Human *Bifidobacterium* strains as adjunct cultures in Spanish sheep milk cheese

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ABSTRACT

Three bifidobacteria strains of human origin (*Bifidobacterium pseudolongum* INIA P2, *Bifidobacterium breve* INIA P734, and *Bifidobacterium longum* INIA P678) were used as potential probiotic adjunct cultures for the manufacture of pasteurized sheep milk cheese. Bifidobacteria were inoculated at 5 to 6 log cfu/mL in milk vats. Microbiological, physicochemical, rheological, color, and sensory characteristics were determined at 7, 28, and 60 d of ripening. Counts of *B. pseudolongum* INIA P2 remained above 6 log cfu/g during 60 d of ripening as well as after further simulated gastrointestinal digestion of cheeses. *Bifidobacterium breve* INIA P734 counts remained stable during 28 d and decreased by less than 1 log unit after simulated digestion. *Bifidobacterium longum* INIA P678 counts dropped sharply during cheese manufacture and ripening and were below detection level after simulated digestion. Addition of bifidobacteria strains did not affect starter viability, cheese pH, dry matter, water activity, or salt content but significantly increased overall proteolysis and the concentration of some free amino acids. Cheeses with bifidobacteria exhibited no significant differences in most sensory characteristics with respect to control cheese. According to our results, *B. breve* INIA P734 and *B. pseudolongum* INIA P2 are promising candidates as probiotic adjunct cultures in fresh and semi-hard sheep milk cheese.

Key words: *Bifidobacterium*, sensory quality, cheese, microbiome

INTRODUCTION

After Greece, Spain is the leading country in sheep milk production in Europe. Most sheep milk is processed into mixed-milk cheese varieties because pure

sheep milk cheese, considered a gourmet product, accounts for only 16.1% of overall Spanish cheese production (Pulina et al., 2018).

Cheese is a fermented food that has historically been an integral part of the human diet and is considered to provide health benefits, which are attributed to metabolic compounds produced by the bacteria responsible for fermentation (Sanlier et al., 2019). Diet is a critical determinant of variation in gut microbial structure and function, outweighing even host genetics (Carmody et al., 2019). The beneficial co-evolution of human–dairy microorganisms is supported by the high survival rates of 2 bifidobacteria strains used in model cheese manufacture under simulated gastrointestinal conditions (Peirotén et al., 2019b). Some strains derived from ancestral or traditional compatible gut healthy microbiota may confer health benefits to humans (Sonnenburg and Sonnenburg, 2019). In this context, incorporation of human bifidobacteria in traditional cheeses offers opportunities to “fertilize” our microbiota and to “re-wild” the industrialized human gut ecosystems (Mills et al., 2019; Sonnenburg and Sonnenburg, 2019).

Bifidobacteria must meet certain technological properties to ensure a minimal viable count (6 log cfu/g) in products to exert a therapeutic effect within the host (Boylston et al., 2004; du Toit et al., 2013). Only a few bifidobacteria strains are robust from a technological point of view (Gomes and Malcata, 1999; Ruiz et al., 2011). In this regard, cheeses present advantages as delivery systems due to their buffering ability, fat content, high pH, and anaerobic environment at the core created by the metabolism of lactic acid bacteria within a few weeks of ripening (Ong et al., 2007; Karimi et al., 2011; Reid, 2015). Studies on cheeses with bifidobacteria have focused mainly on probiotic viability and generally have been based on a few commercial strains alone or in combination with lactobacilli or prebiotics (Barbosa et al., 2016; Song et al., 2017; Cuffia et al., 2018).

Ovine milk is particularly interesting for the manufacture of probiotic cheeses with bifidobacteria in that it permits faster growth than cow milk (Kehagias et al., 2008). In addition, ovine milk seems to be better

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for subjects suffering from cow milk allergy (Albenzio et al., 2013).

Addition of probiotic strains may affect cheese flavor, texture, and appearance (Bergamini et al., 2009; Albenzio et al., 2013), and the commercial success of probiotic products ultimately depends on taste and appeal to the consumer (Granato et al., 2010). Cheese technology influences growth and biochemical activity of starter cultures and probiotic strains (Bergamini et al., 2009). Therefore, sensory studies on cheeses manufactured with bifidobacteria are required to ensure the flavor quality of traditional pure ovine cheese.

To our knowledge, this is the first study on semi-hard Spanish sheep milk cheese made with noncommercial strains of bifidobacteria. In this study, 3 *Bifidobacterium* sp. strains with proven probiotic and technological properties (Rodríguez et al., 2012; Peirotén et al., 2018, 2019b) were assayed as adjunct cultures. The objectives of this work were to evaluate (1) their survival in sheep semi-hard cheese, (2) their further survival during the passage through simulated gastrointestinal conditions, and (3) their influence on cheese physicochemical and sensory characteristics.

MATERIALS AND METHODS

Bacterial Strains and Preparation of Inocula

Bifidobacterium pseudolongum INIA P2, *Bifidobacterium longum* INIA P678 (CECT 8178), and *Bifidobacterium breve* INIA P734 (CECT 8179) were from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) culture collection (Madrid, Spain). *Bifidobacterium breve* INIA P734 (CECT 8178) and *B. longum* INIA P678 (CECT 8179) were selected because of their mother-child pair origin (Peirotén et al., 2018) and are considered robust strains from a technological point of view (Peirotén et al., 2019b). *Bifidobacterium breve* INIA P734 is under patent (Spanish patent P201231747) based on its origin and in vitro antimicrobial properties. *Bifidobacterium pseudolongum* INIA P2 is a promising probiotic strain that is technologically robust and able to produce enterolignans (Peirotén et al., 2019a).

Bifidobacterium pseudolongum INIA P2 was grown in reinforced clostridial medium broth (Difco, Sparks, MD) at 37°C for 48 h in an anaerobic chamber (Whitley DG250, Don Whitley Scientific, Shipley, UK). Then, cells were collected by centrifugation, washed in PBS, and resuspended in milk. Lyophilized concentrated cultures of *B. longum* INIA P678 and *B. breve* INIA P734 were rehydrated in UHT milk (Pascual, Aranda del Duero, Spain). Microbial counts of the inocula were determined before each experiment. Commercial

mesophilic starter culture (Choozit MA 4001 Lyo 5 DCU; Danisco, Laboratorios, Arroyo, Spain) containing MA 016 (*Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, and *L. lactis lactis* biovar *diacetylactis*) and TA 054 (*Streptococcus thermophilus*) was prepared as specified by the manufacturer by resuspending in 200 mL of UHT milk.

