2004); these species are widespread in cheeses and fermented milks (Yvon and Rijnen, 2001). However, cheese flavor may be affected by the entire spectrum of cheese microbiota, which is composed of starter LAB (**SLAB**) and adventitious species including nonstarter LAB (**NSLAB**). Despite several studies that state that the metabolic capabilities of LAB lead to the production of flavor compounds, the effects of NSLAB metabolic products needs further investigation.

In long-ripened cheese, SLAB and NSLAB develop dynamically (Broadbent and Steele, 2005). In Parmigiano Reggiano (**PR**), a raw milk long-ripened Italian cheese, the number of SLAB commonly exceeds 10^9 cfu/g when ripening begins. After PR brining, SLAB cells undergo autolysis (Gatti et al., 2008), and NSLAB (initially fewer than 10^2 cfu/g) grow at cell densities of 10^6 to 10^7 cfu/g (Coppola et al., 1997; De Dea Lindner et al., 2008). At this point, NSLAB become the dominant microflora of cheese until the end of ripening (Gatti et al., 2008). Nonstarter LAB development during ripening can be attributed to their ability to use the major compounds of ripened cheese, such as small peptides and AA that are present at high concentrations, as nutrient sources for growth (Fox and McSweeney, 2004). Additionally, other compounds are found in ripened cheeses that are the products of starter cell autolysis, which include sugars and phospholipids from cell walls, nucleic acids, and peptides. These compounds represent carbon sources for NSLAB (Thomas, 1987; Rapposch et al., 1999; Budinich et al., 2011).

The aim of the current study was to evaluate the ability of NSLAB to produce volatile flavor compounds by using an in vitro system that used only the nutrients available in ripened cheese as the energy source, and to discuss the potential contribution of the NSLAB volatilome on total cheese flavor. For this purpose 2 *Lactobacillus casei* and 2 *Lactobacillus rhamnosus* strains were grown in 2 different media: a cheese-based medium proposed by Neviani et al. (2009) and a medium based on *Lactobacillus helveticus* cell lysate. Growth was monitored through plate counting, and the volatile compounds produced during growth were analyzed through solid-phase microextraction (**SPME**) gas chromatography mass analysis. This is the first work investigating the determination of flavor compounds produced by viable NSLAB growth in an in vitro system, and can be considered a first step toward the selection of a wild NSLAB strain that possess specific aromatic profiles.

MATERIALS AND METHODS

Strains

Four NSLAB strains that were previously isolated from ripened PR cheese, *L. rhamnosus* (1216 and 1473) and *L. casei* (1056 and 1247; Neviani et al., 2009), and 3 SLAB strains that were previously isolated from PR natural whey starter (Neviani et al., 2009), *L. helveticus* (770, 772, and 780), were used. All strains are a part of the Department of Food Science of the University of Parma collection and were maintained as stock cultures at −80°C in de Man, Rogosa, and Sharpe (**MRS**) broth (Oxoid, Basingstoke, UK) supplemented with 15% glycerol (wt/vol).

Preparation of Culture Media

Two different culture media were used: cheese broth medium (**CBM**; Neviani et al., 2009), which mimics cheese, and lysed cell medium (**LCM**) containing only lysed *L. helveticus* cells, which mimics the lysate of SLAB. The CBM was prepared according to the protocol proposed by Neviani et al. (2009) and formulated with 20-mo ripened PR cheese without agar addition. To prepare LCM, 3 *Lactobacillus helveticus* strains (770, 772, and 780) were grown anaerobically (Gas Generating Kit, Oxoid Ltd.) with a 2% initial inoculum in 20 mL of MRS broth at 42°C for 24 h; this growth procedure was repeated. Twenty milliliters of each culture were mixed in 1 L of MRS broth and incubated at 42°C for 24 h. After incubation, the 10^8 cell concentration was determined by microscopic counting (Gatti et al., 2006). The culture was harvested by centrifugation at 10,062 × *g* for 10 min at 4°C (5810R, Eppendorf, Hamburg, Germany), and the cells were resuspended in 60 mL of phosphate buffer (50 m*M*, pH 7; Carlo Erba Reagents, Milan, Italy). The cell suspension was sonicated with a titanium probe operated at 70 W for 30 cycles (30-s pulse on and 1-min pulse off; Sonoplus HD3100, Bandelin, Berlin, Germany) and cooled on ice. Sonication was repeated twice. The MRS agar plate count was performed in duplicate to verify the consistency of sonication and was lower than the limit of detection (0 cfu/mL). As the medium could not be sterilized, the plate counts also helped ensure a lack of contamination. After preparation, LCM was stored at 4°C and was used immediately.

Growth Evaluation

Lactobacillus casei (1056 and 1247) and *L. rhamnosus* (1216 and 1473) were grown anaerobically (Gas Generating Kit, Oxoid Ltd.) with a 2% starting inoculum in 6 mL of MRS broth at 30°C for 24 h; this growth procedure was repeated. To obtain cells for inocula, each viable culture was washed in Ringer solution (Oxoid Ltd.) and harvested at 10,062 × *g* for 10 min at 4°C. Each culture was inoculated to obtain a concentration of 10^6 cfu/mL in vials containing 5 mL of LCM or CBM. Cultures were incubated in CBM and LCM at 30°C for 4 and 6 d, respectively, to reach a similar cell concentration. Before and after incubation in CBM and LCM, microbial counts were carried out in triplicate on MRS agar following anaerobic incubation at 30°C for 48 h. The data are expressed as the mean and standard deviation.

