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Microbial cell-free extracts as sources of enzyme activities to be used for enhancement flavor development of ewe milk cheese

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ABSTRACT

Freeze-dried cell-free extracts (CFE) from *Lactobacillus casei* LC01, *Weissella cibaria* 1XF5, *Hafnia alvei* Moller ATCC 51815, and *Debaryomyces hansenii* LCF-558 were used as sources of enzyme activities for conditioning the ripening of ewe milk cheese. Compared with control cheese (CC), CFE did not affect the gross composition and the growth of the main microbial groups of the cheeses. As shown through urea-PAGE electrophoresis of the pH 4.6-soluble nitrogen fraction and the analysis of free AA, the secondary proteolysis of the cheeses with CFE added was markedly differed from that of the CC. Compared with CC, several enzyme activities were higher in the water-soluble extracts from cheeses made with CFE. In agreement, the levels of 49 volatile compounds significantly differentiated CC from the cheeses made with CFE. The level of some alcohols, ketones, sulfur compounds, and furans were the lowest in the CC, whereas most aldehydes were the highest. Each CFE seemed to affect a specific class of chemical compounds (e.g., the CFE from *H. alvei* ATCC 51815 mainly influenced the synthesis of sulfur compounds). Apart from the microbial source used, the cheeses with the addition of CFE showed higher score for acceptability than the control cheese. Cheese ripening was accelerated or conditioned using CFE as sources of tailored enzyme activities.
Key words: microbial cell-free extract, ripening, ewe milk cheese

INTRODUCTION

The conditioning or even the acceleration of cheese ripening is one of the main current challenges for the dairy industry. Cheese ripening is a slow and, consequently, expensive process that is not fully predictable or controllable. Acceleration of cheese ripening is proposed as the way to produce a fast ripening of curd for processed cheese or to reduce costs that are associated with cheese manufacture. Economic and technology incentives are, therefore, focused to condition and accelerate cheese ripening (Fox et al., 1996).

Over the last 2 decades, several rediscovered or modern strategies were proposed to condition or accelerate cheese ripening. These included the use of exogenous recombinant and encapsulated enzymes, genetically engineered starters, cheese slurries, adjunct and attenuated adjunct cultures, high temperature and pressure (Law et al., 1979), and concentrated source of substrates as enzyme-modified cheese powder (Law et al., 1979; Wilkinson, 1999; Azarnia et al., 2006). Despite this diversity of proposals, the most effective strategy has not yet been defined, as each is associated with some drawbacks. For instance, commercial enzymes are poorly available and expensive, only a few enzymes are selected, and, in general, the preparations are difficult to be homogeneously distributed into the cheese matrix (Azarnia et al., 2006). Considering that cheese ripening is an outcome of several microbiological and biochemical processes, it is unlikely that all processes could be conditioned and accelerated equally, thus avoiding unbalanced and off flavor (Fox et al., 1996). In fact, ripened cheese flavor mainly comes from the balanced activities of a pool of enzymes from milk, coagulants, starters, and nonstarter biota (Forde and Fitzgerald, 2000). The addition of free or encapsulated exogenous enzymes is an accepted method to accelerate Cheddar cheese ripening and it was the most studied strategy (Azarnia et al., 2006). An appropriate and complementary portfolio of enzymes is necessary to ensure a balanced flavor to the cheese, which may result in a very expensive process.

The contribution of the nonstarter biota to cheese ripening cannot be neglected. Recently, deep sequencing approaches have focused on the microbial population of raw milk, highlighting the presence of subdominant mi-

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croorganisms, which include gram-positive and gram-negative bacteria as well as yeasts and molds (Quigley et al., 2013). The exploitation of these subdominant microorganisms as potential sources of suitable enzyme activities was recently hypothesized (Quigley et al., 2013).

Although usually considered as indicators of poor hygiene, some gram-negative bacteria such as *Hafnia alvei*, *Psychrobacter celer*, and *Proteus vulgaris* may play a role in dairy fermentations, thus positively contributing to the synthesis of flavor compounds during cheese ripening (Delbès-Paus et al., 2012). Among gram-positive bacteria, nonstarter mesophilic lactobacilli are undoubtedly those with the highest and proven technological significance (Lynch et al., 1997). Nevertheless, the role on cheese ripening of some nonconventional genera, such as *Weissella*, has not yet been investigated. Molds and yeasts may also positively contribute to cheese ripening because of the release of enzymes that contribute to texture and aroma (Quigley et al., 2013). Based on these premises to accelerate cheese ripening, it could be worthwhile to investigate the potential of cell-free extracts from selected cheese-related and nonrelated microorganisms.