Cheese Manufacture

Cheeses were made in 2 trials carried out on different days. In each trial, whole sheep milk (Castellana de Ganaderos, Campo Real, Spain) was pasteurized (74°C for 20 s), cooled to 30°C, and distributed in four 40-L vats. Progressive heating was applied, and CaCl₂ (40 mL of 20% CaCl₂ solution per vat) was added when the milk reached 30°C. At 33°C, 40 mL of commercial starter culture, prepared as specified above, was added to each of the 4 vats. Bifidobacteria strains were added to vats as follows: no bifidobacteria to vat 1 (control cheese), *B. pseudolongum* INIA P2 to vat 2 (INIA P2 cheese), *B. longum* INIA P678 to vat 3 (INIA P678 cheese), and *B. breve* INIA P734 to vat 4 (INIA P734 cheese). Liquid rennet (5.3 mL of 1:15,000-strength calf rennet per vat; Laboratorios Arroyo, Santander, Spain) was added 20 min later to all the vats. Curds were cut 40 min after rennet addition and heated at 37°C for 20 min. Then, whey was drained off for 30 min and curds were washed by adding 20 L of pasteurized tap water to each vat and distributed into cylindrical molds. Four cheeses of approximately 2 kg were obtained from each vat. Cheeses were pressed overnight at room temperature and 1.5 kg/cm² pressure, salted in 150 g/L NaCl solution for 24 h, and ripened at 12°C and 83% relative humidity for 60 d. Cheeses were sampled for analysis at d 7, 28, and 60.

Microbial determinations were carried out on curd and on cheeses after 7, 28, and 60 d of ripening. A representative sample (5 g) of cheese core was diluted in 45 mL of sterile sodium citrate solution and homogenized for 90 s in a stomacher 400 (A. J. Seward Ltd., London, UK). Decimal dilutions were prepared in sterile 0.1% peptone water and plated in duplicate. *Bifidobacterium* counts were performed on reinforced clostridial medium agar (Difco) supplemented with 50 µg/mL mupirocin (Oxoid, Basingstoke, UK) and incubated at 37°C for 48 h under anaerobic conditions. When necessary, enrichment was performed by incubating 10 g of cheese core homogenized in De Man, Rogosa and Sharpe broth (Biolife, Milano, Italy) supplemented with 0.5 g/L L-cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO) and 50 µg/mL mupirocin for 48 h at the same conditions before plating. Thermophilic and mesophilic starter counts in milk and cheese samples were

determined in M17 agar (Biolife) supplemented with glucose at 0.5 g/mL and incubated for 24 h at 40 and 30°C, respectively. Total aerobic counts in pasteurized milk were determined on duplicate plates of tryptic soy agar (Oxoid) incubated at 37°C for 24 h under aerobic conditions.

Resistance to Gastrointestinal Conditions

Microbial resistance to gastrointestinal conditions was determined at d 7, 28, and 60 of ripening. Cheese core (5 g) was diluted in 45 mL of buffered solution (PBS; pH 3.0) at 37°C and homogenized for 90 s. Homogenates were incubated at 37°C for 1 h under anaerobic conditions. Then, a 1-mL sample was added to 9 mL of bile solution (0.15%, ox-bile desiccated; Oxoid) and kept at the same conditions for 1 h. Decimal dilutions were made in sterile 0.1% peptone water and plated in duplicate on reinforced clostridial medium agar (Difco) supplemented with 50 µg/mL mupirocin (Oxoid).

Physicochemical Determinations

All physicochemical determinations were performed at d 7, 28, and 60 of ripening; in addition, cheese pH was measured at d 1. Cheese pH was measured in duplicate by means of a Crison pH meter (model GPL 22, Crison Instruments, Barcelona, Spain) using a penetration electrode (model 52-3.2). Dry matter content was determined in duplicate by drying to constant weight in an oven at 100°C. Results were expressed as a percentage of DM on total weight.

Water activity was measured using AquaLab Series 3 equipment (Decagon Devices Inc., Pullman, WA). Salt content was determined in duplicate using Quantab chloride titrators (Hach Co., Loveland, CO) according to AOAC (1984). Results were presented as percentage of salt content on total weight.

Cheese proteolysis was determined on duplicate samples using the *o*-phthaldialdehyde test (Church et al., 1983). Free AA (**FAA**) were determined on duplicate samples of cheese as described by Krause et al. (1995) with reversed phase HPLC using a Beckman System Gold chromatograph (Beckman Coulter, Brea, CA) after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Cohen and Michaud, 1993). Results were expressed as milligrams of AA per 100 g of cheese DM.

Organic acids (orotic, citric, pyruvic, lactic, uric, acetic, propionic, and butyric acids) and sugars (lactose, glucose, and galactose) were extracted from duplicate samples of cheese as described by Bouzas et al. (1991) and determined by HPLC. Extracts (20 µL) were injected in duplicate and eluted with 3 mM sulfuric

acid at 65°C and a flow rate of 0.7 mL/min on a 300 × 7.8-mm ion exchange column (HPX-87H Aminex) protected by a cation H⁺ Micro-Guard cartridge (Bio-Rad Laboratories, Hercules, CA) in a Beckman System Gold liquid chromatograph (Beckman Coulter). The system was equipped with 2 detectors connected in series, a diode array detector with detection wavelength of 210 nm for organic acids and a differential refractometer detector module (Knauer, Berlin, Germany) for sugars. Organic acids and sugars were quantified using the external standard method, and the results were expressed as milligrams of organic acid or sugar per 100 g of cheese DM.

Volatile compounds were extracted by automated solid-phase microextraction using a CTC CombiPal autosampler (Agilent, Palo Alto, CA) and analyzed by GC-MS using HP 6890-MSD HP 5973 (Agilent) equipment as described by Gómez-Torres et al. (2015) with minor modifications. Duplicate 10-g cheese samples were homogenized in an analytical grinder (IKA Labortechnik, Staufen, Germany) with 15 g of anhydrous Na₂SO₄ and 30 µL of an aqueous solution of 1,000 µg/mL cyclohexanone (Sigma-Aldrich) as internal standard. Five grams of this mixture was weighed in a glass vial (20-mL headspace) and equilibrated for 20 min at 37°C. Volatile compounds were extracted using a 2-cm × 50/30-µm StableFlex divinylbenzene/carboxen/polydimethylsiloxane-coated fiber (Supelco, Bellefonte, PA) exposed to the headspace for 30 min at 37°C. Desorption into the GC injection port was at 260°C for 9.5 min in splitless mode. Chromatographic separation was carried out in a Zebtron 100% polyethylene glycol capillary column (60 m long, 0.25-mm internal diameter, 0.50-µm film thickness; ZB-WAXplus, Phenomenex, Torrance, CA) with 1 mL/min helium flow. The following temperature program was used: 16 min at 45°C; first ramp 5°C/min to 110°C, 9 min at 110°C; second ramp at 15°C to 220°C, 5 min at 220°C; final ramp to 240°C at 10°C/min and 6 min at 240°C. Mass detection was performed in the scan mode, from 33 to 300 amu at 5.16 scans/s and ionization by electron ionization at 70 eV. Data were collected with the HP ChemStation program (Agilent), and volatile compounds were identified by comparison of spectra with the Wiley 275 library (Wiley and Sons Inc., Weinheim, Germany) and by comparison of their retention times with authentic standards (Sigma-Aldrich). Relative abundances of compounds were calculated as compound peak area × 300/internal standard peak area.