Volatilome Determination

The volatile compounds produced by the 4 strains of NSLAB, following growth in LCM and CBM, were detected through SPME-GC analysis of the headspace of the sealed vials in which they were grown. The LCM and CBM media without inoculum were used as a control and were incubated under the same conditions that were used with media containing inoculum. Experiments were carried out in triplicate. For each media and strain, analysis of the volatile compounds in the vial headspace was performed by GC-MS using an Agilent Technologies 6890 gas chromatograph coupled to an Agilent Technologies 5970 mass spectrometer (Waters, Milford, MA). An SPME fiber assembly with a divinylbenzene-carboxen-polydimethylsiloxane coating 50/30 μm (Supelco Inc., Bellefonte, PA) was used after preconditioning according to the manufacturer's instruction manual. The samples were preheated for 10 min at 45°C. The SPME fiber was exposed to each sample at 40°C for 40 min, and the fiber was inserted into the injection port of the GC for 5 min of sample desorption. A Varian CB 7773 capillary column (50 m × 320 μm × 1.2 μm) was used. Volatile compounds were separated under the following conditions: helium carrier gas (1 mL/min), an initial column temperature of 50°C for 1 min, an increase of temperature to 65°C at 4.5°C/min, an increase of temperature to 230°C at 10°C/min, and maintenance at 230°C for 25 min. The injector, interface, and ion source temperatures were 250, 250, and 230°C, respectively. Each chromatogram obtained contained a high number of peaks, most of which were tentatively identified by comparison of the experimental mass spectral data with those of the NIST Mass Spectral database library (NIST/EPA/NIH version 1998). The relative abundance of each compound was expressed as the percentage of its peak area with respect to the total peak area. All of the volatile compounds identified in LCM and CBM media were grouped by chemical classes (alcohols, ketones, aldehydes, acids, and others). Unidentified peaks were not reported; however, their overall relative abundance was always lower than 5% of the total peak area under all conditions tested. The principal component analysis (**PCA**) was carried out with the statistical package Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy).

RESULTS AND DISCUSSION

Growth Evaluation

Lactobacillus casei and *L. rhamnosus* growth in sealed vials containing CBM and LCM broth was monitored via plate counting. The growth of the 2 lactobacilli strains in both media is described in Table 1. No growth was detected in LCM and CBM without inoculum (control).

Volatilome in LCM

The LCM medium obtained by the mechanical breaking of *L. helveticus* cells simulated autolysis of starter cells and likely contained mainly FA, AA, N-acetylglucosamine, and ribose, all of which can be used as a nutrient source in ripened cheese for NSLAB growth (Thomas, 1987; Adamberg et al., 2005; Budinich et

Table 1. Plate count in de Man, Rogosa, and Sharpe broth (log cfu/mL) of *Lactobacillus casei* 1056, *L. casei* 1247, *Lactobacillus rhamnosus* 1216, and *L. rhamnosus* 1473 inoculated in lysed cell medium (LCM) and cheese-based medium[1]

	LCM		CBM	
Strain	t0	6 d	t0	4 d
Lc 1056	1.8×10^7 (3.8×10^6)	1.5×10^8 (4.1×10^7)	7.5×10^6 (3.7×10^5)	2.8×10^8 (1.1×10^8)
Lc 1247	2.4×10^7 (4.8×10^6)	3.6×10^8 (6.3×10^7)	2.2×10^7 (3.4×10^6)	2.2×10^8 (1.1×10^8)
Lr 1216	5.4×10^6 (1.8×10^5)	9.0×10^7 (1.7×10^7)	2.7×10^7 (1.9×10^6)	8.7×10^7 (5.8×10^7)
Lr 1473	2.3×10^7 (2.2×10^6)	1.3×10^8 (8.2×10^6)	2.8×10^7 (2.6×10^5)	1.3×10^8 (3.2×10^7)

[1]The count was carried out in triplicate before (t0) and after 4 (CBM) and 6 d (LCM) of incubation at 30°C. Data are expressed as mean and SD.

al., 2011). The growth of the inoculated strains demonstrated that LCM components could be used as a nutrient source. Therefore, the volatile substances produced by metabolizing these compounds were analyzed to evaluate if this metabolism, which is based on the consumption of cell lysis products, could lead to the formation of flavor compounds. The volatile compounds identified after growth of *L. casei* 1056, *L. casei* 1247, *L. rhamnosus* 1216, and *L. rhamnosus* 1473 in LCM media are reported in Table 2. Differences in the composition of the vial headspace of LCM samples were observed in the noninoculated (controls) and inoculated samples, and differences in composition were also observed among the samples inoculated with the 4 strains of the 2 different species, *L. casei* and *L. rhamnosus*.

One of the compounds that was most abundant in the headspace of the samples is an alkane, 6,6-dimethyl-undecane. To our knowledge, the production of this molecule through LAB metabolism has not been previously described. The presence of this alkane in the control (approximately 13% of the total volatile area in the head space) suggests that it is also accumulated in the *L. helveticus* strains used for LCM preparation before cell rupture. The 2 NSLAB species studied here demonstrated different behaviors toward 6,6-dimethyl-undecane. *Lactobacillus casei* reduced the amount of this molecule (final relative percentage 7–8%) and *L. rhamnosus* contributed to an increase in 6,6-dimethyl-undecane in the head-space (up to 15–17%). In contrast, another alkane found in the LCM samples (tetramethyl-cyclohexane) did not significantly change in relative percentage with relation to NSLAB presence and growth.

Even if the metabolic pathway that leads to the production of these 2 molecules has not been previously described, their hydrocarbon structure could indicate that the metabolism of FA might be a possible source of their production (Collins et al., 2003). In previous studies, molecules belonging to the alkane group, such as dodecane, hexane, heptane, and octane, were detected in different cheeses (Moio and Addeo, 1998; Curioni and Bosset, 2002). The alkane group molecules are derived from FA that act as precursors for a series of metabolic reactions that lead to the production of several volatile compounds, including alkanes (Collins et al., 2003).

The origin of 3,3-dimethyl-butanamide was even more unclear. The relative percentage of this molecule (between 7 and 11%) was not significantly different with respect to the control (8%) or between the samples following the growth of the different NSLAB species. This compound has a baked note and has been found in PR cheese flavor (Qian and Reineccius, 2002). It is possible to hypothesize that 3,3-dimethyl-butanamide, a nitrogen-containing compound, can be derived from the degradation of Trp or other AA (Curioni and Bosset 2002).

