Article

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Characterization of volatile compounds produced by *Lactobacillus helveticus* strains in a hard cheese model

Facundo Cuffia, Carina V Bergamini, Irma V Wolf, Erica R Hynes and María C Perotti

Abstract

Starter cultures of *Lactobacillus helveticus* used in hard cooked cheeses play an important role in flavor development. In this work, we studied the capacity of three strains of *L. helveticus*, two autochthonous (Lh138 and Lh209) and one commercial (LhB02), to grow and to produce volatile compounds in a hard cheese extract. Bacterial counts, pH, profiles of organic acids, carbohydrates, and volatile compounds were analyzed during incubation of extracts for 14 days at 37 °C. Lactobacilli populations were maintained at 10^6 CFU ml^{-1} for Lh138, while decreases of approx. 2 log orders were found for LhB02 and Lh209. Both Lh209 and LhB02 slightly increased the acetic acid content whereas mild increase in lactic acid was produced by Lh138. The patterns of volatiles were dependent on the strain which reflect their distinct enzymatic machineries: LhB02 and Lh209 produced a greater diversity of compounds, while Lh138 was the least producer strain. Extracts inoculated with LhB02 and Lh 209 were characterized by ketones, esters, alcohols, aldehydes, and acids, whereas in the extracts with Lh138 the main compounds belonged to aromatic, aldehydes, and ketones groups. Therefore, Lh209 and LhB02 could represent the best cheese starters to improve and intensify the flavor, and even a starter composed by combinations of LhB02 or Lh209 with Lh138 could also be a strategy to diversify cheese flavor.

Keywords

Lactobacillus helveticus, strain characterization, volatile compounds, hard cheese model

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INTRODUCTION

Lactobacillus helveticus is a thermophilic lactic acid bacteria (LAB) industrially important in the production of fermented dairy products (Beresford et al., 2001). It is an essential component of the whey starter cultures traditionally used in the manufacture of hard cooked and long-ripened cheeses such as Italian- and Swiss-type cheeses (Parmigiano Reggiano, Grana Padano, Gruyere, Emmental) (Gatti et al., 2003), as well as Reggianito cheese (Reinheimer et al., 1996).

The improvement of cheese quality and the modulation of cheese flavor are topics of great interest, both for industrial and research areas. One of the strategies adopted is to employ selected strains as primary or adjunct starters, based on their ability to enhance cheese flavor through a higher production or diversification of volatile compounds. In particular, strains of *L. helveticus* have a complex and potent proteolytic system, which involves different enzymes able to produce medium and short peptides and amino acids (AAs) from milk proteins (Griffits and Tellez, 2013). In addition, they have demonstrated to possess the ability to produce volatile compounds throughout the AA catabolism (Helinck et al., 2004; Klein et al., 2001;

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Instituto de Lactología Industrial (INLAIN-UNL/CONICET), Santa Fe, Argentina

Corresponding author:
Facundo Cuffia, Santiago del Estero 2829, Santa Fe 3000, Argentina.
Email: fcuffia@unl.edu.ar

Petersen et al., 2010). In this sense, it is known the contribution of *L. helveticus* to the cheese ripening acceleration and the reduction of bitterness in cheeses (Hannon et al., 2007; Johnson et al., 1995). For these reasons, strains of *L. helveticus* have been assayed as adjunct cultures in Cheddar (Nateghi, 2012), Ras (Awad et al., 2007), and Edam cheeses (Tungjaroenchai et al., 2001). On the other hand, even though lipase and esterase activities of LAB remain poorly characterized (El-Soda et al., 1986), several authors emphasize their role in flavor development in long-ripening cheeses (Slattery et al., 2010). Fenster et al. (2000) characterized esterases from *L. helveticus* strains.

Since the 1990s, our research group has worked to know manufacture technology better, as well as the microbiological and biochemical characteristics of Reggianito cheese. First, strains of *L. helveticus* were isolated from whey starters used in Reggianito cheese makings at industrial scale (Reinheimer et al., 1996) and their technological, biochemical, and genetic properties were studied (Quiberoni et al., 1998). In addition, the strains were successfully tested as starters in Reggianito cheese making trials. The results of global composition, proteolysis, and lipolysis demonstrated that these strains contributed to standardize the cheese quality (Candioti et al., 2002; Hynes et al., 2003; Perotti et al., 2005). The peptidolytic activity and AAs production were studied in a hard cheese model (Milesi et al., 2011). Likewise, the ability to produce bioactive peptides with immunomodulatory capacity was detected for one of the strains tested (Burns et al., 2010).

More recently, we focused on exploring the aromatic profiles of autochthonous and commercial thermophilic and mesophilic lactobacilli strains. Model systems that simulate the cheese environment have been widely used with this purpose, as they have the advantage of individualizing the effect of a specific microorganism. For that, in a first step, Peralta et al. (2016a) performed a screening of aminotransferase (AT) and glutamate dehydrogenase activities, enzymes which play a key role in AA catabolism. In order to continue to deepen the knowledge related to metabolic activity of *L. helveticus* strains, in the present work, we evaluated the ability of two autochthonous and one commercial strain (Lh209 and Lh138, and LhB02, respectively), to grow and produce flavor compounds in a model system of hard cooked cheese.