The current study aimed at using freeze-dried cell-free extracts from selected cheese-related and nonrelated microorganisms as sources of diverse enzymes to condition or accelerate the ripening of the Pecorino-type cheese. During manufacture and ripening cheeses were compared based on compositional, microbiological, and biochemical analyses, as well as volatile and sensory profiles.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

Lactobacillus casei LC01 and *Weissella cibaria* 1XF5, belonging to the Culture Collection of Department of Soil, Plant and Food Sciences (University of Bari, Italy), *Hafnia alvei* Moller ATCC 51815 (biosafety level 1) and *Debaryomyces hansenii* LCF-558, from the personal Culture Collection of G. Cardinali (Department of Pharmaceutical Sciences, University of Perugia, Italy), were all used as sources of cell-free extracts (**CFE**) for cheesemaking. *Lactobacillus casei* LC01, previously isolated from cheese and used as attenuated adjunct culture (Di Cagno et al., 2011), and *W. cibaria* 1XF5 were selected based on various proteolytic activities. *Hafnia alvei* and *D. hansenii* were chosen for the potential ability to synthesize flavor compounds and proteolytic activity, respectively (Morales et al., 2004). Recently, *D. hansenii* was proposed as a starter culture for making blue cheese (Gkatzionis et al., 2014). *Lactobacillus casei* LC01 and *W. cibaria* 1XF5 were propagated for 24 h at 30°C on MRS broth (Oxoid, Basingstoke, UK). For *W. cibaria* 1XF5 propagation, the MRS broth was supplemented with the addition of fresh yeast extract (5% vol/vol) and 28 m*M* maltose at the final pH of 5.6. *Hafnia alvei* ATCC 51815 was grown for 24 h at 30°C in nutrient broth (Oxoid). *Debaryomyces hansenii* LCF-558 was propagated for 48 h at 25°C in malt-yeast-peptone-glucose broth, pH 6.5, containing yeast and malt extracts (0.3% wt/vol), bacteriological peptone (0.5% wt/vol), and glucose (1%, wt/vol).

Preparation of CFE

After overnight cultivation (cell count of ~9.0 ± 0.2 log cfu/mL), microorganisms were harvested by centrifugation (10,000 × *g*, 10 min, 4°C), washed twice with sterile 50 m*M* potassium phosphate buffer, pH 7.0, resuspended in the same buffer at a cell density of ~11.0 ± 0.1 log cfu/mL, and subjected to sonication in an ice bath. Sonication was carried out using a Vibra-Cell sonicator (Sonic and Materials Inc., Danbury, CT), equipped with a microtip setting (sonic power 375 W; output control 5) for 45 min (3 cycles, 15 min/cycle, 15-min interval between cycles). Efficiency of the sonication treatment was estimated by plate count and Bradford assay (Bradford, 1976). After treatment, CFE were recovered by centrifugation (15,000 × *g*, 15 min, 4°C) and freeze-dried. Freeze-dried CFE were resuspended into sterile skim milk for enzyme assays or used to inoculate cheese milk.

Enzyme Activities

Enzyme activities were determined in the centrifuged (10,000 × *g*, 10 min, 4°C) sterile skim milk or water-soluble extracts of the cheeses prepared according to a modified method of Kuchroo and Fox (1982). The modifications included the dialysis of the extracts to eliminate interference from salt and peptides, and sterile filtration to avoid interference due to cellular activity (Gobbetti et al., 2002). Aminopeptidase (EC 3.4.11.11) type N activity on Leu-*p*-nitroanilide, proline iminopeptidase (EC 3.4.11.9) activity on Pro-*p*-nitroanilide, and endopeptidase type O (EC 3.4.23) activity on Z-Gly-Pro-NH-trifluoromethyl-coumarin were determined as described by Gobbetti et al. (1999). An arbitrary unit of enzymatic activity was defined as the amount of enzyme that caused an increase in absorbance at 410 nm per minute of 1 (aminopeptidase type N) and 0.1 (proline iminopeptidase and endopeptidase type O) at 37°C and pH 7.0. Glutamate dehydrogenase (EC 1.4.1.2)