Color Determinations

Color determinations were performed at d 7, 28, and 60 of ripening using a CM-700 spectrophotometer and

analyzed with Spectramagic NX VA.9 software (Minolta Camera Co., Osaka, Japan). Cheese samples were cut into 1-cm-thick slices. The reference illuminant was D65 (standard daylight), and the observer angle was 10°. Results are expressed as L^* (lightness), a^* (−a green to +a red), and b^* (−b blue to +b yellow). After the L^* , a^* , and b^* measurements were read, the sample was rotated 90° and read again. This procedure was carried out in quadruplicate. The method of color measurement applied was specular component excluded, where only the diffuse reflectance is measured, which correlates better to the way the observer sees the color of an object (Gómez-Torres et al., 2014).

Textural Determinations

Textural determinations were performed at d 7, 28, and 60 of ripening. After being kept at room temperature (20–22°C) for 2 h, 6 cylinder-shaped samples (17 mm height × 17 mm diameter) from each cheese were compressed to 75% of their original height using an Instron Compression Tester 4301 (Instron Ltd., High Wycombe, UK) with crosshead and chart speeds of 50 and 500 mm/min, respectively. Fracturability (breaking force expressed in newtons, N), hardness (work after 75% compression, expressed in joules, J), and elasticity (apparent elastic module, expressed in newtons per square millimeter, N/mm²) were measured from the compression curves as previously described (del Pozo et al., 1988). Data were analyzed using BlueHill software (BlueHill Materials Testing Software; Norwood, IL).

Sensory Evaluation

Sixteen trained panelists tasted cheeses at d 7, 28, and 60 of ripening. The descriptive test used was based on the guidelines of Bérodiér et al. (1997). Panelists were asked to score cheese appearance, texture, quality and intensity of odor, aroma, and taste on a 0- to 10-point scale using a horizontal line anchored at the middle, at the left end (lowest = 0 points), and at the right end (highest = 10 points). Odor was defined as the olfactory sensation felt directly by the nose. Aroma was defined as the olfactory sensation felt retronasally upon mastication. Panelists were also asked to give a score, on a 0- to 10-point scale, to the taste notes acidic, bitter, sweet, and salty.

Statistical Analysis

Statistical analysis of the data was performed using IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY) by ANOVA using a general linear model, with bifidobacteria strains and ripening time as main ef-

fects. Comparison of means was carried out by Dunnett test (bifidobacteria effect with respect to control) and Tukey test (ripening time or bifidobacteria effects) at $P < 0.01$.

RESULTS AND DISCUSSION

Bacterial Counts in Cheese and Resistance to Simulated Gastrointestinal Conditions

Initial counts of mesophilic and thermophilic commercial starter cultures in milk after inoculation were on average 7.00 and 5.86 log cfu/mL, respectively. Starter counts in cheese (Table 1) were influenced ($P < 0.01$) by ripening time, according to ANOVA results, but not by addition of any of the *Bifidobacterium* strains to cheese.

Bifidobacteria levels in inoculated milk were 6.31 log cfu/mL for *B. pseudolongum* INIA P2, 5.01 log cfu/mL for *B. longum* INIA P678, and 5.77 log cfu/mL for *B. breve* INIA P734, and their respective counts in curd were 6.56, 4.16, and 5.89 log cfu/g. *Bifidobacterium pseudolongum* INIA P2 counts remained constant in cheese until the end of ripening, whereas *B. breve* INIA P734 counts remained stable during the first 28 d and declined to 4.35 log cfu/g after 60 d ($P < 0.01$). On the other hand, *B. longum* INIA P678 counts diminished dramatically to 2.38 log cfu/g on d 7 and were below detection at the end of ripening.

Bifidobacterium longum INIA P678 had shown satisfactory in vitro technological properties in previous studies (Peirotén et al., 2018, 2019b). However, its low survival in cheese seems to indicate injury during freeze-drying and by cheese-making and ripening conditions. Culture viability alone is not an adequate predictor for the strain functionality under adverse conditions, such as in a food product or after a technological process (Saarela et al., 2006; Vinderola et al., 2012). Bifidobacteria viability in cheese was dependent on the strain used, as observed in previous works (Albenzio et al., 2015; Barbosa et al., 2016), and could be influenced by the starter culture, cheese technology, and type of milk. In this study, we tried to maintain cheese pH above 5.0 by using sheep milk with high mineral content and by washing the curd for partial removal of lactose. In addition, using medium-sized cheeses of approximately 2 kg would ensure anaerobic conditions for bifidobacteria as previously demonstrated for *Clostridium* spp. (Ávila et al., 2016). A buffered and anaerobic matrix is crucial for the survival of bifidobacteria.

Digestion of cheeses under simulated gastrointestinal conditions showed high survival rates for *B. pseudolongum* INIA P2 at any time of ripening, with decreases below 0.4 log units in all cases. *Bifidobacterium breve*

Table 1. Counts (log cfu/g) of starter cultures and *Bifidobacterium* sp. strains during cheese ripening and after simulated gastrointestinal conditions (SGIC)¹

Microorganism	Day	Cheese ²			
		Control	INIA P2	INIA P678	INIA P734
Termophilic starter**	7	9.56 ± 0.06 ^a	9.59 ± 0.02 ^a	9.52 ± 0.11 ^a	9.49 ± 0.17 ^a
	28	9.40 ± 0.12 ^a	9.35 ± 0.06 ^a	9.38 ± 0.02 ^a	9.42 ± 0.10 ^a
	60	9.22 ± 0.09 ^a	8.85 ± 0.27 ^a	8.88 ± 0.16 ^a	9.05 ± 0.17 ^a
Mesophilic starter**	7	8.22 ± 1.21 ^a	8.25 ± 1.18 ^a	8.26 ± 1.07 ^a	8.33 ± 1.08 ^a
	28	7.87 ± 0.23 ^a	7.74 ± 0.16 ^a	7.63 ± 0.38 ^a	7.82 ± 0.16 ^a
	60	6.34 ± 0.09 ^a	6.29 ± 0.53 ^a	6.36 ± 0.18 ^a	6.54 ± 0.49 ^a
<i>Bifidobacterium</i> (cheese)	7	—	6.61 ± 0.18 ^a	2.38 ± 0.15 ^b	6.22 ± 0.15 ^a
	28	—	6.66 ± 0.15 ^a	1.27 ± 1.48 ^b	5.75 ± 0.04 ^a
	60	—	6.51 ± 0.20 ^a	ND ^{c,3}	4.35 ± 0.42 ^b
<i>Bifidobacterium</i> (after SGIC)	7	—	6.45 ± 0.09 ^a	ND ^b	6.22 ± 0.46 ^a
	28	—	6.35 ± 0.12 ^a	ND ^c	4.97 ± 0.40 ^b
	60	—	6.12 ± 0.20 ^a	ND ^c	1.74 ± 2.02 ^b

^{a-c}Means at each ripening time with different superscripts differ significantly ($P < 0.01$, Tukey test).

