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Spray-dried adjunct cultures of autochthonous non-starter lactic acid bacteria

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ABSTRACT

Spray-drying of lactic cultures provides direct-to-vat starters, which facilitate their commercialization and use. However, this process may alter the metabolic activity and deteriorate technological features. In this work, we assessed the influence of spray-drying on the survival and aroma production of two strains of mesophilic lactobacilli: *Lactobacillus paracasei* 90 and *Lactobacillus plantarum* 91, which have already been characterized as good adjunct cultures. The spray-drying was carried out using a laboratory scale spray and the dried cultures were monitored during the storage for the survival rate. The dried cultures were applied to two cheese models: sterile cheese extract and miniature soft cheese. The influence on the carbohydrate metabolism and the production of organic acids and volatile compounds was determined. Both strains retained high levels of viable counts in the powder after drying and during the storage at 5 °C for twelve months. In addition, they also remained at high level in both cheese models during incubation or ripening. Similar profiles of carbohydrate fermentation and bioformation of volatile compounds were observed in the cheese extracts for each of the strains when tested as both fresh and dried cultures. In addition, the ability of *Lb. paracasei* 90 to increase the production of acetoin and diacetyl remarkably in cheese models was also confirmed for the spray-dried culture.

1. Introduction

Non-starter lactic acid bacteria (NSLAB) are important components of cheese microbiota because they represent an adventitious and uncontrolled group of lactic acid bacteria that can attain high levels after a few weeks during ripening and prevail or remain stable for several months (Beresford et al., 2001; Crow et al., 2001; Gobbetti et al., 2015; Porcellato and Skeie, 2016). The addition of selected strains of NSLAB to cheese milk is an interesting approach in producing desirable changes in cheese, such as controlling the adventitious flora and improving the flavor or texture (Gobbetti et al., 2015; Johnston et al., 2010). Many strains, mainly mesophilic lactobacilli, isolated from good-quality cheeses, have shown positive effects of their action on cheese quality (Ciocia et al., 2013; Crow et al., 2001; Di Cagno et al., 2012; Randazzo et al., 2008; Settanni and Moschetti, 2010; Settanni et al., 2011; Wouters et al., 2002). However, the commercialization of such NSLAB strains for dairy industry requires a stable and convenient formulation with high cell concentration and ensuring a long-term viability and the preservation of metabolic activity during the storage (Parente and Cogan, 2004). There are very few investigations on the production of adjunct cultures in an appropriate form that can be applied in cheesemaking from the NSLAB strains isolated and characterized (Gobbetti et al., 2015; To and Etzel, 1997).

Lactic cultures are usually preserved by freeze-drying, freezing, or spray-drying. The freeze-dried cultures can be traded and stored at room temperature, although preservation at 4 °C or −20 °C prolongs the survival and improves metabolic activity (Parente and Cogan, 2004). Freezing at a very low temperature (−80 or −196 °C) is the best way to preserve the viability and activity of bacteria; however, the handling of frozen cultures is not practical. In recent years, the interest in spray-drying as a preservation method for bacteria has grown. This technology offers advantages such as high production rates, low operating costs, single-unit processing for particle formation and drying, readily available machinery, and the ease of scaling up (Ghandi et al., 2012; Santivarangkna et al., 2007). Thus the industrial spray dryers, which are used in medium to big-size dairy industries for

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the production of dried skim milk and food ingredients can be exploited to produce spray-dried adjunct cultures (Lavari et al., 2015). However, the survival of bacteria to spray-drying process depends on many factors, such as strain, growth phase, growth conditions, and the parameters of spray-drying. In particular, the spray-drying process involves different parameters, such as outlet and inlet air temperatures, air flow rate, product feed rate, and atomized drop sizes, which could influence the microbial survival (Santivarangkna et al., 2007). Even if the initial attempts to use spray-drying for bacteria were unsuccessful (low survival after spray-drying and during storage, delay in lactic acid production, and the difficulty in rehydration of the product) (Johnson and Etzel, 1995; Teixeira et al., 1995; To and Etzel, 1997), the current technology allows an improved control of the conditions of the spray, and excellent preservation rates have been found for several strains of lactic acid bacteria (Silva et al., 2011). There are some reports related to the production of dehydrated probiotic cultures from different strains of bifidobacteria and lactobacilli, and in these reports the maintenance of the survival and the probiotic functionality have been given the prime importance (Gardiner et al., 2000; Golowczyc et al., 2011; Páez et al., 2012). In addition, there are few studies wherein the spray-drying was applied as a method to prepare attenuated bacteria of *Lb. helveticus* and *Lb. casei* to accelerate Cheddar cheese ripening (Johnson et al., 1995; Madkor et al., 2000). The addition of these adjunct cultures in the cheese led to the enhancement of flavor and reduced the off-flavor, above all when a higher outlet air temperature was applied during the spray-drying process (Johnson et al., 1995). To the best of our knowledge, there are no reports determining the effect of the addition of spray-dried adjunct cultures from autochthonous strains on the volatile compounds production in cheese.

On the other hand, most of the studies about the addition of lactobacilli strains as adjunct cultures in cheese generally considered the assay of a unique dose, while few other studies evaluated different doses of these cultures with an aim to select the optimal dose to produce a desirable effect on cheese (Carunchia Whetstine et al., 2006; Van Hoorde et al., 2010).