MATERIALS AND METHODS

Bacterial strains

Lh209 and Lh138 belong to the culture collection of the Instituto de Lactología Industrial. LhB02 is a freeze-dried commercial strain (Chr. Hansen, Denmark). Stock cultures of the strains were stored frozen at −80 °C in MRS broth (Biokar Diagnostics, Beauvais, France), with the addition of glycerol 15% (v/v) as a cryoprotective. Before use, each strain was cultivated three times in MRS broth at 42 °C overnight.

Preparation, inoculation, and incubation of cheese model

Three different batches of Reggianito cheeses were made in our pilot plant using individual strains of *L. helveticus* (Lh209, Lh138, and LhB02) as primary starter for each one (Candioti et al., 2002). After three months of ripening, cheeses were used for preparing the model according to Milesi et al. (2011). This procedure allowed us to evaluate the individual effect of each *L. helveticus* isolating it from the rest of microflora that coexists in the real matrix of the cheese. Briefly, a representative portion of cheese was processed with distilled water and the resultant slurry was centrifuged. The soluble fraction located between the upper layer of fat and the precipitate of casein was filtered through glass wool, standardized in salt content (4% v/v) and pH (5.10–5.20) and sterilized by heat.

Each extract was inoculated at 6 log CFU ml^{-1} with the lactobacilli strain used as primary starter in cheese making, as corresponds, and distributed in portions of 15 ml into sterile screw-cap tubes and incubated at 37 °C for 14 days (experimental extract, E). Noninoculated extracts were incubated in parallel and served as controls (C).

Bacterial counts and pH were analyzed during incubation (three, seven, and 14 days); the profiles of organic acids, carbohydrates, and volatile compounds were determined at 14 days. All of the experiments were performed in duplicate using two independent cultures of each strain for inoculation.

pH and microbiological analysis

The pH was measured using a digital pH meter (Orion 3 star benchtop, Thermo Fisher Scientific Inc., USA). Viable counts of *L. helveticus* in experimental extracts were counted by plating on MRS (Biokar Diagnostics, France) agar, after incubation at 42 °C for 48 h. Sterility of control extracts was checked by plating on skim milk agar and incubated at 37 °C for 48 h.

Carbohydrates and organic acids

The content of organic acids (lactic, citric, acetic, and butyric) and lactose, glucose, and galactose were analyzed by high-performance liquid chromatography according to Peralta et al. (2016b). Samples were

centrifuged (5000 × g for 10 min), and the supernatants were diluted in the mobile phase (1:4 v/v), filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil) and a volume of 60 μl was injected into the chromatograph.

Analysis of volatile compounds by SPME-CG-FID/MS

Solid-phase microextraction (SPME) technique was used for the isolation and concentration of volatile compounds. Prior to analysis, frozen samples were thawed at 4 °C overnight. Ten milliliters was transferred to a 40 ml screw-top glass vial containing a micro stirring bar and capped with a cap with a silicon rubber/Teflon membrane and a hole enabling SPME sampling. Vial was introduced in an aluminum block placed on a heater/stirrer (IKA, USA) and preheated at 40 °C for 10 min with an agitation speed of 250 r/min. Then, a SPME fiber (DVB/Car/PDMS 50/30 μm) (Supelco, USA) was inserted in the headspace of the vial and exposed at 40 °C for 30 min.

Separation, identification, and semiquantitative analysis of volatile compounds by GC-FID/MS were made according to the procedure described in Oliszewski et al. (2013).

For each volatile compound identified, results were expressed as means ± standard deviations of peak area values of two independent experiments analyzed by triplicate.

Statistical analysis

One-way analysis of variance was applied in order to detect significant differences, at the 5% level of significance, between control and experimental extracts for each tested strain, using SPSS Statistics software (Version 20.0, IBM SPSS Inc., USA).

RESULTS AND DISCUSSION

Microbiological counts

In experimental extracts, lactobacilli counts slightly increased in the first days of incubation to achieve levels of 10^6–10^7 CFU ml^{-1} at three days. Then, the counts in Lh209 and LhB02 extracts decreased approx. 2 log orders reaching approx. 10^5 CFU ml^{-1} at 14 days. On the contrary, in the extract with Lh138, the counts were maintained at 10^6 CFU ml^{-1} during all incubation time. Similar results were obtained by Nejati et al. (2015) for 10 *L. helveticus* strains incubated during 24 h at 40 °C and seven days at 4 °C in skim milk. In effect, these authors reported an increase in the lactobacilli counts during the first day and then the population was maintained or decreased depending on the strains. No microbial growth was detected in the control extracts, indicating that they remained sterile during incubation.

Carbohydrates, organic acids, and pH

Lactose, galactose, and glucose were undetectable in all extracts. These results were expected because the extracts were prepared from hard cheeses with three months of ripening, during which the remaining lactose after cheese making and the monosaccharides produced (glucose and glactose) are usually consumed by microorganisms (Beresford et al., 2001). No modification in pH values was detected during incubation time, which correlated with the absence of fermentable carbohydrates (Table 1).