activity was determined on glutamate by measuring the glutamate-dependent reduction of NADP or NAD at 340 nm, as described by De Angelis et al. (2010). An arbitrary unit of enzymatic activity was defined as the amount of enzyme that gave an increase of absorbance of 0.1 per minute at 37°C and pH 7.0. Cystathionine lyase (EC 4.4.1.1) activity was determined by measuring the amount of ketoacids, ammonia, and free thiols released from cystathionine, as described by De Angelis et al. (2002). An arbitrary unit of enzymatic activity was defined as the amount of enzyme that caused an increase of absorbance (412 nm) of 1 per minute at 37°C and pH 7.0. The esterase activity of CFE was determined on α-naphthyl butyrate as substrate (Medina et al., 2004). A unit of esterase activity was defined as the amount of enzyme that cause the release of 1 μmol of α-naphthol per minute at 37°C and pH 7.0 (560 nm).

Manufacture of Cheese

The ewe milk used for the manufacture of cheeses had the following characteristics: protein 4.3%, fat 5.6%, salt 0.08%, and pH 6.62. Same batches of ewe milk were used for making the 5 variants of cheese as follows: control cheese, without addition of CFE (**CC**); and cheeses with the additions of CFE from *L. casei* LC01, (**LC**), *W. cibaria* 1XF5, (**WC**), *H. alvei* ATCC 51815, (**HA**), or *D. hansenii* LCF-558 (**DH**). Cheesemaking was carried out at an industrial plant (Molino a Vento, Biccari, Foggia, Italy) on 3 consecutive days (total of 3 batches for each variant of cheese) using ewe milk from 3 daily milkings. After heating (62°C for 15 s), ewe milk was cooled at 37°C and inoculated with commercial primary starters *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (initial cell density ~7.0 ± 0.2 log cfu/mL; Mediterranea Biotecnologie srl, Termoli, Campobasso, Italy). The inoculated milk was held at 37°C for 45 min, liquid calf rennet type P (25 mL/100 L; Caglio Bellucci Srl, Modena, Italy) was added, and coagulation took place within 30 min. After cutting (size of ~0.5–1.0 cm), the curd-whey mixture was held at 40°C for ~10 min. After whey drainage, CFE were added individually (0.3% wt/wt) and manually mixed with the curds (Law and Kirby, 1987). After mixing, curds were molded, stored for ~24 h at room temperature, and dry salted once. Ripening was done at ~11°C with a relative humidity of 70% for 60 d. The weight of the cheeses was approximately 1 kg. After CFE addition curds were collected and cheese was collected after 1 (post-dry salting), 15, 30, 45, and 60 d of ripening from each batch; 2 or 3 subsamples were then analyzed. All samples were transported to the laboratory under refrigerated conditions (~4°C) and analyzed immediately or frozen (−80°C).

Compositional, Microbiological, and Biochemical Analyses

Samples of curd and cheese were analyzed for protein (macro-Kjeldahl; IDF, 1964), fat (Gerber method; IIRS, 1955), moisture (oven drying at 102°C; IDF, 1982), and salt (Fox, 1963). The pH was measured by Foodtrode electrode (Hamilton, Bonaduz, Switzerland). Microbiological analyses were carried out as described previously (De Pasquale et al., 2014). Twenty grams of sample were homogenized with 180 mL of sterile sodium citrate (2% wt/vol) solution. Presumptive mesophilic lactobacilli and cocci were enumerated on MRS and M17 agar (Oxoid), respectively, under anaerobiosis at 30°C for 48 h. Presumptive thermophilic lactobacilli and cocci were enumerated on MRS and M17 agar (Oxoid), respectively, under anaerobiosis at 42°C for 48 h. Enterococci were counted on Slanetz-Bartley agar (Oxoid) at 37°C for 48 h. The number of yeast cells was estimated at 30°C for 48 h using Sabouraud dextrose agar medium (Oxoid) supplemented with chloramphenicol (0.1 g/L). The number of molds was estimated on Wort Agar (Oxoid) at 25°C for 5 d. Total coliforms were counted using Violet Red Bile Lactose (Oxoid) at 37°C for 24 h. Except for enterococci, the media for plating of bacteria were supplemented with cycloheximide at 0.17 g/L. The pH 4.6-insoluble and pH 4.6-soluble nitrogen fractions of the samples were analyzed by urea-PAGE and reverse phase (**RP**) HPLC, as described by Andrews (1983) and Gobbetti et al. (2002), respectively. Total and individual free amino acids (**FAA**) from the water-soluble extracts were determined by a Biochrom series 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK), as described by Di Cagno et al. (2011).