In previous investigations, we isolated and characterized two mesophilic lactobacilli strains: *Lactobacillus paracasei* 90 and *Lactobacillus plantarum* 91. These autochthonous NSLAB obtained from good quality semi-hard cheese showed desirable properties as adjunct cultures when they were tested as fresh cultures in Pategrás and Cremoso cheese varieties (semi-hard and soft cheese, respectively). In these cheese varieties, both strains led to beneficial changes during ripening: increase of peptidolysis, enhancement of flavor and control of adventitious microflora (Milesi et al., 2009, 2010).

The aim of this work was to obtain spray-dried adjunct cultures from *Lactobacillus paracasei* 90 and *Lactobacillus plantarum* 91, and to assess their ability to produce flavor-related biochemical events in two cheese models. The performance of the dehydrated versus fresh cultures was compared and the best dose of dehydrated *Lb. paracasei* 90 was assessed.

2. Materials and methods

2.1. Strains and culture conditions

Lb. paracasei 90 and *Lb. plantarum* 91 were obtained from the culture collection of Instituto de Lactología Industrial (INLAIN, Santa Fe, Argentina). The technological and biochemical features of both strains have been documented previously (Milesi et al., 2008; Peralta et al., 2014, 2016a, 2016b). The stock cultures of the strains were stored at − 80 °C in MRS broth (Biokar Diagnostics, Beauvais, France) with the addition of glycerol 15% (v/v) as a cryopreservative. Each strain was revived (2% v/v) twice in the broth overnight at 37 °C prior to the use.

2.2. Spray-drying of lactobacilli strains

Each lactobacilli strain was grown in MRS broth (Biokar Diagnostics) at 37 °C for 16–18 h. Then, the cultures were centrifuged at 6000 $\times g$ for 15 min at 5 °C, the pellets were washed twice with 50 mM potassium phosphate buffer (pH 7), and then resuspended in 20% (w/v) skim milk. The cell suspensions were spray-dried in a laboratory scale spray dryer (Buchi mini spray dryer model B290, Flawil, Switzerland) with a constant inlet air temperature of 140 °C, an outlet temperature of 82 °C, and a flux of 600 L/h. The cell suspensions were atomized and sprayed into the drying chamber using a two-fluid nozzle. The drying process was almost instantaneous and the residence time was negligible. The powders were vacuum sealed in individual packs. The residual moisture content (% w/w) of the powders was determined by drying at 101 ± 1 °C until a constant weight was achieved (FIL-IDF, 1993b). The cell counts of the lactobacilli in the suspensions before spray-drying, in the powders immediately after the process, and after 8 and 12 months of storage at 5 °C were determined on MRS agar (48 h of incubation at 37 °C). The powders were reconstituted in 0.1% casein peptone water to the original liquid volume (i.e., 0.2 g powder/mL) for the cell count determination.

2.3. Cheese models

The ability of the strains to produce flavor compounds was assayed for both fresh and dried cultures in two cheese models. *Lb. plantarum* 91 and *Lb. paracasei* 90 were tested in a sterile extract of soft cheese (Peralta et al., 2016b), while *Lb. paracasei* 90 was also assessed in miniature soft cheese (Milesi et al., 2010).

2.3.1. Sterile cheese extract

Soft cheeses were manufactured to obtain the extract; cheesemaking was made according to Milesi et al. (2009). After 20 days of ripening at 5 °C, the cheeses were grated and homogenized with distilled water (1:1), and the resultant slurry was centrifuged (17,000 $\times g$, 15 min, 10 °C). The soluble fraction was extracted, and standardized with sodium chloride at 1.5% (w/v), filtrated through PVDF membranes of 0.4 μm (Millipore, Sao Paulo, Brazil) and heated 30 min at 70 °C. The initial pH value of the extracts was noted to be 5.20.

The experimental extracts were individually inoculated with each strain in order to reach an initial concentration of 10^6 CFU/mL and then incubated at 37 °C for 48 h. The cultures were added in three different forms: as fresh (F), spray-dried (D), and reactivated spray-dried (R) cultures. An aliquot of a fresh overnight culture of the strains revived on MRS (after two successive overnight incubations) was inoculated in F extracts. For D extracts, a quantity of the spray-dried powder was added directly to the extract, and for R extracts, the spray-dried powder (0.5 g) was reactivated by two successive overnight incubations in MRS broth. The non-inoculated extracts served as the controls. Microbiological counts, pH, carbohydrates, organic acids, and volatile compounds were determined in the extracts during the incubation. All of the extracts were prepared in duplicate using two independent cultures of the strains.

2.3.2. Miniature soft cheese

The spray-dried culture of *Lactobacillus paracasei* 90, which demonstrated the best performance for the production of cheese flavor compounds in the cheese extract, was tested as adjunct culture in miniature soft cheese. Four types of cheese were manufactured: one control cheese (C) containing *Streptococcus thermophilus* as the primary starter, and three experimental cheeses (E) with the addition of the same primary starter and *Lactobacillus paracasei* 90 (Lb90). Lb90 was added at three different levels to reach 5×10^3, 1×10^5, and 5×10^6 CFU/mL in the cheese milk and the cheeses so made were named as E1, E2, and E3, respectively. On each cheesemaking day, the milk was batch-pasteurized at 65 °C for 20 min (Briggiler-Marcó et al.,

Table 1
Moisture content of spray-dried powders and cell counts (CFU/mL) in the suspensions before spray-drying, in the powders immediately after this process, and after 8 and 12 months of storage at 5 °C).