Lactic acid was the predominant organic acid in all extracts; in fact, this compound is the main product of carbohydrates fermentation by LAB. Butyric, citric, and acetic acids were also detected, although at lower concentrations compared to lactic acid. The inoculation of Lh138 produced an increase in the levels of lactic acid, while the inoculation of Lh209 and LhB02

Table 1. pH values during incubation time and concentrations of organic acids at the end of incubation time (14 d) in control (C) and experimental (E) extracts inoculated with individual strains of *L. helveticus*: Lh138, Lh209, and LhB02. Values are means ± standard deviation

	pH			Organics acids (mg/100 ml)			
Extracts	3d	7d	14d	Lactic	Citric	Acetic	Butyric
C_{B02}	5.07 ± 0.03	5.03 ± 0.01	5.03 ± 0.05	1368.5 ± 88.0	86.6 ± 8.5	33.9 ± 0.2b	160.4 ± 10.1
LhB02	5.04 ± 0.02	5.01 ± 0.02	4.98 ± 0.03	1447.6 ± 139.8	81.9 ± 3.3	37.0 ± 0.2a	166.3 ± 18.1
C_{209}	5.02 ± 0.02b	5.03 ± 0.02	5.07 ± 0.00	1290.6 ± 58.4	121.9 ± 1.8	49.8 ± 1.7b	288.1 ± 10.8
Lh209	5.08 ± 0.01a	5.03 ± 0.00	5.08 ± 0.01	1281.9 ± 3.3	122.7 ± 0.7	70.1 ± 0.2a	295.3 ± 3.3
C_{138}	5.04 ± 0.00a	4.96 ± 0.00b	4.94 ± 0.01	1431.5 ± 25.5b	74.9 ± 2.9	28.5 ± 2.1	286.5 ± 0.2
Lh138	5.00 ± 0.01b	5.00 ± 0.01a	5.01 ± 0.01	1555.4 ± 39.6a	73.9 ± 0.4	28.6 ± 0.2	291.4 ± 5.7

Values with different letters within the same column for each strain studied are significantly different ($p < 0.05$).

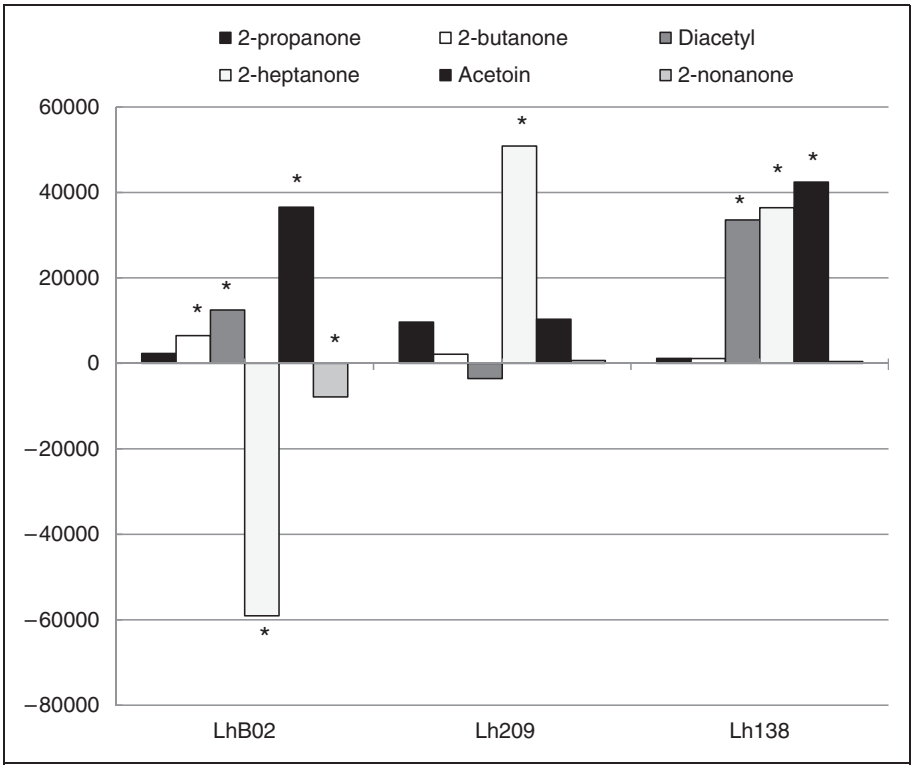

Figure 1. Differences between the values of areas for the ketones from extracts inoculated with different strains of *L. helveticus* and their respective control extracts. *$p < 0.05$.

increased the concentration of acetic acid. The levels of butyric and citric acids were not modified by any strain of lactobacilli. Indeed, thermophilic lactobacilli are usually considered citrate negative (Marilley and Casey, 2004).

Volatile compounds

A total of 32 volatile compounds were identified in the headspace of extracts and grouped by chemical families: ketones, esters, aldehydes, alcohols, acids, and compounds containing an aromatic ring.

For each strain, the difference of the area values for each compound between experimental extracts and the corresponding controls was calculated. The positive values indicate a net production of the compounds due to the metabolic activities of the strains, whereas the negative values mean that the compounds were present in higher quantities in controls than in the experimental extracts, probably due to their degradation by the inoculated strain (Imhof et al., 1995). Figures 1 to 6 show the results obtained for each compound grouped by chemical family for the three lactobacilli strains.