¹Mean ± SD of 2 determinations on 2 cheese-making trials.

²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.

³ND = below detection level.

**Significant effect ($P < 0.01$) of ripening time.

INIA P734 remained fully viable after digestion of 7-d cheese, decreased by 0.8 log unit in the case of 28-d cheese, and fell sharply, by 2.6 log units, after digestion of 60-d cheese. No digestion assays were performed on *B. longum* INIA P678 because its counts in cheese were too low or even undetectable.

Bifidobacterium pseudolongum INIA P2 showed a higher resistance in cheese than in previous studies in skim milk (Rodríguez et al., 2012). This fact confirms the protective role of cheese in simulated digestion that may let the strain reach the human colon. To our knowledge, this is the first time that a *B. pseudolongum* strain has been used for cheese manufacture. Reports on *B. pseudolongum* isolation from human infant feces or breast milk are scarce (Rodríguez et al., 2012; Souza et al., 2013). However, this species has been described as a widely distributed gut commensal in the animal kingdom, including the human gut, where it could play a beneficial role due to its metabolic activities (O'Callaghan and van Sinderen, 2016; Lugli et al., 2019).

Our results for *B. breve* INIA P734 and *B. longum* INIA P678 demonstrated that cheese manufacturing and ripening conditions affected their survival. Injured cells became more sensitive to further gastrointestinal conditions (Collado et al., 2006; Granata et al., 2013). Bile tolerance, and hence resistance to gastrointestinal passage, has been described as strain dependent. In this sense, several genes and proteins conferring bile resistance, which include bile salt hydrolases and bile efflux transporters, were identified and characterized in vitro (Kim et al., 2004; Ruiz et al., 2013). High resistance

to these conditions is essential for the probiotic strains to reach the colon and exert the claimed probiotic effect. In this regard, only *B. breve* INIA P 734 in short-ripened cheeses and *B. pseudolongum* INIA P2 even in long-ripened cheeses would meet those criteria.

Cheese Physicochemical Characteristics

Cheese pH, DM, Water Activity, and Salt Content. Values of pH, DM, water activity, and salt content (Table 2) were influenced by ripening time ($P < 0.01$) and not by *Bifidobacterium* addition to cheese according to ANOVA results. Cheese pH value increased with time of ripening ($P < 0.01$); values were 0.23 to 0.39 pH unit higher on d 60 than on d 1. Dry matter and salt content increased ($P < 0.01$) and water activity values decreased ($P < 0.01$) from d 7 to d 60 of ripening.

Proteolysis and FAA. Overall proteolysis was significantly ($P < 0.01$) influenced by *Bifidobacterium* addition to cheese and by ripening time according to ANOVA results (Table 3). Proteolysis values were 5- to 6-fold higher in 60-d cheeses than in 7-d cheeses. At d 7, there were no significant differences in proteolysis between cheeses. Proteolysis was higher at d 28 ($P < 0.01$) for cheese with *B. longum* INIA P678 than for control cheese and at d 60 for cheeses with *B. longum* INIA P678 and *B. breve* INIA P734 than for control cheese. Cheese with *B. pseudolongum* INIA P2 did not show differences in proteolysis with respect to control cheese at any ripening time. High cheese pH values could enhance proteolysis, as the optimum pH for the

Table 2. Values of pH, DM, water activity, and salt content during ripening in control cheese and in cheeses made using bifidobacteria as adjunct cultures¹

Characteristic	Day	Cheese ²			
		Control	INIA P2	INIA P678	INIA P734
pH**	1	5.03 ± 0.04	5.00 ± 0.06	5.07 ± 0.02	5.07 ± 0.02
	7	5.24 ± 0.10	5.14 ± 0.07	5.19 ± 0.12	5.18 ± 0.08
	28	5.17 ± 0.04	5.15 ± 0.06	5.03 ± 0.04	5.20 ± 0.02
	60	5.42 ± 0.06	5.35 ± 0.01	5.45 ± 0.12	5.30 ± 0.06
DM** (%)	7	54.44 ± 4.12	55.57 ± 2.27	54.33 ± 1.06	54.31 ± 2.10
	28	54.91 ± 1.83	57.71 ± 2.59	56.42 ± 0.95	57.53 ± 0.81
	60	59.82 ± 2.51	61.29 ± 1.93	59.95 ± 1.08	59.78 ± 0.89
Water activity**	7	0.972 ± 0.006	0.968 ± 0.011	0.969 ± 0.002	0.969 ± 0.004
	28	0.960 ± 0.007	0.958 ± 0.005	0.959 ± 0.006	0.959 ± 0.004
	60	0.946 ± 0.003	0.947 ± 0.007	0.949 ± 0.003	0.947 ± 0.005
Salt** (%)	7	1.27 ± 0.19	1.11 ± 0.32	1.37 ± 0.39	1.30 ± 0.27
	28	1.68 ± 0.22	1.65 ± 0.11	1.61 ± 0.12	1.78 ± 0.12
	60	1.07 ± 0.31	0.95 ± 0.01	0.95 ± 0.00	0.95 ± 0.01

¹Mean ± SD of 2 determinations on 2 cheese-making trials.²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.**Significant effect ($P < 0.01$) of ripening time.

activity of most bacterial proteinases and peptidases in cheese is close to neutrality (Garde et al., 1997). Also, proteolytic and peptidolytic enzymes from bifidobacteria could influence proteolysis in cheese (Bergamini et al., 2009). In this sense, the increase in proteolysis of Scamorza sheep milk cheese made with bifidobacteria was associated with enzymes of *B. longum* BL-46 and *B. lactis* BB12 (Albenzio et al., 2013), and a similar result was obtained in Cheddar cheese made with a mix of lactobacilli and bifidobacteria (Ong et al., 2007). Other studies have reported reduced proteolytic activities of bifidobacteria in dairy products (Roy, 2005), of *B. lactis* in a semi-hard cheese model (Bergamini et al., 2009), and of *Bifidobacterium bifidum* and *B. longum* in Canestrato Pugliese cheese (Corbo et al., 2001).