Strain	Moisture (%)	Cell counts (log CFU/mL)			
		Before spray drying	After spray-drying (months)[a]		
			0	8	12
Lb 90	3.23 ± 0.76	9.46 ± 0.15	9.37 ± 0.27	9.48 ± 0.43	9.03 ± 0.25
Lb 91	3.70 ± 0.68	9.48 ± 0.25	9.44 ± 0.50	9.63 ± 0.18	8.95 ± 0.17

[a] The powders were reconstituted in 0.1% casein peptone water to the original liquid volume (i.e., 0.2 g powder/mL) for the cell count determination.

2007), then cooled down to 36 °C, and stored in four 5 L vats. Calcium chloride was added (Merck, Darmstadt, Germany, final concentration 0.02% w/v) and the cultures were inoculated in the cheese milk at 10^6 CFU/mL (primary starter) or as per the appropriate dose (adjunct culture). Then, 15 min later, coagulant was added (Maxiren® 150, France, 0.014 g/L), and when the curd reached the appropriate strength, it was cut into ~1 cm^3 pieces. The whey was drained and the curd was molded. The molds were stored at 45 °C until the pH of the cheeses reached ~5.20. After that, the cheeses were brined (20% NaCl w/v) at 5 °C for 30 min and were put into the molds again for 3 days at 5 °C to achieve further drying, then vacuum-packed and ripened at the same temperature up to 30 days. Each cheese was manufactured in duplicate on different cheesemaking days with different milk batch. Gross composition, microbiological counts, pH, carbohydrates, organic acids, and volatile compounds were determined in the cheeses during ripening.

2.4. Chemical and microbiological analysis of extracts and cheeses

2.4.1. Gross composition, pH, and microbiological counts

For the cheeses, pH (Bradley et al., 1993), fat (FIL-IDF, 1997), protein (FIL-IDF, 1993a), and moisture content (FIL-IDF, 1982) were determined after 3 days of ripening. Microbial counts after 3, 15, and 30 days of ripening were determined in the cheese samples on skim milk agar and MRS agar for the starter and lactobacilli cultures (adjunct cultures or NSLAB), respectively (Milesi et al., 2010).

For the extracts, pH and lactobacilli counts on MRS agar were determined at 0 h and 48 h of incubation.

2.4.2. Carbohydrates and organic acids profiles

The concentrations of organic acids, lactose, and galactose were determined by high-performance liquid chromatography (HPLC) according to Peralta et al. (2016b). HPLC consisted of a quaternary pump, an on-line degasser, a column oven, a UV–visible detector (all Series 200), and a refractive index detector (Series Flexar) (Perkin Elmer, USA). The chromatographic separation was carried out at 65 °C on an Aminex HPX-87H column (300 × 7.8 mm) equipped with a cation H^+ microguard cartridge (Bio-Rad Laboratories, USA), using a mobile phase of 0.01 mol/L H_2SO_4 at a flow rate of 0.6 mL/min.

The samples of cheese extracts were diluted (1/3) with the mobile phase, filtered through 0.45 µm membranes, and injected into HPLC. The samples of miniature cheeses (5 g) were homogenized with distilled water (15 mL) using an Ultraturrax® homogenizer (model T25, IKA, Staufen, Germany) and applying three cycles at 17,000 rpm for 1 min each one. The suspensions were incubated at 40 °C for 1 h and then centrifuged (3000 × g, 30 min, 10 °C) and filtered through fast flow filter paper (Deltalab, Barcelona, Spain). The filtered solution was adjusted to a final volume of 25 mL, filtered again through 0.45 µm membranes (Millex, Millipore, São Paulo, Brazil), and injected into HPLC (Bergamini et al., 2010).

2.4.3. Volatile compounds profiles

Volatile compounds were analyzed by SPME-GC-FID/MS according to Peralta et al. (2014). The refrigerated samples of cheese extracts (10 mL) and the miniature cheese samples (5 g) were transferred into GC vials and the vials were heated to 40 ± 1 °C for 10 min and then CAR/PDMS 75 µm (Supelco Inc. Bellefonte, PA, USA) fiber was exposed into the headspace for 30 min. A gas chromatography system (Perkin Elmer model 9000, USA) with a HP INNOWax column (60 m × 0.25 mm × 0.25 µm) (Agilent J & W, Agilent Technologies, USA) and an FID detector set at 290 °C was used. The oven temperature program was set as follows: 45 °C for 5 min, the temperature increased to 150 °C for 3 min at a ramp of 8 °C/min and, finally, increased to 250 °C for 5 min at a ramp of 10 °C/min. Hydrogen was used as the carrier gas at a flow rate of 2.0 mL/min.

2.4.4. Statistical analysis

One-way ANOVA was applied to compare the means of carbohydrates and organic acids using the SPSS software (SPSS Inc., Chicago, USA). Principal components analysis (PCA) was applied to reduce the dimensionality of volatile compounds using the PAST software (Hammer et al., 2001).

3. Results

3.1. Microbiological counts and moisture content of the spray-dried cultures

The moisture content in the spray-dried powder was lower than 4.00% (w/w) (Table 1). The cell counts in the suspensions before spray-drying and in the reconstituted powders are shown in Table 1. Both strains exhibited good resistance to spray-drying; cell counts in the powders reconstituted to the original liquid volume were similar to those recorded in the suspensions before spray-drying. The cell counts in the powder were around 10^{10} CFU/g immediately after drying and were maintained without a significant loss during the 12 months of storage at 5 °C.