Determinations of Volatile Components and Volatile Free FA

Cheeses at 60 d of ripening were analyzed in duplicate for volatile components by using 2 methods of extraction, purge and trap (**PT**) and solid-phase microextraction (**SPME**). Both methods were coupled with GC-MS. Before PT analysis, 10 mL of a suspension consisting of 1 g of cheese homogenized in 9 mL of UHQ-desodorized water by using an Ultra-Turrax homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany), were poured into a glass extractor connected to the PT apparatus (Tekmar 3000, Agilent Instruments, New York, NY). Extraction was carried out at 45°C for 45 min with helium at a flow rate of 40 mL/min on a Tenax trap (Agilent Technologies, Les Ulis, France) at 37°C. Trap desorption was at 225°C and injection into the chromatograph was performed with

a cryo-cooldown. For SPME extraction, 2 g of grated cheese was placed into a 10-mL sealed flask and the flask was placed into a bath at 40°C for 30 min. An SPME polydimethylsiloxane fiber (Supelco, Sigma Chemical Co., L'Isle d'Abeau, France) was introduced into the flask and held in the headspace for 30 min. Then, it was removed and desorbed for 5 min in a splitless chromatograph injector at 250°C. The chromatograph (Agilent Instruments) was equipped with a DB5-like capillary column (RTX5 Restek, Agilent Instruments), 60 m long, 0.32 μm i.d., and 1 μm thick. The helium flow rate was 2 mL/min and the oven temperature was 40°C during the first 6 min and then increased at 3°C/min to 230°C. The mass detector (MSD5973, Agilent Instruments) was used in scan mode, from 29 to 206 atomic mass volts at 70 eV. Quantification of compounds was expressed in log arbitrary units of area. Extraction and GC analysis of volatile free FA was also carried out as described by Di Cagno et al. (2011).

Sensory Analysis

Sensory analysis of cheeses after 60 d of ripening was carried out by 10 panelists (5 male and 5 female, mean age: 35 yr old, range: 21–40 yr). Cheeses were scored (Madkor et al., 2000) from 0 to 10 for attributes, which included aroma intensity, flavor (0 = none, 10 = aged), aftertaste (the persistence of positive taste sensations perceived after ingestion; 0 = none, 10 = intense), friability (the capacity of a sample to break up into numerous pieces from the beginning of mastication; 0 = no breaking, 10 = intense breaking), solubility (a sensation which emerges when sample melts extremely fast in the saliva; 0 = no melting, 10 = intense melting; Bàrcenas et al., 2007), and overall acceptability (0 = dislike very much, 10 = like very much). The sensory attributes were discussed with the assessors during the introductory sensory training sessions; paper ballots were used. Samples were served in random order and evaluated in 2 replicates by all panelists. Cheeses were cut into aliquots of 20 g, presented to the panel at 18 to 20°C together with unsalted crackers and water to remove any aftertaste between samples, and coded with a randomly selected 2-letter code.

Statistical Analyses

A randomized complete-block split-plot design with 3 replicates for each variants of cheese was used for the analyses. Except for volatile compounds and volatile free FA (**VFFA**), for which the analyses were carried out in duplicate, all the other analyses were carried out thrice. Data were subjected to one-way ANOVA (version 9.1; SAS Institute Inc., Cary, NC); pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$ using the statistical software Statistica for Windows (Statistica 6.0; StatSoft Inc., Tulsa, OK). Biochemical data (number of peaks of the pH 4.6-soluble nitrogen fractions, FAA, enzyme activities), VFFA, volatile components that mainly ($P < 0.05$) differentiated cheeses after 60 d of ripening, and overall acceptability score were used as variables for principal component analysis. All data were standardized before principal component analysis using the statistical software (Statistica 6.0).

RESULTS AND DISCUSSION

In Vitro Enzyme Activities

Several enzyme activities related to secondary proteolysis and catabolism of FAA (Gobbetti et al., 1999), which mainly contribute to cheese flavor, were assayed in vitro using freeze-dried CFE that were singly resuspended into sterile skim milk (Table 1). No viable cells were found in the freeze-dried CFE (data not shown). Except for aminopeptidase type N and cystathionine lyase, which were the highest for *L. casei* LC01 (Di Cagno et al., 2011), all the other enzyme activities were found at the highest levels in the CFE from *D. hansenii* LCF-585. Overall, these preliminary findings confirmed that freeze-dried CFE were suitable sources of enzyme activities (Nongonierma et al., 2013).