Ketones. Six compounds were identified: 2-propanone, 2-butanone, 2,3-butanedione or diacetyl, 2-heptanone, 3-hydroxy-2-butanone or acetoin, and 2-nonanone. They have been reported as important components of the volatile fraction of grana-type cheeses, including Reggianito (Bellesia et al., 2003; Ceruti et al., 2016; Moio and Addeo, 1998; Wolf et al., 2010). Ketones have unique notes (buttery, fruity, musty, and cheesy) and low perception threshold (Qian and Reineccius, 2003); thus, their contribution to overall flavor is expected.

In relation to the production of methyl ketones with odd-number carbon atoms, Lh209 and Lh138 exhibited the ability to produce only significant quantities ($p < 0.05$) of 2-heptanone. The rest of methyl ketones identified, 2-propanone and 2-nonanone, reached similar or lower values in the inoculated extracts than in the respective controls. In this sense, Pogačić et al. (2015) reported that the levels of methyl ketones detected in a curd-based slurry medium inoculated with *L. helveticus* strains did not differ with the control. Similarly, Petersen et al. (2010) did not detect a net production of methyl ketones in a cheese model inoculated with different strains of *L. helveticus*. The presence of these compounds is related to lipase/esterase activities of cheese microorganisms that act on acyl-lipids releasing fatty acids, which are further catabolized by a β-oxidation pathway (Collins et al., 2003).

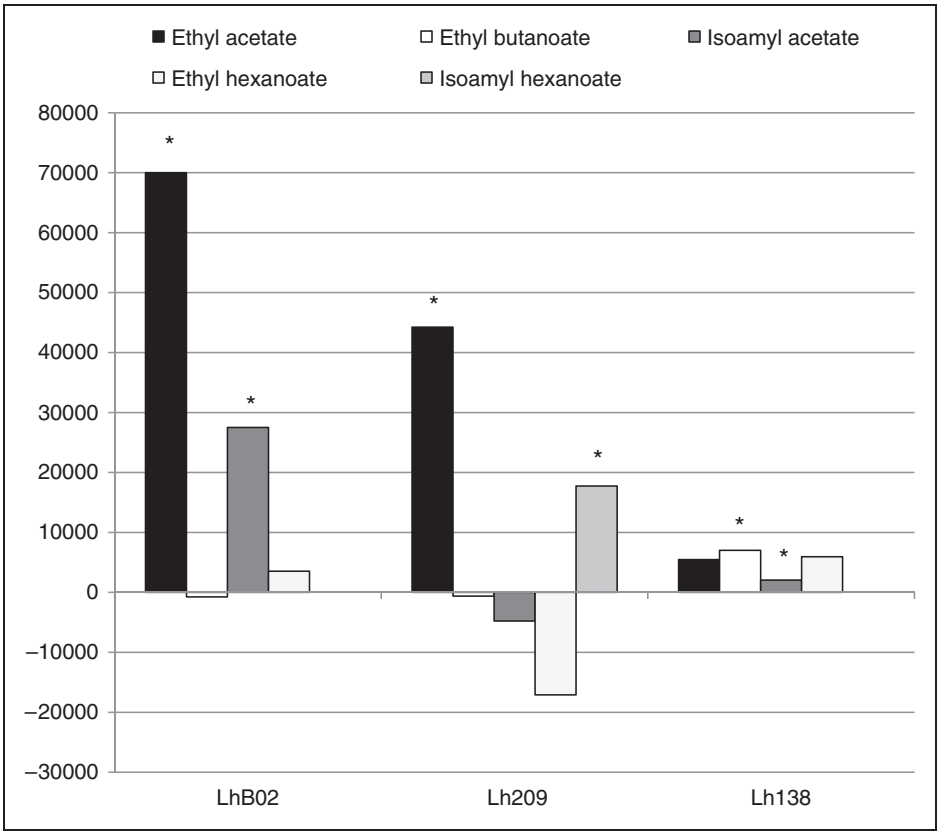

Figure 2. Differences between the values of areas for the esters from extracts inoculated with different strains of *L. helveticus* and their respective control extracts. *$p < 0.05$.

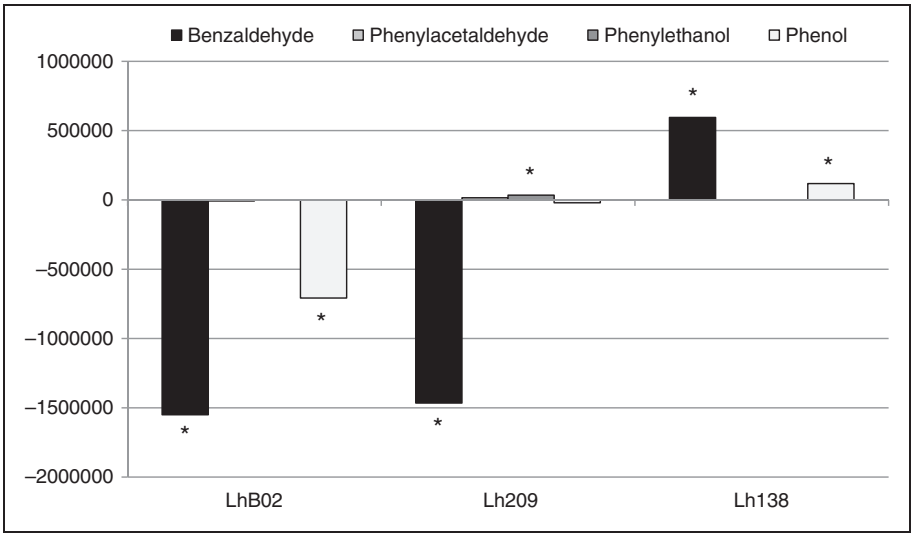

Figure 3. Differences between the values of areas for the aromatics compounds from extracts inoculated with different strains of *L. helveticus* and their respective control extracts. *$p < 0.05$.