3.2. Cheese model: sterile cheese extract

3.2.1. pH and microbiological counts

The pH of the extracts inoculated with lactobacilli decreased significantly during incubation; it was between 4.11 and 4.27 for the extracts inoculated with Lb90 and between 4.21 and 4.49 for the extracts inoculated with Lb91 after 48 h. Concomitantly, an increase in the count of lactobacilli was verified. The initial cell load in all experimental extracts was between 6.2 and 6.7 log CFU/mL, and after 48 h, the number increased approximately 2 log orders for all experimental extracts; the levels were between 8.0 and 8.7 log CFU/mL. Control extracts remained sterile during incubation and the pH stayed at the initial value of 5.20.

3.2.2. Organic acids and carbohydrates

Six organic acids (citric, pyruvic, lactic, acetic, propionic, and butyric acid) and two carbohydrates (lactose and galactose) were detected and quantified in the cheese extracts (data not shown). Lactic and citric acids showed the highest levels, between 920 and

1457 mg/100 mL and 104 and 150 mg/100 mL, respectively. The other organic acids showed lower levels with a value ranging from 2.3 to 5.7 mg/100 mL for pyruvic acid, from 17.4 to 60.2 mg/100 mL for acetic acid, from 18.4 to 102.7 mg/100 mL for butyric acid, and from 9.5 to 12.1 mg/100 mL for propionic acid. The concentration of citric, pyruvic, and lactic acids was higher ($p \leq 0.05$) in the experimental extracts than in the control extracts. In particular, the increase of lactic acid was lower in D extracts than in F and R extracts for both strains. In addition, butyric and acetic acids were also increased ($p \leq 0.05$) in the extracts with Lb91.

Regarding to the levels of carbohydrates in the control extracts, the lactose concentration was very low (< 35 mg/100 mL) while that of galactose was high (571 mg/100 mL). The concentration of both carbohydrates significantly diminished in the extracts due to the addition of lactobacilli; this effect was more prominent with the strain Lb90. In addition, the carbohydrate fermentation, as well lactic acid production, was slightly higher in F and R extracts in comparison to D extract for both strains.

3.2.3. Volatile compounds

Twenty-seven compounds were detected in the headspace of the cheese extracts consisting of one aldehyde, ten alcohols, seven ketones, six acids, two esters, and one lactone (Table S1 in Supplementary Material).

In order to analyze the variability between the volatile profiles of the samples, Principal Component Analysis (PCA) was carried out. The volatile compounds in the extracts after 48 h of incubation were considered as variables for the PCA; the area of the peak of each compound was determined, and the correlation matrix was applied. The first five principal components (PC) were extracted (eigenvalue higher than 1), which explained 85% of total variance. Biplot (scores and loading) for PC1 vs. PC2 which explained 64% of the variance is shown in Fig. 1. A clear discrimination between the control and experimental cheese extracts (with the strain Lb90 or Lb91) was detected in the score plot. The control extracts had negative scores on PC1, while all experimental extracts had positive or near zero scores on the same PC. In addition, there was a difference between the extracts with the strain Lb90 and Lb91 along the PC2: all extracts with the strain Lb90 showed negative scores, while all extracts with the strain Lb91 had positive scores. The influence of the incorporation of each lactobacilli strain was independent of its physiological state at the time of addition, because the extracts with fresh (F), spray-dried powder (D) or reactivated spray-dried powder (R) for each strain were not different in the score plot. The compounds positively associated with PC1, such as some alcohols (3-methylbutanol, 2-heptanol, and 1-hexanol) and some acids (acetic, butyric, hexanoic, and octanoic) characterized all extracts with lactobacilli. On the other hand, the extracts with Lb91 were also characterized by variables positively associated with PC2 such as 2-propanone, 2-butanone, ethylbutanoate, and alcohols (1-propanol, 1-butanol), while the extracts with Lb90 were associated mainly with higher levels of diacetyl, acetoin, and pentanoic acid, the compounds negatively associated with PC2. In particular, the peak area for diacetyl was ten times higher in these extracts than that in the rest of samples (Table S1 in Supplementary Material). ANOVA for the scores of the samples showed significant differences ($p \leq 0.05$) on PC1 and PC2 according to the type of extract, which agreed with the interpretation made for the score plot. In effect, the values of the scores along PC1 were classified in three groups of homogeneous means by Duncan test: 1) C, 2) 90D, 90F, 90R, 91D, 91R, and 3) 90F, 90R, 91D, 91R, 91F. In addition, the values of the scores along PC2 were classified in two groups of homogeneous means: 1) 90F, 90D, 90R, and 2) C, 91F, 91D, 91R.

3.3. Cheese model: miniature soft cheese

3.3.1. Gross composition and pH

The mean values of pH and the contents of protein, fat, and moisture of all cheese samples were 5.20, 18.5%, 23.4% and 55.5% (w/w), respectively. The values were similar for the control and experimental cheeses (data not shown).