The relative percentage of ketones in the vial headspace generally increased following the growth of the inoculated *L. casei* and *L. rhamnosus* samples. The increase in ketone relative percentage ranged from approximately 2% in the control to approximately 5 to 6% in the inoculated samples independent of strain. The most abundant ketones in the vial headspace were 2-heptanone, 4-methyl-2-heptanone, 2-tridecanone, and 2-pentadecanone. Ketones are common constituents of most dairy products and are known for their contribution to cheese aroma due to their odor and low perception threshold (Curioni and Bosset, 2002). Among the ketones detected, 2-heptanone is known to be an important flavor compound in Emmental and natural and creamy Gorgonzola (Moio et al., 2000; Curioni and Bosset, 2002). Moreover, 2-nonanone only increased in samples inoculated with the *L. rhamnosus* 1473 strain and is a predominant methyl-ketone in natural Gorgonzola and ripened Ragusano cheeses (Curioni and Bosset, 2002). Furthermore, 2-heptanone, 2-nonanone, and 2-tridecanone were found in Grana Padano (Moio and Addeo, 1998). Ketones are also an important class of flavor compounds in Fiore Sardo and Pecorino Romano (Di Cagno et al., 2003). The general pathways of ketone production were reported by McSweeney and Sousa (2000): after the release of FFA by lipolysis they are oxidated to β-ketoacids and decarboxylated to ketones or alkan-2-ones with 1 less carbon atom. Therefore, the ketones found in LCM medium are related to the NSLAB metabolism of FA. For this reason, it is possible to hypothesize that the FA, and the lipids derived from those FA, that arose from the cellular membrane of SLAB were metabolized through this pathway.

Among aldehydes, benzaldehyde deserved specific consideration, as it was the only aldehyde found in the noninoculated control (approximately 2%). The relative percentage of benzaldehyde did not significantly vary after *L. casei* growth; however, its relative percentage increased by approximately 6% in the presence of the 2 *L. rhamnosus* strains. Benzaldehyde is produced as a result of the catabolism of aromatic AA, particularly phenylalanine and tyrosine. Benzaldehyde arises from the chemical conversion of α-ketoacids, such as phenyl pyruvic acid and *p*-hydroxy phenyl pyruvic acid, which are produced by the transamination of phenylalanine and tyrosine respectively (Yvon and Rijnen, 2001; Marilley and Casey, 2004; Fernández and Zuniga, 2006). However, no evidence exists of direct energy production from the metabolic steps involved in production of benzaldehyde.

Table 2. Volatile compounds identified in lysed cell media before (control) and after growth of *Lactobacillus casei* 1056, *L. casei* 1247, *Lactobacillus rhamnosus* 1216, and *L. rhamnosus* 1473[1]

	Control		Lc 1056		Lc 1247		Lr 1216		Lr 1473	
Compound	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
4,4-Dimethyl-2-pentanone	0.87	0.15	0.72	0.62	0.64	0.41	1.20	0.59	1.09	0.60
2-Tetradecanone	—[2]	—	1.42	0.16	—	—	—	—	—	—
2-Heptanone	—	—	—	—	1.40	0.30	—	—	1.83	0.71
4-Methyl-2-heptanone	—	—	2.27	0.20	2.35	0.16	—	—	0.80	0.43
2-Nonanone	0.86	0.07	0.96	0.11	0.68	0.44	0.73	0.34	1.41	0.27
2-Tridecanone	—	—	0.40	0.30	—	—	1.50	0.84	—	—
2-Penta-decadenone	—	—	—	—	—	—	1.51	1.39	—	—
Ketones[3]	1.73	0.19	5.78	0.78	5.06	0.90	4.95	1.72	5.13	1.15
8-Octadecenal	—	—	—	—	2.00	0.50	1.78	0.88	1.57	0.84
Nonanal	—	—	—	—	—	—	—	—	2.72	0.44
Benzaldehyde	1.91	0.45	1.36	0.56	1.58	0.13	5.76	1.36	7.29	1.15
2-Decenal	—	—	—	—	—	—	1.28	0.54	0.82	0.45
Aldehydes[3]	1.91	0.45	1.36	0.56	3.58	0.58	8.82	1.63	12.39	1.71
Ethyl alcohol	10.92	1.21	19.58	1.15	22.00	0.57	11.29	0.39	7.23	0.46
3-Methyl-1-butanol	1.16	0.05	1.52	0.49	3.07	0.12	—	—	1.51	0.82
3-Methyl-1-pentanol	6.12	0.24	1.14	0.23	2.35	0.58	3.30	0.45	2.68	0.27
6,6-Dimethyl-1,3-heptadien-5-ol	1.34	0.83	1.60	0.27	—	—	—	—	—	—
1-Hexanol	8.65	0.67	—	—	3.33	0.20	—	—	—	—
2-Heptanol	—	—	0.47	0.35	2.40	0.65	3.06	0.26	2.91	0.58
2-Ethyl-1-hexanol	3.63	0.36	3.15	0.21	—	—	3.06	0.13	3.80	0.46
1-Octanol	5.22	0.20	2.53	0.29	4.50	0.48	3.54	0.45	3.43	0.71
4-(Methylthio) phenol	0.73	0.51	1.10	0.48	0.93	0.60	—	—	—	—
1-Nonanol	1.30	0.07	1.79	0.06	2.77	0.52	1.93	0.27	1.62	0.38
1-Undecanol	1.00	0.11	3.54	0.02	3.64	0.23	1.81	0.34	1.91	0.31
Phenylmethanol	0.73	0.45	—	—	—	—	—	—	—	—
Dodecanethiol	1.50	0.02	2.68	0.21	2.92	0.45	1.51	0.90	2.08	0.22
2,4-bis-(1,1-dimethylethyl)-phenol	3.25	0.33	3.72	0.04	4.86	0.22	—	—	—	—
Phenol-4-(1,1,3,3-tetramethylbutyl)	6.07	1.03	4.97	1.10	1.38	1.02	3.52	2.20	8.65	2.46
Alcohols[3]	51.63	4.56	47.78	3.23	54.15	2.71	33.02	3.33	35.82	4.15
Benzoic acid	—	—	—	—	—	—	—	—	—	—
2,2-Dimethyl-propanoic acid	—	—	1.46	0.06	1.28	0.18	—	—	—	—
Acetic acid	—	—	1.72	0.61	1.28	0.05	3.30	3.11	2.91	0.05
3-Methyl-pentanoic acid	1.82	0.07	—	—	—	—	—	—	—	—
Acids[3]	1.82	0.07	3.19	0.63	2.55	0.21	3.30	3.11	2.91	0.05
Hexadecanoic acid methyl ester	1.37	1.03	—	—	—	—	—	—	—	—
2,4-Dimethyl heptene	—	—	4.47	0.74	3.01	0.31	—	—	—	—
Dimethyl-disulfide	2.62	0.61	1.06	0.06	1.14	0.76	2.44	0.67	2.07	1.11
Heptene	1.58	0.24	1.62	0.28	0.79	0.51	1.38	0.44	1.32	0.72
6,6-Dimethyl-undecane	12.85	3.39	7.92	2.94	7.68	1.98	17.02	3.07	15.31	5.16
3,3-Dimethyl-butanamide	8.40	2.18	11.03	4.84	7.99	3.43	9.71	2.93	9.49	5.53
2-Pentyl furan	3.07	0.52	2.46	1.32	3.03	0.95	2.82	0.98	2.68	0.66
2-Methyl-1,2,3-tricholo-propane	1.71	0.69	1.64	0.23	1.63	0.35	2.24	0.19	1.85	0.37
1,3-bis (1, 1-dimethylethyl)-benzene	1.81	0.66	3.34	1.01	3.15	0.80	3.19	0.81	2.66	0.51
Tetra-methyl-cyclohexane	3.63	0.25	3.59	0.47	4.44	0.60	4.08	0.74	4.51	0.85
Hexaoxacyclooctadecane	0.89	0.55	0.61	0.47	—	—	2.09	1.53	0.94	0.68
Other compounds[3]	37.92	5.12	37.73	6.32	32.84	5.63	44.97	4.59	40.84	7.15
Total percentage of identified compounds	95.02	8.64	95.84	9.89	98.19	7.56	95.05	9.61	97.09	8.44
Total area of identified compounds[4]	15,599	1,347	16,232	1,605	15,040	1,137	14,665	1,409	13,011	1,098