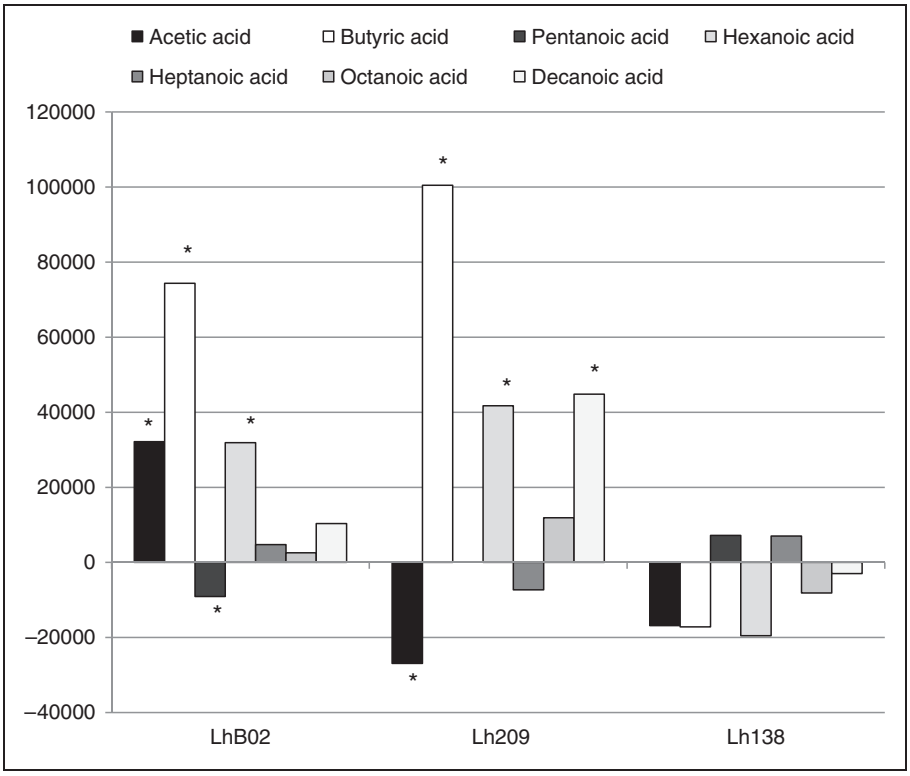

Figure 4. Differences between the values of areas for the acids from extracts inoculated with different strains of *L. helveticus* and their respective control extracts. *$p < 0.05$.

On other hand, the extracts with LhB02 were characterized by higher levels ($p < 0.05$) of acetoin, diacetyl, and 2-butanone than controls, whereas the extracts with Lh138 showed a net production ($p < 0.05$) of acetoin and diacetyl. On the contrary, the levels of these compounds were similar in extracts with Lh209 and their respective controls (Figure 1). In this sense, Petersen et al. (2010) reported the production of diacetyl and acetoin by different strains of *L. helveticus* in a cheese model.

Diacetyl, acetoin, and 2-butanone are of particular interest in different types of cheeses (Le Bars and Yvon, 2008; Qian and Reineccius, 2003). The metabolic routes for the diacetyl biosynthesis and their subsequent reduction products: acetoin, 2-butanone, 2,3-butanediol, and 2-butanol, include the citrate metabolism, the lactose fermentation, and the catabolism of AA (Ala and Asp) (Le Bars and Yvon, 2008). However, there are low levels of citrate and lactose in ripened cheeses and only a few bacterial genera have the enzymes for these pathways. Therefore, the Asp catabolism has been suggested for the production of diacetyl and acetoin for some lactobacilli strains (Kieronczyk et al., 2004; Le Bars and Yvon, 2008). In our study, extracts did not contain residual carbohydrates and the citrate was not consumed by any strain. Thus, AA catabolism could be the most likely pathway for the production of diacetyl and acetoin. In this way, the conversion of AA to aroma compounds by LAB is a multiple step pathway which is initiated with a transamination reaction, catalyzed by specific ATs. In this first step, AA are converted into their corresponding α-ketoacids. These α-ketoacids are intermediate compounds in the aroma development as can be transformed into aldehydes, alcohols, and carboxylic acids via enzymatic reactions catalyzed by α-ketoacid decarboxylase (KADC), alcohol dehydrogenase (ADH), aldehyde dehydrogenase, and α-ketoacid dehydrogenase (KADH). In addition, chemical conversion of α-ketoacids into different volatile compounds is also possible (Yvon and Rijnen, 2001). In particular, the three strains tested demonstrated AT activity toward Asp (Peralta et al., 2016a). The transamination of Asp produces oxaloacetate (OAA) that in turn could be converted to pyruvate in a reaction catalyzed by the OAA decarboxylase. After that, pyruvate could be metabolized to acetolactate, acetoin, diacetyl, and 2,3-butanediol in several steps catalyzed by different enzymes (Le Bars and Yvon, 2008).