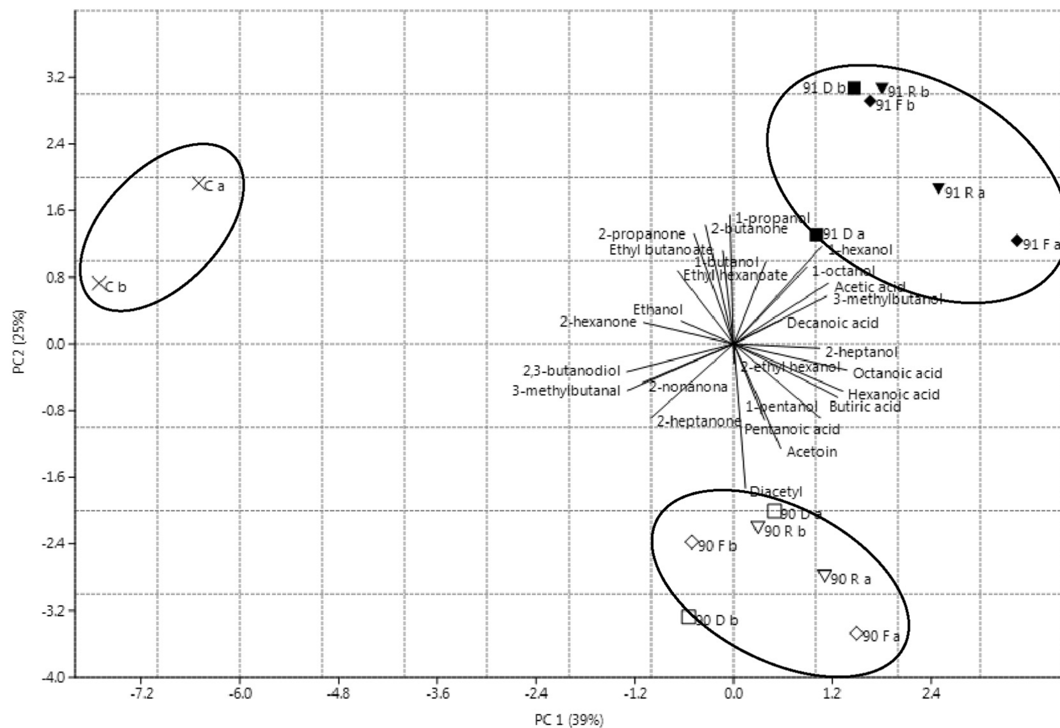

Fig. 1. Biplot of scores and loading of PCA of volatile compounds in control (C) and experimental extracts with the strain Lb90 or Lb91 added as fresh (F) cultures, directly as spray-dried powder (D) or reactivated spray-dried powder (R). a and b: replicas of experiences. Ellipses enclose Control and Experimental extracts with the strain Lb90 or Lb91. Cheese extract code: C (cross), 90F (diamond), 90D (square), 90R (inverted triangle), 91F (filled diamond), 91D (filled square), 91R (filled inverted triangle).

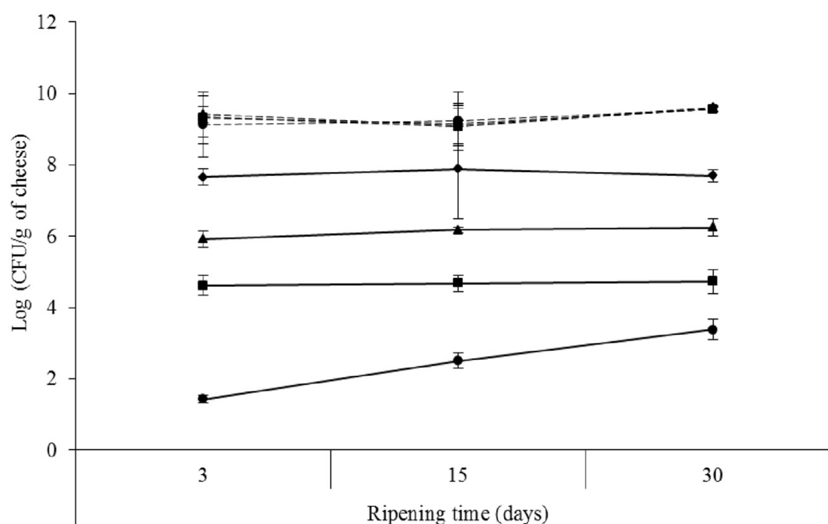

Fig. 2. Counts of starter (dashed lines) and mesophilic lactobacilli (solid lines) in miniature cheeses, after 3, 15 and 30 days of ripening. C (circle): control cheese without adjunct culture addition. E1 (square), E2 (triangle), and E3 (diamond): experimental cheeses with addition of the spray-dried culture of *Lb. paracasei* 90 to reach 5×10^3, 1×10^5 and 5×10^6 CFU/mL in the cheese milk, respectively. The results are means of duplicate analyses and 2 cheesemaking trials.

3.3.2. Microbiological counts

The microbiological counts of the starter and mesophilic lactobacilli are shown in Fig. 2. Streptococci maintained over 9 log CFU/g during all ripening times in all cheese samples, while initial counts of mesophilic lactobacilli were around 4.6, 5.9, and 7.7 log in E1, E2, and E3 cheeses remaining constant during 30 days. In C cheese, lactobacilli remained lower than 10^3 CFU/g during all ripening time (30 days).

3.3.3. Organic acids and carbohydrates

The values of organic acids (citric, pyruvic, lactic, and propionic) and carbohydrates (lactose and galactose) were similar for all cheeses during the ripening (Table 2). Lactic and citric acids were found at the highest level among the organic acids in all cheeses, while lower levels were observed for pyruvic and propionic acids. These results were similar to those obtained for cheese extracts. Significant differences were found only for lactic and propionic acids at 30 days of ripening and the levels of these acids were higher for E3 cheese. On the other hand, low levels of residual lactose, in general < 0.1%, were detected in the cheese samples while the values of galactose were around eight times higher than lactose (between 0.65 and 0.83%). No significant differences were found for the levels of lactose and galactose between control and experimental cheeses.