[1]The data are expressed as relative abundance percentages and are reported as the mean of triplicate experiments.

[2]Under the detection limit.

[3]Sum of volatile compounds of the same chemical classes.

[4]Expressed as arbitrary units $\times 10^4$.

The aldehydes in the vial headspace changed in relation to the strains, particularly 2-decenal, nonanal, and 8-octadecenal. These aldehydes were found in relative percentages of between approximately 2 and 6% after microbial growth. Nonanal was found only following the growth of the *L. rhamnosus* 1473 strain, and 2-decenal was present only following the growth of the 2 *L. rhamnosus* strains. The aldehyde 8-octadecenal was found following the growth of both of the *L. rhamnosus* strains and the *L. casei* 1247 strain. Nonanal, 2-decenal, and 8-octadecenal are aliphatic aldehydes; therefore, they can be produced by β-oxidation of

unsaturated FA (Curioni and Bosset, 2002). In fact, straight-chain aldehydes are presumably produced by FA metabolic pathways, whereas branched-chain aldehydes are produced through the catabolism of AA (Belitz et al., 2004). Some aldehydes that were found in the experimental samples are quite common among the cheeses. Nonanal is usually considered among the most important, and it is characterized by a green grass and herbaceous aroma (Curioni and Bosset, 2002).

Among alcohols, ethanol was found at high relative percentages in the control sample (10%) and at similar relative percentages in samples inoculated with *L. rhamnosus* (11 and 7%). After growth with *L. casei*, ethanol increased at least 2 times with respect to the control sample, and the relative percentages was determined to be 19 and 22% in the presence of the strains Lc1056 and Lc1247, respectively. Ethanol mainly originates from acetaldehyde in the lactate metabolic pathway. Under limiting nutritional conditions, NSLAB can metabolize lactate through different metabolic pathways (McSweeney and Sousa, 2000; Marilley and Casey, 2004). In LCM, ethanol production through the metabolism of lactate can be excluded due to the low concentration of lactate present in the medium. However, ethanol can also be generated from the pentose sugar pathway (Mozzi et al., 2010). Furthermore, ethanol can be produced by the catabolism of AA. It is known that threonine catabolism leads to the production of acetaldehyde (and glycine) with the concomitant oxidation of NADPH. Acetaldehyde can then be further reduced to ethanol in the presence of alcohol dehydrogenase (Ardö, 2006). Following NSLAB growth, other alcohols that were present in the control sample decreased with respect to the control levels. This is observed in the relative percentage of 3-methyl-pentanol (6.12% in the control sample). After the growth of all experimental strains, the 3-methyl-pentanol decreased to approximately 1 to 3%; a similar trend was observed for 1-octanol. Furthermore, 1-hexanol (8% in the control sample) was absent in the experimental samples after NSLAB growth, with the exception of *L. casei* Lc1247, which was unable to completely metabolize this alcohol.

Conversely, 2-heptanol was present in all experimental samples following NSLAB growth; 2-heptanol is found in the flavor of Gorgonzola (Moio et al., 2000) and Grana Padano cheeses (Moio and Addeo, 1998), and its odor is herbaceous, green, and oily (Curioni and Bosset, 2002). Secondary alcohols, such as 2-heptanol, are formed by the enzymatic reduction of the corresponding methyl ketones that arise from FA by β-oxidation or from β-ketoacids (Molimard and Spinnler, 1996).