In the present study, total FAA concentration was significantly ($P < 0.01$) influenced by the *Bifidobacterium* strain and ripening time (Table 4). In control cheese, total FAA increased significantly ($P < 0.01$)

during ripening. Addition of *B. longum* INIA P678 and *B. breve* INIA P734 resulted in higher concentrations of total FAA at 28 and 60 d compared with control cheese. Similarly, contents of free alanine, glutamine, histidine, isoleucine, proline, serine, threonine, tyrosine, and valine at 60 d were higher ($P < 0.01$) in cheeses with *B. longum* INIA P678 and *B. breve* INIA P734 than in control cheese. Higher leucine and lysine concentrations were detected at 60 d in all experimental cheeses compared with control cheese, whereas higher methionine concentration at 60 d was observed only for *B. longum* INIA P678 cheese. Despite the low or undetectable counts of this strain, cheese with *B. longum* INIA P678 presented significantly higher overall proteolysis and total FAA levels than control cheese. Bifidobacteria are capable of hydrolyzing products from primary proteolysis to smaller peptides and AA (Ong et al., 2007). Similar to our results, higher levels of free glutamine, serine, arginine, isoleucine, and leucine were reported

Table 3. Overall proteolysis during ripening in control cheese and in cheeses made using bifidobacteria as adjunct cultures¹

Variable	Day	Cheese ²			
		Control	INIA P2	INIA P678	INIA P734
Overall proteolysis**	7	0.264 ± 0.017 ^a	0.277 ± 0.015 ^a	0.283 ± 0.018 ^a	0.278 ± 0.008 ^a
	28	0.665 ± 0.021 ^a	0.730 ± 0.054 ^{ab}	0.800 ± 0.055 ^b	0.741 ± 0.047 ^{ab}
	60	1.400 ± 0.053 ^a	1.459 ± 0.065 ^{ab}	1.702 ± 0.082 ^c	1.543 ± 0.057 ^b

^{a-c}Means at each ripening time with different superscripts differ significantly ($P < 0.01$, Tukey test).¹Mean ± SD of 4 determinations on 2 cheese-making trials.²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.**Significant effect ($P < 0.01$) of ripening time.

for ripe Scamorza cheese made with a mix of *B. longum* and *B. lactis* than for control cheese (Albenzio et al., 2013).

Organic Acids and Sugar Content. Significant differences ($P < 0.01$) in organic acids content during ripening (Table 5) were recorded between cheeses with

Table 4. Free AA (mg/100 g of cheese DM) during ripening in control cheese and in cheeses made using bifidobacteria as adjunct cultures¹

AA	Day	Cheese ²			
		Control	INIA P2	INIA P678	INIA P734
Ala**	7	11.7 ± 0.7	10.9 ± 2.1	12.5 ± 0.2	11 ± 1
	28	18.2 ± 1.2	14.9 ± 2.4	20.2 ± 4.2	17 ± 4.9
	60	35.6 ± 4.2	28.5 ± 3.2	46.5 ± 3.7 ^a	44.4 ± 8.8 ^a
Arg**	7	54.8 ± 11.5	48.9 ± 10.2	44.3 ± 8.8 ^a	46.7 ± 5.1 ^a
	28	73.2 ± 21.9	65.5 ± 20.4	60 ± 12.7	68.8 ± 15.8
	60	135.7 ± 35.2	134.7 ± 23.9	170.5 ± 45.7	164.5 ± 78.4
Cys**	7	234.1 ± 18.3	230.2 ± 11.5	235.4 ± 28.7	246.1 ± 12.9
	28	271.8 ± 13.1	287.1 ± 18.9	383.1 ± 56.2 ^a	341.5 ± 30.5 ^a
	60	346.5 ± 93.8	254.4 ± 23.3	390.5 ± 70.2	376 ± 98.4
Glu**	7	9.7 ± 2.1	10 ± 1.4	13.2 ± 2.6 ^a	11.1 ± 3
	28	16.9 ± 5.9	18 ± 1.8	34.7 ± 4.1 ^a	22.4 ± 3.4 ^a
	60	80.8 ± 11.3	86.6 ± 15.3	158.9 ± 13.5 ^a	108.1 ± 13.7 ^a
His**	7	13.3 ± 3	16.4 ± 2.1	15.8 ± 3.5	16 ± 2.6
	28	29.5 ± 5.4	26.7 ± 2.8	39 ± 2.4 ^a	31.5 ± 3.4
	60	75 ± 9.5	75.8 ± 9.4	114 ± 14 ^a	91.1 ± 14.5 ^a
Ile**	7	2.1 ± 0.5	2.3 ± 0.4	2.7 ± 0.2 ^a	2.1 ± 0.3
	28	12.2 ± 1.8	11.8 ± 2.1	17.3 ± 1.6 ^a	13.9 ± 0.6 ^a
	60	45.5 ± 2.9	44.8 ± 8.7	66.7 ± 7.1 ^a	54.4 ± 7.6 ^a
Leu**	7	8.3 ± 1.2	9.5 ± 1.3 ^a	11.6 ± 0.3 ^a	9.4 ± 0.8 ^a
	28	38.2 ± 0.6	44.8 ± 1.7 ^a	59.6 ± 4.1 ^a	46.8 ± 0.6 ^a
	60	145.7 ± 12	164.2 ± 6 ^a	210.8 ± 12.2 ^a	178.2 ± 7.2 ^a
Lys**	7	22.9 ± 5.8	20.1 ± 4	21.9 ± 5.2	18.1 ± 7.2
	28	56.8 ± 9.1	61.2 ± 19.6	85.8 ± 6.4 ^a	78.1 ± 2.3 ^a
	60	204.2 ± 7.5	227.8 ± 24.7 ^a	261.4 ± 29.1 ^a	231.6 ± 34.2 ^a
Met**	7	3 ± 0.4	3.1 ± 1	4.6 ± 0.9 ^a	3.6 ± 0.8
	28	15.5 ± 2.2	18.2 ± 2.4 ^a	28.3 ± 6.9 ^a	21.2 ± 3.9 ^a
	60	67.7 ± 3.4	72.2 ± 13.4	98.9 ± 12.7 ^a	71.3 ± 13.5
Phe**	7	ND ³	ND	ND	ND
	28	ND	ND	ND	ND
	60	141.9 ± 15.2	159.6 ± 15.5	206.5 ± 23.3 ^a	145.1 ± 33.5
Pro**	7	33.7 ± 3.5	34.7 ± 2	35 ± 2.1	32.9 ± 4
	28	43.6 ± 0.9	36.2 ± 10.5 ^a	37.4 ± 13.2 ^a	36.6 ± 13.8 ^a
	60	70.9 ± 10.3	62.6 ± 2.1	97.8 ± 1.9 ^a	87.7 ± 15.7 ^a
Ser**	7	8.8 ± 1.2	10.9 ± 4	12 ± 1.4 ^a	8.9 ± 1.7
	28	24.5 ± 4.8	25.5 ± 1.1	41 ± 3.3 ^a	28.4 ± 2.8
	60	94.1 ± 9	92.5 ± 15	154 ± 15.7 ^a	110.6 ± 4.4 ^a
Thr**	7	6.8 ± 1.4	7.5 ± 0.8	6.1 ± 0.7	5.9 ± 1
	28	11.3 ± 3.2	9.2 ± 1.6 ^a	11.5 ± 0.8	9.7 ± 0.7 ^a
	60	27.7 ± 8.5	26.1 ± 8.6	39.7 ± 9 ^a	35.7 ± 14.2 ^a
Tyr**	7	10.2 ± 1.1	9.9 ± 1.7	11.4 ± 0.8	10.9 ± 1.3
	28	20.9 ± 0.8	21.2 ± 0.8	26.7 ± 2.3 ^a	22.8 ± 0.7 ^a
	60	54.9 ± 4.4	58.2 ± 7.5	79 ± 7.3 ^a	61.6 ± 4.7 ^a
Val**	7	10.6 ± 1.9	11.2 ± 2	15.3 ± 0.6 ^a	12.1 ± 1.2 ^a
	28	38 ± 2.7	40 ± 2.2 ^a	63.4 ± 1.1 ^a	49.1 ± 5.1 ^a
	60	110.3 ± 9.3	110.7 ± 4.3	165.6 ± 4.8 ^a	141.5 ± 4.3 ^a
Total**	7	430 ± 40.9	425.6 ± 26.7	442 ± 30.3	434.9 ± 20.7
	28	670.5 ± 49.4	680.2 ± 29.4	908.1 ± 63.9 ^a	787.7 ± 39.4 ^a
	60	1,636.5 ± 97.6	1,598.6 ± 122.2	2,260.7 ± 165 ^a	1901.9 ± 181.4 ^a