3.3.4. Volatile compounds in cheese

The spray-dried culture of Lb90 was used in miniature cheese owing to its good performance in producing volatile compounds (such as diacetyl and acetoin) desirable in soft cheese.

Twenty compounds were detected in the headspace of the cheese samples, including one aldehyde, five alcohols, seven ketones, six acids, and one ester (Table S2 in Supplementary Material).

In order to analyze the variability among the volatile profiles of the samples, Principal Component Analysis (PCA) was applied. The variables were the volatile compounds in the cheese samples after 3 and 30 days of ripening; the peak area for each compound was recorded and the correlation matrix was applied. The first four principal components (PC) were extracted (eigenvalue higher than 1), and explained 80.0% of the total variance. Biplots (scores and loading) for PC1 vs. PC2 and PC3 vs. PC4 are presented in Fig. 3A and B, respectively. These plots showed a grouping of the samples according to the ripening time and replica of cheesemaking along PC1 and PC2, respectively. In addition, the effect of the incorporation of the spray-dried adjunct culture was observed in the fourth PC, which explained 10% of total variance. This influence was observed only when the adjunct culture was added at the highest dose; in effect, E3 cheeses were clearly distinct from the rest of the cheeses on PC4, while C, E1, and E2 cheeses were similar. The ANOVA confirmed our interpretation of the significance of PCs, as it showed significant differences ($p \leq 0.05$) in the scores of the samples on PC1, PC2, and PC4 according to ripening time, replica of cheesemaking, and type of cheese, respectively (Table 3). The variables acetoin, diacetyl, α-dodecalactone, acetic and decanoic acids showed high positive loading values on PC4, and consequently were those that characterized and differentiated E3 cheese from others.

4. Discussion

During the last decade, several investigations assessed the impact of adjunct cultures of lactobacilli on flavor development of different cheese varieties such as Cheddar (Ciocia et al., 2013; Madkor et al., 2000), Swiss (Kocaoglu-Vurma et al., 2008), Pecorino Siciliano

Table 2
Levels of organic acids and carbohydrates (mg/100 g cheese) in control (C) and experimental (E) miniature cheeses, at 3 and 30 days of ripening. Values are means (± standard deviations) of two cheese replicates manufactured in different days with different milk.

	3 days				30 days			
	C	E1	E2	E3	C	E1	E2	E3
Citric acid	152.6 ± 8.0	153.2 ± 3.2	156.7 ± 14.2	151.5 ± 4.0	148.9 ± 4.2	136.4 ± 5.5	165.1 ± 19.7	180.8 ± 14.3
Pyruvic acid	9.4 ± 2.7	9.5 ± 3.9	10.4 ± 1.9	10.2 ± 3.4	8.4 ± 1.1	7.8 ± 1.3	9.0 ± 0.4	7.6 ± 0.6
Lactic acid	1296.2 ± 26.5	1291.3 ± 75.2	1304.6 ± 32.7	1270.6 ± 123.7	1211.8 ± 10.5[ab]	1082.0 ± 48.8[a]	1295.5 ± 85.9[bc]	1416.4 ± 103.8[c]
Propionic acid	8.8 ± 3.9	10.7 ± 0.8	10.3 ± 1.3	10.7 ± 1.5	9.6 ± 0.2[a]	8.1 ± 0.3[a]	9.8 ± 1.3[a]	12.8 ± 0.9[b]
Lactose	95.0 ± 23.3	79.5 ± 32.6	82.8 ± 27.7	82.7 ± 31.2	103.3 ± 9.3	89.8 ± 35.0	77.9 ± 17.1	100.4 ± 1.5
Galactose	827.2 ± 30.9	808.3 ± 62.6	800.4 ± 14.8	771.5 ± 85.6	721.1 ± 22.9	650.5 ± 28.2	771.6 ± 43.6	769.8 ± 31.9