It is interesting to note that the 3-methyl-1-butanol did not change following NSLAB growth with the exception of the *L. casei* 1247 strain, which produced 3-fold its initial amount. The 3-methyl-1-butanol present was most likely derived from the degradation of leucine, and it confers a pleasant aroma of fresh cheese (Moio et al., 1993; Randazzo et al., 2007).

Many metabolic pathways are involved in the biosynthesis of alcohols: lactose or lactate metabolism, aldehyde reduction, and AA catabolism, which includes the degradation of linoleic and linolenic acids (Molimard and Spinnler, 1996). In LCM, it is possible that the catabolism of AA is the major metabolic pathway responsible for the production of alcohols by NSLAB. This hypothesis was confirmed by the lack of detection of alcohol-related aldehydes.

The relative percentage of aromatic alcohols in the samples tested showed interesting differences. The relative percentage of 2,4-bis-dimethyl-ethyl-phenol was comparable in the control and in the samples following *L. casei* growth, but it was not detected following *L. rhamnosus* growth. Additionally, 1,1,3,3-tetramethyl-4-butyl-phenol was present at a relative percentage of more than 6% in control samples and demonstrated a strain-dependent trend. Phenolic compounds often contribute positively to cheese flavor can also confer an unpleasant note when their concentrations exceed certain limits. The sensory quality ranges from sharp, medicinal, sweet, and aromatic to smoky, charred, caramel, unpleasant, and "sheep-yard" (Curioni and Bosset, 2002). In the literature, only the production of phenol molecules without methyl groups has been reported (Curioni and Bosset, 2002; McSweeney, 2004).

Finally, acetic acid was absent in the control but was found in relative percentages from approximately 1% following *L. casei* growth to approximately 3% following *L. rhamnosus* growth. The presence of acetic acid can be explained through several metabolic pathways. Under poor nutritional conditions, lactate can be metabolized (McSweeney, 2004) and leads to the formation of pyruvate, which can then be converted into acetyl-phosphate and ultimately converted into acetic acid. In the metabolism of citrate (Palles et al., 1998; McSweeney, 2004), citrate lyase can catalyze the cleavage of citrate to oxaloacetate and acetic acid. Furthermore, the catabolism of some AA can lead to acetic acid formation (Liu et al., 2003). Due to the presumable absence of lactate, it is possible to hypothesize that acetic acid production in LCM can be due to the presence of acetate kinase, which leads to the formation of acetic acid and ATP from acetyl-phosphate.

Volatilome in CBM

The volatile compounds identified following the growth of *L. casei* 1056, *L. casei* 1247, *L. rhamnosus*

1216 and *L. rhamnosus* 1473 in CBM media are reported in Table 3. The volatile profile trend of the headspace samples following NSLAB growth in CBM demonstrated some similarities with the profiles that were observed following growth in LCM. This is not surprising, given that LCM is trying to mimic the lysed cells composition of CBM and, therefore, is already naturally present in CBM.

Compared with LCM, 6,6-dimethyl-undecane and 3,3-dimethyl-butanamide were found at lower relative percentages (approximately 6 and 3%, respectively) in the control samples and after microbial growth. In addition, pentadecane and 4-cyclohexyl-undecane were found to be present at a relative percentage of approximately 2 to 3%, which did not significantly change following NSLAB growth. As previously discussed, the presence of alkanes may be due to FA metabolic pathways (Collins et al., 2003). The most significant differences between the volatile profiles of control samples and CBM samples following NSLAB growth were observed for the aldehydes and ketones. With respect to ketones, a significant presence of acetone, diacetyl, and acetoin was observed. Acetone was produced in greater quantity by *L. casei* 1056 (4%) compared with the other strains (1–2%). High relative percentages of diacetyl and acetoin were found following all NSLAB growth,

Table 3. Volatile compounds identified in cheese-based media before (control) and after growth of *Lactobacillus casei* 1056, *L. casei* 1247, *Lactobacillus rhamnosus* 1216 and *L. rhamnosus* 1473[1]