^aMeans at each ripening time followed by a superscript are significantly different ($P < 0.01$, Dunnett test) from the control cheese.

¹Mean ± SD of duplicate determinations in 2 cheese-making experiments.

²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.

³ND = not detected.

**Significant effect ($P < 0.01$) of ripening time.

Bifidobacterium strains and control cheese. Cheeses with *B. pseudolongum* INIA P2 showed higher levels of citric, uric, and butyric acids and lower levels of pyruvic and acetic acids than control cheese at d 7 or 28 but not at d 60. Cheeses with *B. longum* INIA P678 had higher levels of acetic and butyric acids and lower levels of citric and lactic acids at d 60 with respect to control cheese. Cheeses with *B. breve* INIA P734 showed higher levels of citric and uric acids all throughout ripening and of butyric acid at d 28 compared with control cheese and lower levels of acetic acid until d 28 and of lactic acid at d 60. Contents of lactic, citric, and acetic acids were similar to values reported in Manchego cheese by Garde et al. (2012), although in our work a higher amount of butyric acid was observed and propionic acid was not detected. Higher concentrations

of acetic acid during ripening of cheeses containing bifidobacteria were also observed by Ong et al. (2007) and related to their metabolism. In our study, there was not a marked increase of the level of acetic acid in cheeses with bifidobacteria, which is important in terms of sensory properties (Madureira et al., 2013). On the other hand, the increase in short-chain fatty acids observed in cheeses with *Bifidobacterium* strains revealed their ability to produce these compounds in food and possibly in the intestine. Short-chain fatty acids produced by gut microorganisms play a pivotal role in gastrointestinal functionality, immune regulation, and host metabolism and could counteract stress-induced brain-gut axis alteration (van de Wouw et al., 2018).

Lactose and glucose were below detection level in all cheeses as early as d 7 (Table 5), which makes these

Table 5. Organic acids and sugars (mg/100 g of cheese DM) during ripening in control cheese and in cheeses made using bifidobacteria as adjunct cultures¹

Compound	Day	Cheese ²			
		Control	INIA P2	INIA P678	INIA P734
Citric acid**	7	268.68 ± 7.25	298.53 ± 8.01 ^a	266.33 ± 31.89	304.78 ± 14.74 ^a
	28	129.32 ± 17.82	193.13 ± 7.74 ^a	116.94 ± 21.69	190.66 ± 25.65 ^a
	60	111.06 ± 14.91	115.26 ± 4.18	94.95 ± 11.04 ^a	132.48 ± 13.38 ^a
Pyruvic acid**	7	43.99 ± 2.14	42.11 ± 2.26	39.73 ± 1.03 ^a	42.70 ± 6.27
	28	44.32 ± 4.41	39.37 ± 3.79 ^a	47.59 ± 6.19 ^a	46.29 ± 1.45
	60	78.16 ± 10.56	82.90 ± 5.40	83.42 ± 4.63	81.11 ± 3.13
Lactic acid**	7	4,895.21 ± 375.90	4,919.74 ± 70.27	4,685.32 ± 96.59	4,901.89 ± 228.80
	28	4,832.92 ± 323.88	4,879.52 ± 66.19	4,611.93 ± 427.21 ^a	4,882.75 ± 366.56
	60	4,577.27 ± 504.51	4,412.93 ± 131.02	3,833.25 ± 211.71 ^a	4,219.02 ± 223.39 ^a
Uric acid**	7	0.43 ± 0.36	0.66 ± 0.48 ^a	0.30 ± 0.24 ^a	0.63 ± 0.28 ^a
	28	0.07 ± 0.03	0.15 ± 0.09 ^a	0.05 ± 0.03	0.18 ± 0.12 ^a
	60	0.05 ± 0.04	0.07 ± 0.05	0.04 ± 0.03	0.12 ± 0.12 ^a
Orotic acid**	7	ND ³	ND	ND	ND
	28	ND	ND	ND	ND
	60	ND	ND	ND	ND
Acetic acid**	7	110.30 ± 5.94	104.25 ± 4.40	119.70 ± 3.36 ^a	102.08 ± 17.90 ^a
	28	137.87 ± 9.19	125.75 ± 20.29 ^a	164.73 ± 7.99 ^a	130.20 ± 22.17 ^a
	60	118.71 ± 12.87	115.72 ± 7.62	127.26 ± 14.47 ^a	123.99 ± 10.20
Propionic acid	7	ND	ND	ND	ND
	28	ND	ND	ND	ND
	60	ND	ND	ND	ND
Butyric acid**	7	27.17 ± 5.27	23.97 ± 6.38	30.83 ± 9.34	30.97 ± 13.88
	28	38.65 ± 4.94	49.72 ± 4.72 ^a	71.64 ± 7.68 ^a	56.52 ± 5.90 ^a
	60	154.61 ± 13.04	149.91 ± 24.86	197.40 ± 21.50 ^a	140.07 ± 14.61
Lactose	7	ND	ND	ND	ND
	28	ND	ND	ND	ND
	60	ND	ND	ND	ND
Glucose	7	ND	ND	ND	ND
	28	ND	ND	ND	ND
	60	ND	ND	ND	ND
Galactose**	7	1,450.23 ± 571.18	928.00 ± 77.03 ^a	1,014.96 ± 412.63 ^a	1,285.77 ± 523.26 ^a
	28	1,013.02 ± 345.06	716.84 ± 43.53 ^a	507.09 ± 196.16 ^a	699.54 ± 38.81 ^a
	60	573.10 ± 117.92	594.15 ± 77.63	484.27 ± 37.08 ^a	570.44 ± 48.41

^aMeans at each ripening time followed by a superscript in each row are significantly different ($P < 0.01$, Dunnett test) from the control cheese.