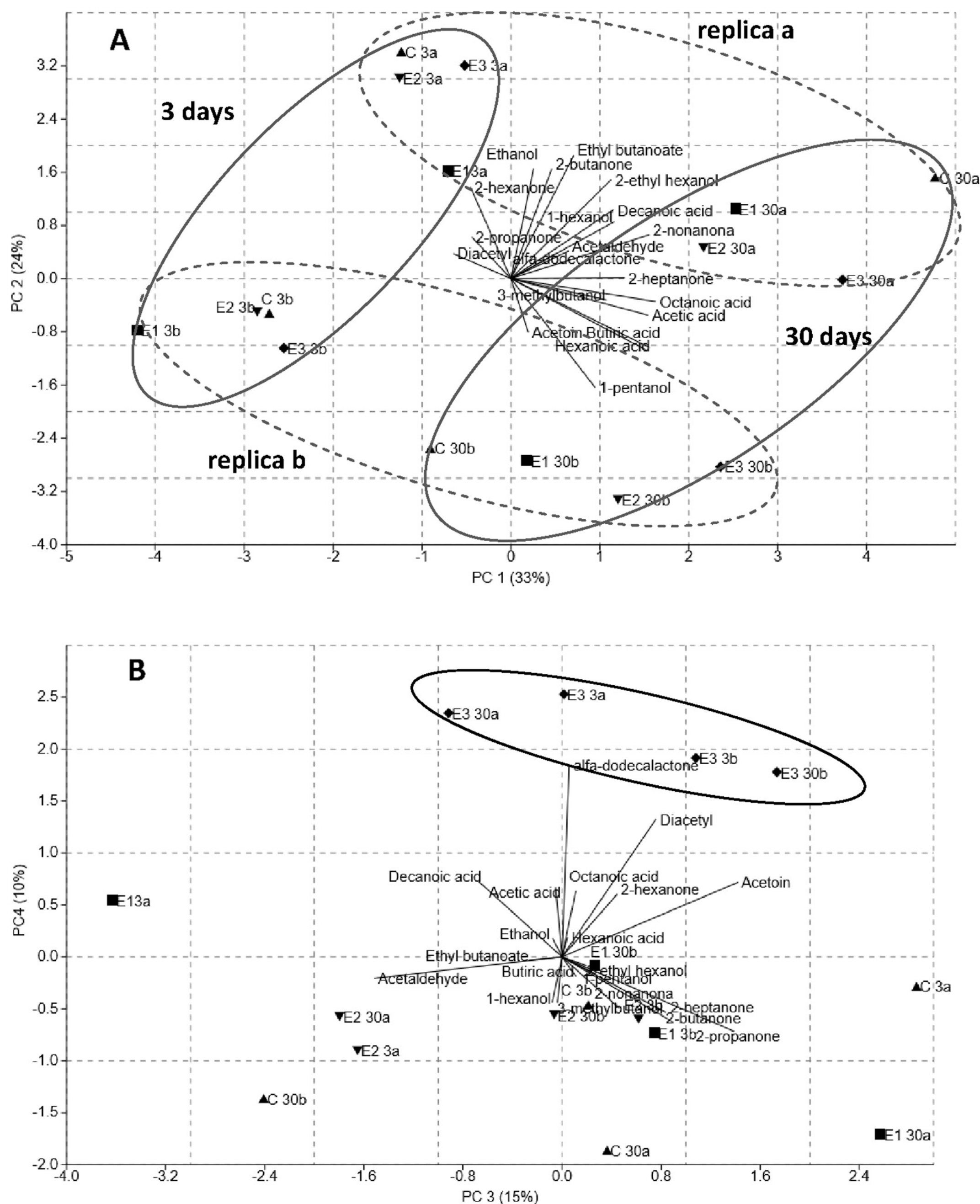

Fig. 3. Biplot of scores and loading of PCA of volatiles compounds in control (C: triangle) and experimental (E1: square, E2: inverted triangle and E3: diamond) cheeses at 3 and 30 days of ripening. a and b: replicas of cheesemaking. A) PC1 vs. PC2. Ellipses enclose cheese samples at 3 and 30 days of ripening (solid lines), and cheese samples from replicas a and b (dashed lines). B) PC3 vs. PC4. Ellipse encloses E3 cheese samples.

Table 3
Significance of the addition of adjunct cultures, ripening time and replicas of cheesemaking on the first four principal components (PC) of the principal component analysis of volatile compounds of cheeses C, E1, E2 and E3 at 3 and 30 days of ripening.

PC	Addition of adjunct cultures	Ripening time	Replica of cheesemaking
1	NS	*	NS ($p = 0.062$)
2	NS	NS ($p = 0.052$)	*
3	NS	NS	NS
4	*	NS	NS

NS = not significant, $p > 0.05$.
* $p \leq 0.05$.

(Randazzo et al., 2008), Caciocavallo Pugliese (Di Cagno et al., 2012), and Cremoso (Burns et al., 2012; Milesi et al., 2008). However, no studies pursued the production of concentrated adjunct cultures from these NSLAB strains. In the present work, we obtained spray-dried cultures from the well-known NSLAB strains *Lb. paracasei* 90 and *Lb. plantarum* 91 and assessed their performance in cheese models.

Since very few reports are available on the NSLAB conservation by spray-drying, we compared our results with those obtained on probiotic lactobacilli, taking into account that several factors, both technological and strain-dependent, may influence bacterial survival. *Lb. paracasei* 90 and *Lb. plantarum* 91 showed a good survival after spray-drying and during storage, similar to those reported by Páez et al. (2012) for *Lb. acidophilus* A9, *Lb. plantarum* com, *Lb. plantarum* 8329, *Lb. paracasei* A13 and *Lb. casei* Nad, which were dried and stored in similar conditions. A

higher decrease in the cell counts during storage was reported by Lavari et al. (2014), with reduction from 1.6 to 2.5 log orders for *Lb. gasseri* 37, *Lb. paracasei* JP1 and *Lb. rhamnosus* 64, stored for six months at 5 °C. Some earlier reports showed that a strain of *Lb. paracasei* (NFBC 338) could maintain high viability through spray-drying and storage for two months at 4 °C, while another strain of *Lb. salivarius* UCC 118 was found to be more sensitive, with a loss of 1 log (Gardiner et al., 2000; Gardiner et al., 2002). A viability loss of 34% to 86% for *Lactococcus lactis* ssp. *lactis* C2 was reported after spray-drying and storing the powder for three months at 4 °C (Fu and Etzel, 1995).

In this work, we aimed at assessing the performance of dried cultures of *Lb. paracasei* 90 and *Lb. plantarum* 91. We had previously demonstrated they are able to survive and prevail in cheese when added as fresh adjunct cultures (Milesi et al., 2008; Milesi et al., 2009). This property was also observed for the dehydrated cultures that survived in the extracts similarly to fresh or reactivated spray-dried cultures. In addition, the spray-dried powder of Lb90 showed good survival in cheeses during ripening; the levels of lactobacilli in E cheeses were one log higher than the cell load in the cheese milk, indicating an increase in the concentration during the cheesemaking (Bergamini et al., 2005).