	Control		Lc 1056		Lc 1247		Lr 1216		Lr 1473	
Compound	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Acetone	—[2]	—	4.33	0.16	1.56	0.12	1.25	0.12	1.60	0.57
2,3-Butanedione	—	—	7.39	0.14	17.29	0.41	13.73	0.59	—	—
2-Pentanone	—	—	—	—	—	—	—	—	1.81	0.57
2-Heptanone	17.56	0.26	11.68	0.73	9.01	0.19	8.34	0.26	12.25	1.44
3-Hydroxy-2-butanone	—	—	14.53	5.17	5.61	1.25	19.88	1.00	—	—
2-Nonanone	2.03	0.08	3.40	0.05	1.50	0.11	4.34	0.91	4.37	1.03
2-Undecanone	1.15	0.02	2.04	0.04	0.82	0.03	1.98	0.46	1.86	2.02
2-Tridecanone	1.06	0.06	2.22	0.28	0.72	0.01	1.95	0.06	1.71	2.42
Ketones[3]	21.80	0.38	45.59	6.07	36.52	1.63	51.47	2.01	23.60	4.15
3-Methyl butanal	2.49	0.19	—	—	—	—	—	—	—	—
Hexanal	6.69	0.02	—	—	—	—	—	—	—	—
8-Octadecenal	4.68	0.14	3.72	0.03	2.93	0.01	2.15	0.02	3.46	1.09
Nonanal	7.66	0.08	—	—	1.47	0.04	—	—	—	—
2-Nonenal	4.28	0.15	—	—	1.16	0.07	—	—	—	—
Decanal	1.59	0.01	—	—	0.71	0.07	0.26	0.15	—	—
Benzaldehyde	6.58	0.10	5.55	0.75	26.95	1.34	23.42	3.76	32.06	6.53
Aldehydes[3]	33.95	0.45	9.27	0.76	33.22	1.43	25.82	3.90	35.52	7.02
1-Hexadecanol	3.45	0.05	3.10	0.09	2.25	0.11	1.46	0.24	3.28	0.97
1-Octanol	1.59	0.03	—	—	1.44	0.03	—	—	1.09	2.06
4-Methylthio-phenol	1.74	0.08	1.10	0.34	1.49	0.07	0.86	0.16	1.62	0.41
2-Furanmethanol	—	—	0.73	0.23	0.40	0.15	—	—	—	—
Phenylethyl alcohol	—	—	1.23	0.04	—	—	—	—	—	—
Alcohols[3]	6.77	0.10	6.16	0.50	5.58	0.26	2.32	0.31	5.99	2.88
Acetic acid	—	—	5.29	0.65	1.71	0.21	1.43	0.15	3.47	1.34
n-Decanoic acid	—	—	—	—	—	—	—	—	2.07	0.51
Acids[3]	—	—	5.29	0.65	1.71	0.21	1.43	0.15	5.53	1.53
6,6-Dimethyl-undecane	8.25	0.84	9.01	1.09	5.44	0.97	5.10	0.87	7.54	0.83
3,3-Dimethyl-butanamide	4.65	0.85	4.94	0.89	2.36	0.84	2.27	0.96	3.90	0.64
4-Cyclohexyl-undecane	3.22	0.06	3.15	0.03	2.54	0.01	1.98	0.14	2.68	1.33
Pentadecane	4.08	0.01	3.42	0.18	3.19	0.02	2.02	0.13	2.65	1.59
2-Pentyl-furan	5.87	0.07	4.28	0.42	3.17	0.32	1.63	0.41	4.40	0.74
Tetradecane	1.33	0.08	0.86	0.27	0.56	0.21	0.71	0.01	0.80	0.20
1,3-bis (1,1-dimethylethyl) benzene	1.37	0.06	0.76	0.24	0.49	0.19	0.79	0.04	0.94	0.30
2(2,2 Dimethylvinyl) thiophene	1.97	0.03	1.26	0.04	1.35	0.02	0.72	0.02	1.11	0.28
Heptane, 1 chloro	1.77	0.27	1.39	0.03	0.50	0.18	0.46	0.19	1.52	1.06
Other compounds[3]	32.51	1.52	29.10	2.01	19.59	1.65	15.67	1.85	25.52	4.53
Total % of identified compounds	95.04	2.52	95.41	7.68	96.62	5.98	96.70	8.64	96.16	9.46
Total area of identified compounds[4]	24,175	609	24,762	1,902	22,533	1,347	27,288	2,358	22,341	2,113

[1]The data are expressed as relative abundance percentages and are reported as the mean of triplicate experiments.

[2]Under the detection limit.

[3]Sum of volatile compounds of the same chemical classes.

[4]Expressed as arbitrary units $\times 10^4$.

with the exception of *L. rhamnosus* 1473, which was unable to produce these aromatic compounds. These ketones were not detected in LCM.

Acetone has been reported in Cheddar (McSweeney and Sousa, 2000) and other cheeses. Acetone is a volatile compound that is likely produced by acetyl-CoA metabolic pathways in *L. casei* (Budinich et al., 2011) and *L. rhamnosus* (Ramzan et al., 2010).

Diacetyl is derived from pyruvate, which is produced from the metabolism of lactate, citrate, and AA, and it is appreciated for its buttery and nut-like notes. Diacetyl is a key aroma component of most cheeses, which include Camembert, Cheddar, and Emmental (Curioni and Bosset, 2002). It has been widely reported that NSLAB can metabolize citrate to acetoin and diacetyl (Palles et al., 1998; McSweeney, 2004). It is hypothesized that the citrate and lactate present in CBM were metabolized, which led to the formation of these compounds (Careri et al. 1996; Neviani at al. 2009). The relative percentage of 2-heptanone, the major constituent of the headspace of the control sample, decreased following the growth of all NSLAB samples. Additionally, the relative percentage of 2-nonanone increased 2-fold with respect to the control in all of the inoculated samples, with the exception of *L. casei* Lc1247. Because 2-nonanone and 2-heptanone are methyl-ketones, it is reasonable to assume that, similar to other methyl-ketones, they are derived from the catabolism of FA.

Among the aldehydes detected in the control sample, 3-methyl-butanal, hexanal, 8-octadecenal, nonanal, 2-nonenal, and decanal decreased or were not detected following *L. casei* and *L. rhamnosus* growth. Therefore, it is possible to hypothesize that aldehydes can be rapidly reduced to alcohols or oxidized to the corresponding acids (Curioni and Bosset, 2002).

Aldehydes such as 8-octadecenal, nonanal, and 2-decenal increased following bacterial growth in LCM, which was not observed in CBM. In LCM, the benzaldehyde consistently increased following NSLAB growth with the exception of *L. casei* 1056.

In CBM, the total alcohol increased following NSLAB growth, with the exception of *L. casei* 1056. The alcohol present at the highest relative percentage (approximately 2–3%) was 1-hexadecanol, and it did not significantly change following NSLAB incubation.

Finally, acetic acid was absent in the control sample and increased following NSLAB growth, particularly in *L. casei* 1056 (5%). The higher quantity of acetic acid found following growth in CBM, as opposed to LCM, could be due to lactate and citrate metabolism. Additionally, LAB are able to produce acetic acid from pyruvate under poor nutrient conditions (McSweeney and Sousa, 2000).

PCA of Volatile Compounds

To better evidence the relationships between the LAB strains and the volatilome a PCA was carried out using the relative percentages of the volatile compounds for LCM and CBM, reported in Tables 2 and 3, respectively.