¹Mean ± SD of duplicate determinations in 2 cheese-making experiments.

²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.

³ND = not detected.

**Significant effect ($P < 0.01$) of ripening time.

cheeses of interest for people with lactose intolerance and is in agreement with their abundance of organic acids. Galactose concentration was lower ($P < 0.01$) at d 7 and 28 in all cheeses containing *Bifidobacterium* than in control cheese and at d 60 only in cheese made with *B. longum* INIA P678.

Volatile Compounds. Volatile analysis detected 32 compounds, including aldehydes, ketones, alcohols, acids, esters, alkanes, hydrocarbons, and sulfur compounds (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18203>), of which 29 compounds varied significantly ($P < 0.01$) with ripening time. Several volatile compounds considered key odorants in cheese (e.g., 2-butanone, acetic acid, hexanoic acid, 1-propanol, and 2-pentanol) were detected in this work, suggesting their importance in the distinctive flavor of sheep semi-hard cheese (Ávila et al., 2017). The same volatile compounds were detected in cheeses made with *Bifidobacterium* strains and in control cheese. There were no significant differences in the levels of volatile compounds between cheeses at d 28 and 60 except for 2-pentanol, which exhibited significantly ($P < 0.01$) lower values in cheeses made with *Bifidobacterium* strains than in control cheese.

Although a significant increase in FAA concentration was observed in cheeses with *B. longum* INIA P678 and *B. breve* INIA P734, this increase had no unfavorable effect on the levels of volatile compounds. On the other hand, cheeses with *B. longum* INIA P678 presented higher ($P < 0.01$) concentrations of pyruvic, acetic, and butyric acids than control cheese throughout ripening. The increase in pyruvic and butyric acids could be considered a potential probiotic finding of interest in the human colon. Pyruvate is the central point of butyrate synthesis and precursor for acetate formation. Butyrate is the main energy source of colonic epithelial cells and plays an important role in gastrointestinal homeosta-

sis and as a preventive agent of intestinal pathologies (Muñoz-Tamayo et al., 2011).

Sensory Evaluation

Color Characteristics. Values of L^* , a^* , and b^* color parameters after 7, 28, and 60 d of ripening were not affected by the addition of any of the *Bifidobacterium* strains (Supplemental Table S2, <https://doi.org/10.3168/jds.2020-18203>). The L^* and a^* values of cheeses decreased ($P < 0.01$) during ripening because of the increase in DM content, as described previously (Ávila et al., 2008). All cheeses showed negative a^* values in all cases, in the greenish direction, whereas b^* values were positive, in the yellowish direction, and increased with ripening time due to chemical reactions (Mortensen et al., 2004).

Instrumental Texture. Fracturability, hardness, and elasticity of cheeses (Table 6) increased significantly ($P < 0.01$) during ripening according to ANOVA results, probably because of the strengthening effect of moisture loss during ripening, as reported previously (Picon et al., 2010). The 3 rheological characteristics were also influenced by the addition of *Bifidobacterium* strains ($P < 0.01$) according to ANOVA results. Only INIA P734 cheese showed significant differences ($P < 0.01$) with respect to control cheese for the 3 texture parameters after 7 or 28 d of ripening.

Sensory Evaluation. Trained panelists noticed a significant ($P < 0.01$) effect of ripening time with increases in odor intensity, aroma intensity, and taste intensity as ripening progressed according to the ANOVA, which was accompanied by a significant ($P < 0.01$) effect on aroma quality, taste quality, and texture quality, the values of which also increased over ripening (Table 7). There were also significant ($P < 0.01$) effects of ripening time on acidic taste, which decreased, and

Table 6. Texture parameters during ripening in control cheese and in cheeses made using bifidobacteria as adjunct cultures¹

Parameter	Day	Cheese ²			
		Control	INIA P2	INIA P678	INIA P734
Fracturability** (N)	7	29.170 ± 8.380	26.318 ± 1.364	27.626 ± 3.931	22.738 ± 3.219
	28	32.454 ± 7.869	31.881 ± 7.310	33.223 ± 6.414	26.188 ± 6.522
	60	36.842 ± 6.130	35.789 ± 6.216	35.268 ± 5.484	31.877 ± 6.522
Hardness** (J)	7	0.198 ± 0.057	0.181 ± 0.034	0.161 ± 0.024	0.152 ± 0.027
	28	0.283 ± 0.073	0.280 ± 0.063	0.256 ± 0.043	0.211 ± 0.048
	60	0.328 ± 0.058	0.324 ± 0.057	0.296 ± 0.043	0.286 ± 0.062
Elasticity** (N/mm ²)	7	0.410 ± 0.137	0.376 ± 0.110	0.315 ± 0.079	0.289 ± 0.057
	28	0.719 ± 0.329	0.742 ± 0.339	0.558 ± 0.190	0.522 ± 0.206
	60	1.028 ± 0.290	1.064 ± 0.301	0.817 ± 0.288	0.889 ± 0.308

¹Mean ± SD of 6 determinations on 2 cheese-making trials.

²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.

**Significant effect ($P < 0.01$) of ripening time.

Table 7. Sensory evaluation during ripening in control cheese and in cheeses made using bifidobacteria as adjunct cultures¹