Esters. Five esters were identified in the extracts, particularly, ethyl and isoamyl esters: ethyl acetate, ethyl

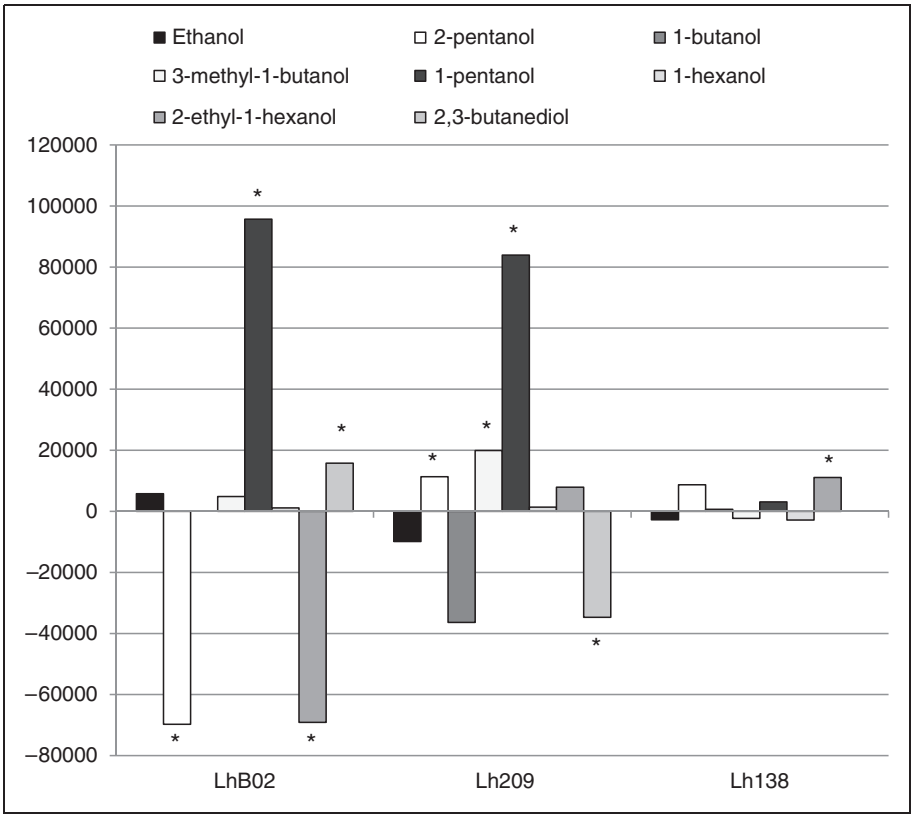

Figure 5. Differences between the values of areas for the alcohols from extracts inoculated with different strains of *L. helveticus* and their respective control extracts. *$p < 0.05$.

butanoate, ethyl hexanoate, isoamyl acetate, and isoamyl hexanoate. Ethyl acetate reached high levels ($p < 0.05$) in extracts inoculated with LhB02 and Lh209; in addition, significant levels ($p < 0.05$) of isoamyl acetate and isoamyl hexanoate were produced by LhB02 and Lh209, respectively. Regarding extracts inoculated with Lh138, low but significant levels respect to controls were detected for ethyl butanoate and isoamyl acetate (Figure 2).

Two main mechanisms of ester biosynthesis by LAB have been proposed: esterification reactions between carboxylic acids and alcohols and alcoholysis between acylglycerols and alcohols (Liu et al., 2004). Several strains of *S. thermophilus* have demonstrated ability to synthesize ethyl esters (Liu et al., 2004); however, the data reported on the formation of esters by *L. helveticus* strains are scarce. Different authors have highlighted the presence of ethyl esters in the volatile profile of Italian grana cheeses (Bellesia et al., 2003; Qian and Reineccius, 2003) and Reggianito (Ceruti et al., 2016; Wolf et al., 2010). Esters play an important role in the flavor of cheeses, giving the typical fruity character of grana-type cheeses (Moio and Addeo, 1998).

Aromatic compounds. Four compounds containing an aromatic ring derived from AA catabolism were identified in the extracts: benzaldehyde, phenylacetaldehyde, and phenylethanol from Phe, and phenol from Tyr (Tavaria et al., 2002) (Figure 3). According to Peralta et al. (2016a), the three strains tested in our study showed AT activities toward Phe and Tyr, suggesting the possible conversion of the AA precursors into the corresponding volatile compounds, as was mentioned above. However, only a marked production ($p < 0.05$) of phenol and benzaldehyde in the extracts inoculated with Lh138 and a mild production ($p < 0.05$) of phenylethanol by Lh209 were observed. By contrast, lower area values ($p < 0.05$) for benzaldehyde and phenol in LhB02 extracts and for benzaldehyde in Lh209 extracts, in comparison to the respective controls, were detected. The biochemical pathway for benzaldehyde production by LAB includes a microbial and chemical conversion from Phe. The reaction begins with the transformation of Phe to phenylpyruvic catalyzed by an AT and then this α-keto acid is converted to benzaldehyde by chemical oxidation. This latter step is favored by heat, oxygen, alkaline pH, and the presence of divalent cations (Mn^{+2}) (Klein et al., 2001).