Figure 1a reports the PCA loadings plots on the 2 first factors relative to the volatiloma composition of the 4 strains grown on LCM and the medium not inoculated. Factor 1 accounted for 40.10% of the variability and factor 2 for 29.54%. Figure 1b reports the variable factor coordinates for the first 2 factors, which represent the correlations of the respective variables with each factor. The not inoculated control was different from the inoculated media because of the importance of the compounds that contributed to factor 2 with a negative sign, such as 3-methyl-1-pentanol, 1-octanol, phenylmethanol, hexadecanoic acid methyl ester, and 3-methyl-pentanoic acid. By contrast, the inoculated media were all characterized by a positive sign of factor 2. In addition, the strains belonging to the 2 species were grouped according to the signs of the contributions of volatile compounds to factor 1. In particular, *L. rhamnosus* strains were clustered because of their higher content of benzaldheyde, 2-decenal, 6,6-dimethyl-undecane, which are present in factor 1 with a positive sign. By contrast, the strains of *L. casei* were grouped according to their high contents of 4-methyl-2-heptanone, ethanol, 3-methyl-1-butanol, 1-undecanol, 2,2-dimethyl-propanoic acid, and 2,4-dimethyl heptene, which characterized factor 1 with a negative sign.

In Figure 2a are the PCA loadings plots of the 2 first factors relative to the volatilome composition of the 4 strains grown on CBM and the medium not inoculated; these 2 factors accounted for about 75% of variability (50.92 and 24.99 for factor 1 and factor 2, respectively). Differences between inoculated and not inoculated samples are explained mainly by the factors having a negative sign in factor 1. In fact, compounds such as 2-heptanone and the aldehydes 3-methyl-2-butanal, hexanal, 8-octadecenal, nonanal, 2-nonenal, and decanal are mainly present in the not inoculated medium, as well as pentadecane, tetradecane, 2-pentylfurane, and 2-(2,2 dimethylvynil) thiophene. By contrast, the volatilome after bacterial growth is characterized by the presence of compounds with a positive sign in factor 1, such as acetoin, diacetyl, acetone, and other ketones (nonanone, 2-undecanone, 2-tridecanone), as well as benzaldehyde and acetic acid. Differences have been seen among the strains of the same species. The different position of *L. rhamnosus* strains can be explained by the different behaviors showed by the absence of

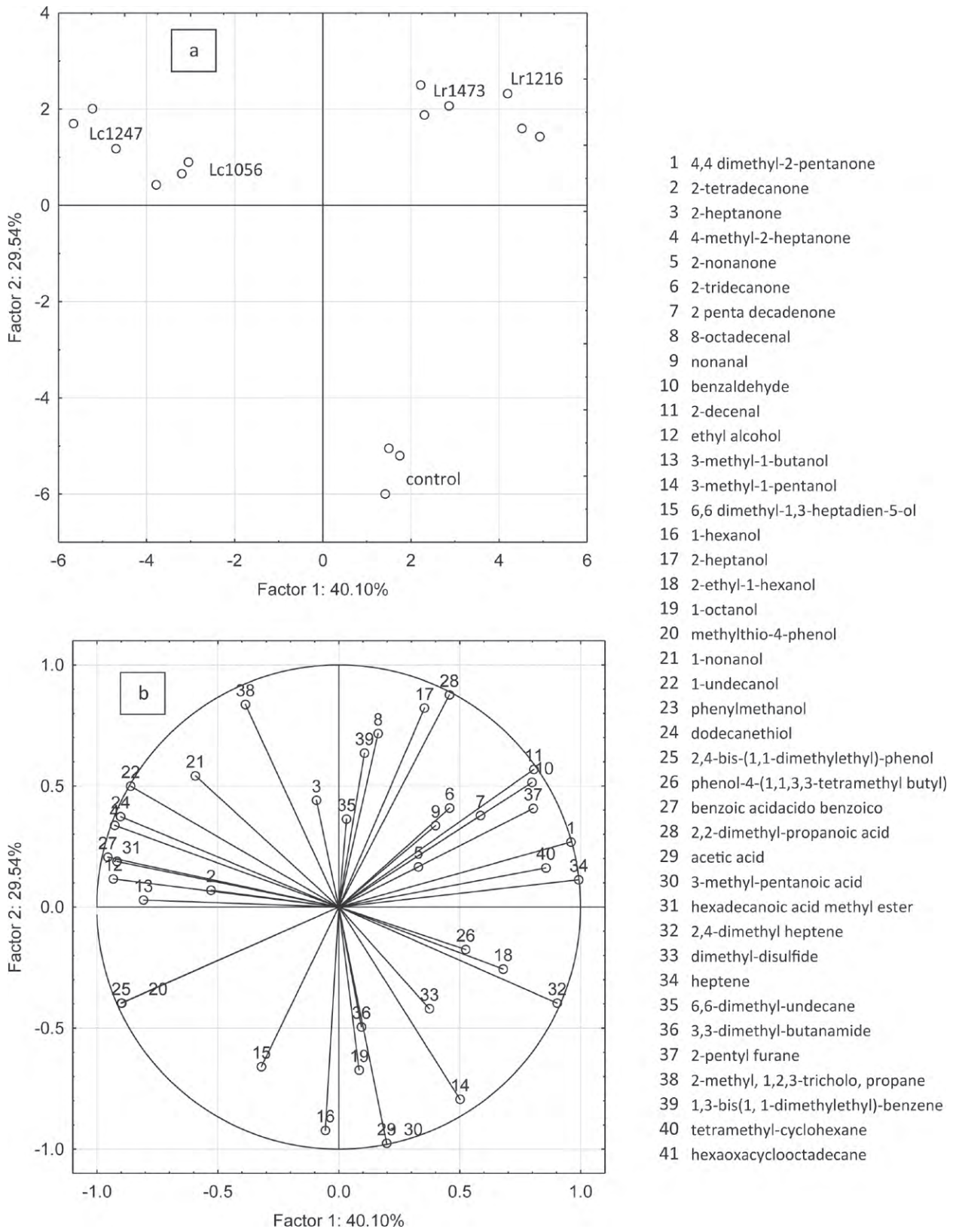

Figure 1. Principal component analysis loadings plots on the 2 first factors relative to the volatiloma composition of the 4 strains grown on lysed cell medium and the medium not inoculated (a) and variable factor coordinates for the first 2 factors (b); the numbers correspond to the volatile compounds reported on the right side of the figure. Lc = *Lactobacillus casei*; Lr = *Lactobacillus rhamnosus*.