Sensory characteristic	Day	Cheese ²			
		Control	INIA P2	INIA678	INIA P734
Visual appearance ³	7	5.73 ± 1.12 ^a	5.64 ± 1.17 ^a	5.78 ± 1.21 ^a	5.44 ± 1.19 ^a
	28	5.70 ± 0.93 ^a	5.64 ± 1.05 ^a	5.74 ± 0.89 ^a	5.52 ± 1.04 ^a
	60	5.65 ± 1.26 ^{ab}	5.34 ± 1.45 ^a	5.86 ± 1.05 ^b	5.62 ± 1.22 ^{ab}
Odor intensity ^{4**}	7	4.44 ± 1.54 ^a	4.50 ± 1.41 ^a	4.13 ± 1.59 ^a	4.45 ± 1.44 ^a
	28	4.94 ± 1.31 ^a	5.17 ± 1.15 ^a	5.03 ± 1.17 ^a	5.24 ± 1.30 ^a
	60	5.55 ± 0.95 ^a	5.69 ± 0.89 ^a	5.50 ± 0.92 ^a	5.67 ± 0.95 ^a
Odor quality ³	7	5.77 ± 1.17 ^b	5.87 ± 0.91 ^b	5.33 ± 1.10 ^a	5.88 ± 1.01 ^b
	28	5.63 ± 0.70 ^a	5.76 ± 0.71 ^a	5.58 ± 0.71 ^a	5.65 ± 0.92 ^a
	60	5.82 ± 0.80 ^a	5.80 ± 0.85 ^a	5.68 ± 0.85 ^a	5.88 ± 0.80 ^a
Aroma intensity ^{4**}	7	4.05 ± 1.60 ^a	3.98 ± 1.71 ^a	3.87 ± 1.89 ^a	4.02 ± 1.58 ^a
	28	5.04 ± 1.31 ^a	5.41 ± 1.19 ^a	5.28 ± 1.16 ^a	5.35 ± 1.09 ^a
	60	5.91 ± 0.78 ^a	6.10 ± 0.96 ^a	5.80 ± 1.17 ^a	5.89 ± 0.92 ^a
Aroma quality ^{3**}	7	5.01 ± 0.92 ^b	5.06 ± 0.96 ^b	4.31 ± 1.32 ^a	5.20 ± 0.90 ^b
	28	5.44 ± 0.79 ^a	5.59 ± 0.74 ^a	5.19 ± 1.01 ^a	5.49 ± 0.85 ^a
	60	5.75 ± 0.77 ^b	5.88 ± 0.92 ^b	5.26 ± 1.04 ^a	5.77 ± 0.94 ^b
Texture quality ³	7	5.18 ± 1.42 ^a	5.13 ± 1.26 ^a	4.88 ± 1.41 ^a	5.19 ± 1.19 ^a
	28	5.54 ± 0.86 ^a	5.57 ± 0.99 ^a	5.58 ± 0.80 ^a	5.52 ± 0.75 ^a
	60	5.91 ± 1.03 ^a	5.84 ± 1.10 ^a	5.84 ± 0.99 ^a	5.88 ± 0.83 ^a
Taste intensity ^{4**}	7	4.74 ± 1.54 ^a	4.53 ± 1.38 ^a	4.61 ± 1.80 ^a	4.66 ± 1.50 ^a
	28	5.66 ± 1.21 ^a	5.75 ± 1.09 ^a	5.65 ± 1.18 ^a	5.73 ± 1.14 ^a
	60	6.39 ± 0.90 ^a	6.56 ± 0.92 ^a	6.25 ± 1.18 ^a	6.38 ± 0.92 ^a
Taste quality ^{3**}	7	5.06 ± 1.35 ^b	5.18 ± 1.05 ^b	4.30 ± 1.40 ^a	5.29 ± 0.80 ^b
	28	5.50 ± 0.91 ^b	5.67 ± 0.96 ^b	5.06 ± 1.14 ^a	5.58 ± 0.96 ^b
	60	5.77 ± 0.82 ^b	5.80 ± 0.92 ^b	5.09 ± 1.31 ^a	5.82 ± 0.91 ^b
Acidic taste ^{4**}	7	3.18 ± 2.19 ^a	2.87 ± 2.09 ^a	2.95 ± 2.18 ^a	3.04 ± 2.12 ^a
	28	2.19 ± 2.20 ^a	2.20 ± 2.15 ^a	1.98 ± 2.11 ^a	2.08 ± 2.19 ^a
	60	2.04 ± 2.19 ^a	2.12 ± 2.22 ^a	1.84 ± 2.25 ^a	2.03 ± 2.21 ^a
Bitter taste ^{4**}	07	0.77 ± 1.31 ^a	0.82 ± 1.29 ^a	0.88 ± 1.45 ^a	0.62 ± 1.15 ^a
	28	0.95 ± 1.38 ^a	1.15 ± 1.47 ^a	1.17 ± 1.61 ^a	1.16 ± 1.58 ^a
	60	1.45 ± 1.76 ^a	1.57 ± 1.92 ^a	1.70 ± 2.05 ^a	1.55 ± 1.84 ^a
Salty taste ^{4**}	7	5.63 ± 0.89 ^a	5.31 ± 0.72 ^a	5.34 ± 0.95 ^a	5.44 ± 0.76 ^a
	28	5.25 ± 0.44 ^{ab}	5.35 ± 0.49 ^b	5.14 ± 0.42 ^a	5.27 ± 0.50 ^{ab}
	60	5.24 ± 0.60 ^a	5.30 ± 0.52 ^a	5.08 ± 0.56 ^a	5.23 ± 0.50 ^a
Sweet taste ^{4**}	7	0.75 ± 1.50 ^a	0.68 ± 1.43 ^a	0.72 ± 1.49 ^a	0.67 ± 1.40 ^a
	28	0.57 ± 1.21 ^a	0.51 ± 1.10 ^a	0.57 ± 1.15 ^a	0.55 ± 1.15 ^a
	60	0.62 ± 1.26 ^a	0.57 ± 1.20 ^a	0.76 ± 1.60 ^a	0.61 ± 1.22 ^a

^{a,b}Means at each ripening time with different superscripts differ significantly ($P < 0.01$, Tukey test).

¹Mean ± SD from 16 trained panelists of duplicate determinations on 2 cheese-making trials.

²Initial inoculum of bifidobacteria in milk was 6.31 ± 0.17 log cfu/mL for INIA P2, 5.01 ± 0.17 log cfu/mL for INIA P678, and 5.77 ± 0.06 log cfu/mL for INIA P734.

³Scale from 0 ("I dislike it") to 10 ("I like it").

⁴Scale from 0 (very weak) to 10 (very strong).

**Significant effect ($P < 0.01$) of ripening time.

on bitter taste, which increased. No significant effect of ripening time was found for visual appearance or for the other taste descriptors. Our results agree with those obtained by Bergamini et al. (2009) in semi-hard cheeses made with mixed cultures, which included *B. lactis*.

In the present study, *B. longum* INIA P678 counts markedly declined during ripening, and the cheeses made with this strain received significantly ($P < 0.01$) lower aroma quality and taste quality scores than the rest at almost all sampling times, which may hinder its use as an adjunct probiotic culture. *Bifidobacterium breve* INIA P734 exhibited a high survival rate until d 28, and the sensory characteristics of cheeses made with

this strain did not differ from those of control cheese. Therefore, it may be considered of technological interest as an adjunct probiotic culture for cheese. *Bifidobacterium pseudolongum* INIA P2, which showed the highest survival rates during cheese ripening, and even after simulated gastrointestinal conditions, did not affect the sensory characteristics of cheese at any of the sampling times. Therefore, it offers considerable interest for use as an adjunct probiotic culture for cheese.

CONCLUSIONS

Marked differences in the survival of the 3 *Bifidobacterium* strains during cheese ripening and after further

simulated gastrointestinal digestion were observed in the present study, the highest survival rates being those of *B. pseudolongum* INIA P2. There were also differences between the 3 *Bifidobacterium* strains regarding the chemical changes in cheeses leading to the formation of FAA, organic acids, and volatile compounds. Those differences influenced the sensory characteristics of cheeses with *Bifidobacterium* with respect to control cheese. Taking into account the highest survival rates and the sensory characteristics of cheeses, *B. pseudolongum* INIA P2 was considered the most adequate strain for use as an adjunct probiotic culture in the manufacture of fresh and semi-hard sheep milk cheeses. Further work is needed to evaluate the probiotic activity of sheep milk cheeses made using these *Bifidobacterium* strains as adjunct cultures in assays carried out with animals and humans.

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