High levels of galactose are common in the cheese made with the starters composed only of strains of *S. thermophilus* (St-Gelais et al., 2009). The ability of an adjunct culture to metabolize residual carbohydrates in cheese is a desirable characteristic because it can indirectly inhibit the growth of NSLAB. In the present work, Lb90 and Lb91 fermented galactose; however, the spray-dried cultures were slightly slower than fresh cultures in fermenting galactose. This trend was reverted when dehydrated cultures were reactivated before inoculation. Similar to our results, To and Etzel (1997) reported a delay in the lactic acid production from lactose by spray-dried cultures of three different lactic acid bacteria species.

In contrast to the extracts, Lb90 was not able to ferment galactose in cheeses, which may be due to the spray-drying, but most probably due to the ripening temperature. In the cheesemaking experiments, lactobacilli were kept at their optimal temperature only during the cheesemaking process (approx. 6 h) and then they were ripened at 5 °C for 30 days, while in the extracts they were incubated for 48 h at 37 °C. In order to overcome this difficulty and check the origin of the delay, we suggest incubating the spray-dried cultures in the cheese milk prior to cheesemaking.

On the other hand, the dehydrated strains were not able to metabolize citrate in the extract or in the cheese, similarly to the fresh cultures in Cremoso cheese (Milesi et al., 2010). However, the same strains as fresh cultures metabolized citrate in a galactose- and lactose-free extract derived from a hard cooked cheese (unpublished results). In this regard, Díaz-Muñiz et al. (2006) suggested that citrate serves as energy source to *Lb. casei* during cheese ripening only when the residual levels of post fermentation carbohydrates are limited (< 2.5 mM).

The analysis of the volatile profiles of the extracts showed that the contribution of both strains tested for the production of aroma-related compounds was independent of their physiological states (F, D and R cultures), which demonstrated that spray-drying did not affect their performance as flavor enhancers. The differences between Lb91 and Lb90 observed as fresh cultures were also maintained in their dehydrated forms. In particular, the ability of the strain Lb90 to increase diacetyl and acetoin, established earlier (Milesi et al., 2010; Peralta et al., 2014), was also verified for the dehydrated culture during the extract fermentation, making this spray-dried adjunct culture suitable for further evaluation in the miniature cheese. In this case, the increased diacetyl and acetoin contents characterized the E3 cheeses, i.e., the experimental cheeses with the highest dose of the spray-dried culture of Lb90. The production of diacetyl and acetoin by Lb90 can be attributed to Asp catabolism by the Asp-AT activity (Peralta et al., 2016a, 2016b), similarly to lactococci (Le Bars and Yvon, 2008) and other mesophilic lactobacilli (Kieronczyk et al., 2004), especially taking into account that citrate content remained constant in the extracts and cheeses.

While comparing the effects of Lb90 in both cheese models tested in this work, we found that the differences in the production of diacetyl, in particular, and in volatile compounds, in general, were more pronounced in the extracts than in the cheese samples. This observation can be explained on the basis of several reasons. The temperature of incubation, as mentioned earlier, might have favored metabolic transformations. In addition, it is easier monitor biochemical changes in extracts than cheese owing to their simple composition.

There are very few studies available discussing the impact of the different doses of the adjunct culture. Carunchia Whetstine et al. (2006) assayed the addition of a malty *Lactococcus lactis* adjunct culture at two levels (10^4 and 10^5 CFU/mL) in Cheddar cheese in order to favor the development of the nutty flavor, which is correlated with the production of 2/3-methyl butanal and 2-methyl propanal. These authors found greater changes (chemical and sensory) at a higher level of addition. On the other hand, Van Hoorde et al. (2010) verified that the addition of the strain *Lb. paracasei* R-40926 in Gouda-type cheese produced different changes in the volatile profiles according to the dose employed. In effect, the cheeses with the lower dose (10^3 CFU/mL) were mainly characterized by volatiles derived from proteolysis, while the cheese made with the higher dose of the adjunct (10^5 CFU/mL) was dominated by fat-related compounds next to some amino acid-related compounds. In our work, the changes in the volatile profiles of cheese due to the addition of the strain Lb90 were detected only in the cheeses at the higher dose of this adjunct.

5. Conclusions

Spray-drying was found to be an appropriate technology for the production of dehydrated adjunct cultures from autochthonous strains. The spray-dried powders obtained from *Lactobacillus paracasei* 90 and *Lactobacillus plantarum* 91 retained high levels of viable counts which were stable during the 12 months of storage at 5 °C. In addition, the strains maintained viability and high counts in cheese models during incubation and ripening. Furthermore, the spray-drying process did not exert any negative effect on the metabolic activity of the strains with regard to carbohydrate metabolism and the production of organic acids and volatile compounds, except only for a slight delay in the fermentation of carbohydrates.

Finally, the positive characteristic of *Lb. paracasei* 90 to increase markedly the production of acetoin and diacetyl in cheese through aspartate catabolism, as reported earlier for the fresh culture, was confirmed for the spray-dried culture in cheese extracts and in cheeses, when a high dose was used.

The findings of this work suggest that autochthonous strains could be dried relatively easily, even in dairy facilities, in order to use them in the same industry or commercialize as adjunct cultures. The production of these adjunct cultures will allow revalorizing autochthonous strains while preserving biodiversity and cheese diversity.

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Appendix A. Supplementary data.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2017.05.014.

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