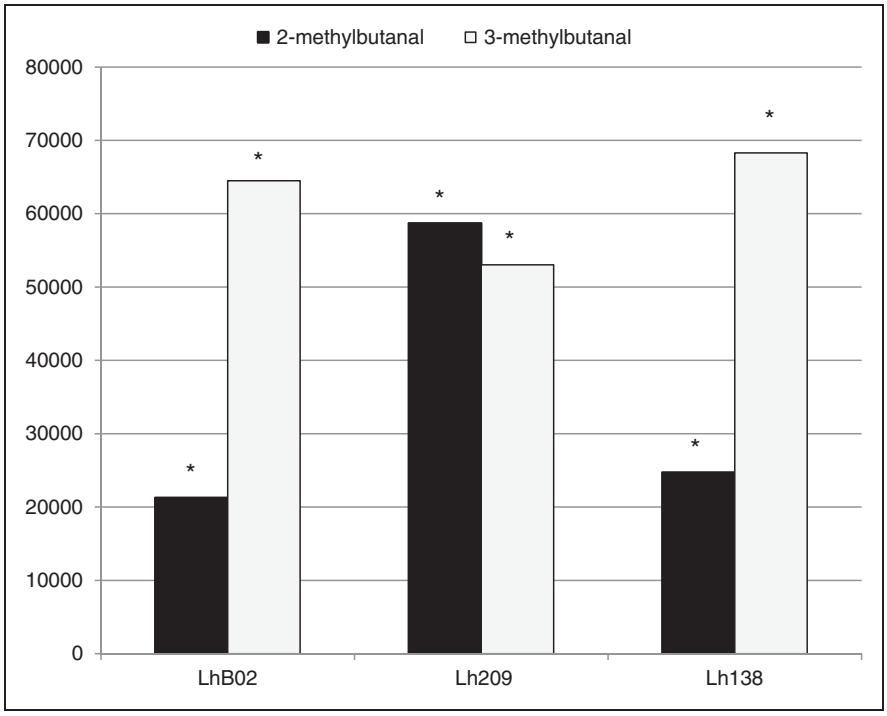

Figure 6. Differences between the values of areas for the aldehydes from extracts inoculated with different strains *of L. helveticus* and their respective control extracts. *$p < 0.05$.

A high level of benzaldehyde was detected in a reaction mixture containing AAs inoculated with cell-free extract of *L. helveticus* strains (Klein et al., 2001). Pogačić et al. (2015) also reported higher amounts of benzaldehyde in a curd-based slurry medium inoculated with *L. helveticus* strains than in controls, whereas Petersen et al. (2010) found lower values in a cheese model inoculated with strains of *L. helveticus* respect to controls. In preliminary experiments, we observed that benzaldehyde levels in control extracts increased with the incubation time, reaching very high values at 14 days (data not shown). Thus, our results suggest that the presence of LhB02 and Lh209 in the extracts clearly avoided the chemical conversion from phenylpyruvic to benzaldehyde. It is probable that the growth of LhB02 and Lh209 during incubation diminished the redox potential of the extracts, which could have influenced on this chemical reaction (Kieronczyk et al., 2004).

The role of *L. helveticus* in the production of phenol, phenylmethanol, phenylethanol, and phenylacetaldehyde is little known. Nevertheless, the presence of these aromatic compounds in the volatile profile of grana-type cheeses, as well as the contribution to flavor cheese, has been largely reported (Ceruti et al., 2016; Moio and Addeo, 1998; Wolf et al., 2010). Overall, aromatic compounds appear to make a positive contribution to cheese flavor at low concentrations but tend toward an unpleasant note as their level increases (Curioni and Bosset, 2002). In fact, solvent and bitter almond notes attributed to benzaldehyde were detected by a sniffing assay in reaction mixtures containing cell-free extract of *L. helveticus* strains (Klein et al., 2001).

Acids. Seven acids were identified in the extracts: five saturated (fatty) acids of even number of carbon atoms (C_2 to C_{10}) and two acids of odd number of carbon atoms (C_5 and C_7). As shown in Figure 4, significant increases ($p < 0.05$) were found in the levels of acetic, butyric, and hexanoic acids in LhB02 extracts, while the volatile fraction of Lh209 extracts was characterized by increased levels ($p < 0.05$) of butyric, hexanoic, and decanoic acids. For extracts with Lh138, no differences were observed compared to controls. Fatty acids play an important role in the flavor of hard cheeses, not only by their high levels, as usually represent the most important fraction of the volatile profile (Ceruti et al., 2016; Wolf et al., 2010), but also by the low perception threshold, especially for the short-chain acids (Collins et al., 2003; Curioni and Bosset, 2002). In particular, butyric and hexanoic acids have been reported as key odorants in Italian grana cheeses (Moio and Addeo, 1998; Qian and Reineccius, 2003). Milk fat lipolysis by lipase/esterase activity is considered the main

biochemical event for the formation of fatty acids (from C_4 to C_{18}) in cheeses; although, butyric and hexanoic acids could also derive from AA and lactose metabolism (Tavaria et al., 2002). Acetic acid is produced from different pathways that include lactose/citrate metabolism and AA catabolism (Collins et al., 2003; Marilley and Casey, 2004). The strains assayed in the present work clearly showed to produce different fatty acid profiles. Although, LAB are considered weakly lipolytic agents, the presence of lipase/esterase activity has been demonstrated for different strains of lactobacilli, including *L. helveticus* (El-Soda et al., 1986; Fenster et al., 2000). However, the specific biosynthesis of acetic acid by *L. helveticus* has not been yet reported.