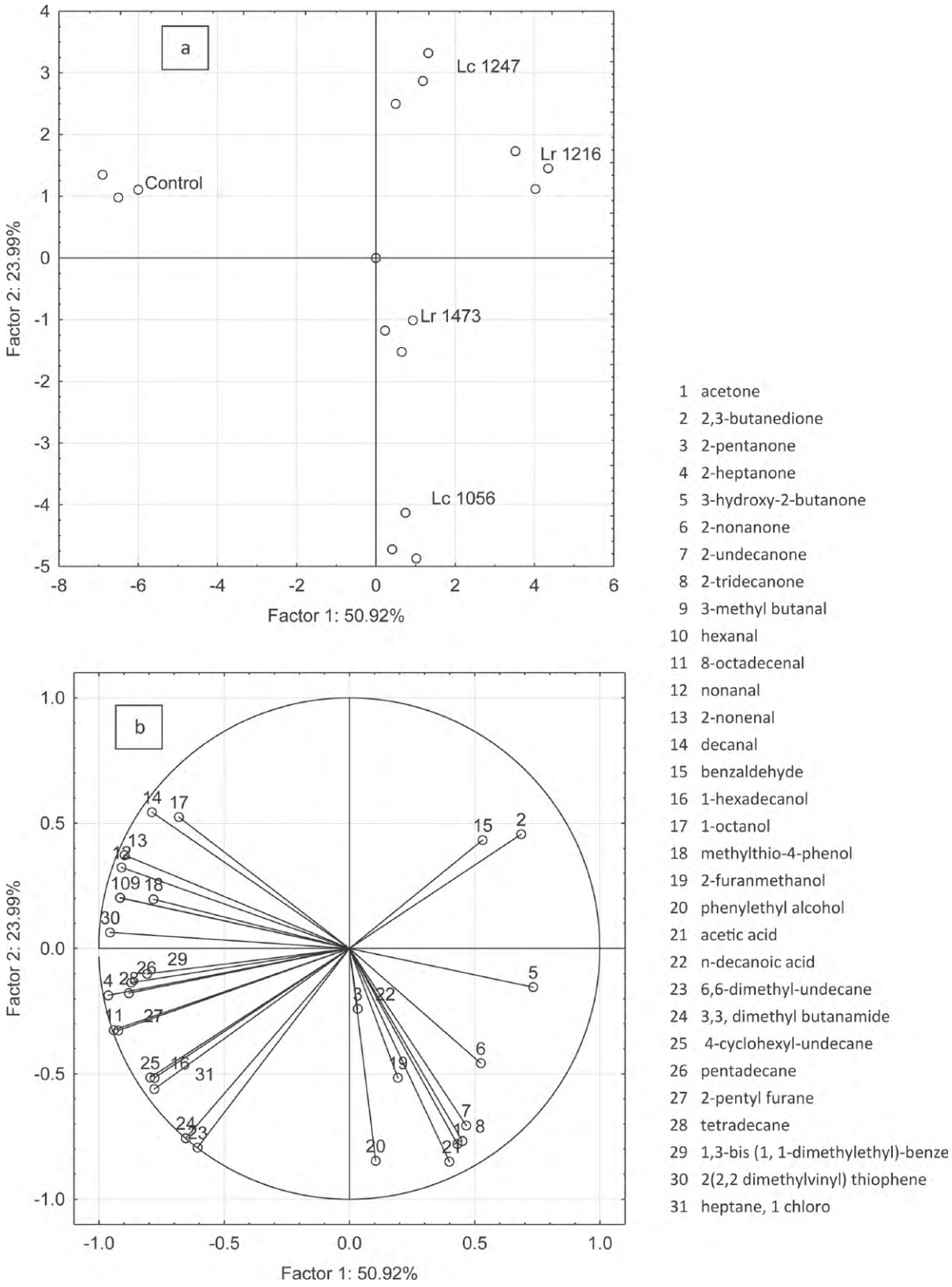

Figure 2. Principal component analysis loadings plots on the 2 first factors relative to the volatiloma composition of the 4 strains grown on cheese-based medium and the medium not inoculated (a) and variable factor coordinates for the first 2 factors (b); the numbers correspond to the volatile compounds reported on the right side of the figure. Lc = *Lactobacillus casei*; Lr = *Lactobacillus rhamnosus*.

the citric acid pathway in the strain Lr1473. The same pathway seems to be able to discriminate also the strains of *L. casei*. In fact, both strains were even able to produce aromatic compounds with 4 carbon atoms, whereas Lc1247 accumulated mainly diacetyl, Lc1056 produced mainly its reduced form (acetoin). In addition, Lc1056 accumulated reduced amounts of benzaldehyde compared with all the other strains.

CONCLUSIONS

The research presented in this study can be considered a first step toward a deeper insight into the ripening process and the development of cheese flavor. This study exploits the potential of volatiles produced by using the products of lysed cells or cheese as substrate. The results obtained following growth on CBM indicate that pyruvate could be a common precursor for all compounds produced; its presence in CBM could be explained by the presence of lactate and citrate in this medium. In LCM, these organic acids were not present; therefore, NSLAB mainly used FFA, free AA, or sugars linked on the cellular wall. Taking into account the differences in the volatilome among the strains, further insights into the metabolic capabilities and the volatilome of the NSLAB strains isolated from a raw milk long-ripened cheese, such as Parmigiano Reggiano, could allow the selection of wild NSLAB that possess specific aromatic profiles to be used as adjunct cultures.

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