Alcohols. A total of eight alcohols were identified: four linear-chain primary alcohols (ethanol, 1-butanol, 1-pentanol, and 1-hexanol), one linear-chain secondary alcohol (2-pentanol), two branched-chain primary alcohols (3-methyl-1-butanol, 2-ethyl-1-hexanol), and 2,3-butanediol (Figure 5). In spite of the diversified alcoholic profile observed in the extracts, only a few compounds showed a net production by inoculated strains.

The main alcohol produced ($p < 0.05$) by Lh209 and LhB02 was 1-pentanol. Besides, 2,3-butanediol was significantly increased ($p < 0.05$) by LhB02 and 2-pentanol and 3-methyl-1-butanol by Lh209. On the contrary, the alcoholic profile of Lh138 extracts showed only a slight increase ($p < 0.05$) in the production of 2-ethyl-1-hexanol. Some studies indicate the ability of *L. helveticus* strains for producing primary and branched-chain alcohols (Helinck et al., 2004; Petersen et al., 2010). Alcohols detected in the extracts have been also reported as normal constituents of the volatile profiles of hard cooked cheeses (Bellesia et al., 2003; Qian and Reineccius, 2003).

Primary alcohols are produced by the reduction of aldehydes derived from AA and fatty acids, while secondary alcohols are from methyl ketones (Collins et al., 2003). The branched-chain alcohols are generated from the reduction of the corresponding aldehydes, which in turn come from branched-chain AA by means of Strecker degradation or enzymatic processes. In particular, α-ketoacids formed in the first step of AA catabolism could be transformed to aldehydes and further reduced to alcohol by ADHs (Yvon and Rijnen, 2001). Meanwhile, 2,3-butanediol is produced from the acetoin reduction catalyzed by acetoin reductase (Marilley and Casey, 2004).

Aldehydes. Only two branched-chain aldehydes were identified in the extracts: 2-methylbutanal and 3-methylbutanal (Figure 6). Aldehydes generally represent a minority volatile fraction of cheeses, which it has been associated to the strong reducing environment prevailing in aged cheeses and the presence of reductases (Mallia et al., 2005). 2-methylbutanal and 3-methylbutanal have been detected in the volatile profile of grana-type cheeses (Bellesia et al., 2003; Ceruti et al., 2016; Wolf et al., 2010), being responsible for malty and green notes (Qian and Reineccius, 2003). These compounds are formed from isoleucine and leucine metabolism, respectively. After the transamination of the AA, the conversion of α-keto acids to aldehydes takes place either by a direct pathway using KADC enzyme or by an indirect pathway comprising KADH enzyme (Tavaria et al., 2002; Yvon and Rijnen, 2001).

In the present work, the three lactobacilli strains were able to produce ($p < 0.05$) both compounds; similar proportions were found in Lh209 extracts, while higher levels of 3-methylbutanal than 2-methylbutanal were detected in LhB02 and Lh138 extracts. In agreement with these results, all strains presented AT activity toward leucine and isoleucine (Peralta et al., 2016a).

Summarizing, the three *L. helveticus* strains studied demonstrated the ability to produce volatile compounds in a cheese model, but the profiles obtained were different among them. LhB02 and Lh209 produced a greater diversity of volatile compounds, while Lh138 was the least producer strain. Therefore, the results reflect the distinct enzymatic machineries of the strains. In addition, the composition of the medium is another important factor to take into account as it defines the profile of available substrates for the multiple reactions and it could also regulate the enzymatic activities.

CONCLUSION

In the present work, we revealed marked differences in the volatile compounds formed in the cheese extracts by the three studied strains of *L. helveticus*, two of autochthonous origin and the other one commercial, which is closely related to their machinery enzymatic and the substrates available in the medium. All compounds detected are related to the volatilome of Reggianito cheese. LhB02 and Lh209 produced a greater diversity of volatile compounds, while Lh138 was the least producer strain. In particular, the compounds produced in higher levels by LhB02 were 2-butanone; diacetyl; acetoin; ethyl acetate; isoamyl acetate; 1-pentanol; 2,3-butanediol; 2- and 3-methylbutanal; acetic, butyric, and hexanoic acids. Significant amounts of 2-heptanone; ethyl acetate; isoamyl hexanoate; 2-pentanol; 3-methyl-1-butanol; 1-pentanol; phenyl ethanol; butyric, hexanoic, and decanoic acids; and 2- and 3-methylbutanal were detected for Lh209. Finally, Lh138 was characterized by the formation of diacetyl, acetoin, 2-heptanone, 2- and 3-methylbutanal, ethyl butanoate,

isoamyl acetate, 2,3-butanediol, benzaldehyde, and phenol, at low levels. Therefore, Lh209 and LhB02 could represent the best cheese starters to improve, intensify and modulate the flavor, and even a starter composed by combinations of LhB02 or Lh209 with Lh138 could also be a strategy to diversify cheese flavor.

DECLARATION OF CONFLICTING INTERESTS

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