Cheese Manufacturing and Compositional and Microbiological Analyses

Addition of CFE was carried out directly into the curd, after cutting and whey drainage. This technology option was chosen to minimize the loss of enzyme activities in the whey. Preliminarily, CFE from *L. casei* LC01 was added either to the milk or directly to the curd. The aminopeptidase type N activity was 4.78 ± 0.18 and 11.15 ± 0.43 U/kg of curd in the curd coming from milk added with CFE and in that directly added with CFE, respectively. A considerable loss of enzyme activity (21.18 ± 0.88 U/kg of curd) was found in the whey when CFE was added to the milk. A similar trend was found for all the other enzyme activities and for the other microbial CFE (data not shown). Without addition of CFE (CC), the aminopeptidase type N activity when CFE was added to milk was no significantly ($P < 0.05$) higher than in the control cheese (4.50 ± 0.16 U/kg of curd), indicating no retention in the curd when CFE was added to the milk. Very relevant losses of enzyme activities during whey drainage were also found by other authors (Law and Kirby, 1987) when CFE were added to the milk before coagulation.

Table 1. Enzyme activities[1] of freeze-dried cell-free extracts (CFE) from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) suspended into sterile skim milk

Enzyme	LC	WC	DH	HA
Aminopeptidase type N	162.3 ± 4.31[a]	56.0 ± 1.18[b]	52.0 ± 0.92[b]	63.0 ± 0.71[b]
Proline iminopeptidase	2.64 ± 0.04[c]	2.55 ± 0.04[c]	8.88 ± 0.16[a]	7.16 ± 0.13[b]
Endopeptidase type O	0.93 ± 0.02[c]	1.56 ± 0.03[b]	1.84 ± 0.08[a]	0.97 ± 0.02[c]
Glutamate dehydrogenase	1.2 ± 0.02[b]	0.9 ± 0.01[c]	2.4 ± 0.03[a]	2.4 ± 0.04[a]
Cystathionine lyase	18.2 ± 0.23[a]	9.2 ± 0.12[b]	2.2 ± 0.05[d]	6.1 ± 0.11[c]
Esterase	4.0 ± 0.07[b]	1.5 ± 0.03[d]	5.7 ± 0.14[a]	3.1 ± 0.08[c]

[a–d]Data in the same row with different letters are significantly different ($P < 0.05$).

[1]Arbitrary units; values represent the average of 3 triplicates (±SD).

Encapsulation of enzyme preparations in liposomes or in fat globules, or their direct surface distribution in the curd before salting were considered during the manufacture of Cheddar cheese to limit losses of enzyme activities (Walstra et al., 1999). As expected, the use of CFE did not affect the pH or the gross composition of the cheeses compared with CC (Table 2). This is one of the main prerequisites to successfully use CFE. As recently shown (Nongonierma et al., 2013; Yarlagadda et al., 2014), the gross composition of Cheddar cheese did not vary when CFE from lactic acid bacteria were entrapped in yeasts or encapsulated in liposomes to enhance flavor development. The pH values of all ewe milk cheeses markedly decreased from the curd during

Table 2. Main chemical composition[1] during manufacture and ripening of control cheese (CC) without addition of freeze-dried cell-free extract (CFE) and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA)

Item	Day of ripening	pH	Moisture content (%)	Fat content (%)	Protein content (%)	NaCl content (%)
CC	Curd[2]	6.77 ± 0.3[a]	70.3 ± 2.1[a]	11.9 ± 0.7[e]	13.1 ± 0.3[d]	0.3 ± 0.0[f]
	1[3]	5.22 ± 0.2[bc]	57.0 ± 1.8[b]	14.8 ± 1.2[d]	18.3 ± 1.1[c]	1.8 ± 0.1[e]
	15	5.15 ± 0.2[bc]	49.2 ± 1.7[c]	18.3 ± 0.7[c]	23.6 ± 1.2[ab]	3.2 ± 0.1[d]
	30	5.07 ± 0.1[c]	42.9 ± 1.4[d]	20.3 ± 1.3[b]	24.9 ± 0.9[a]	3.5 ± 0.2[c]
	45	5.2 ± 0.2[bc]	40.2 ± 1.2[e]	22.9 ± 1.1[ab]	25.6 ± 1.1[a]	4.1 ± 0.2[b]
	60	5.45 ± 0.2[b]	37.2 ± 0.9[f]	24.2 ± 1.2[a]	26.3 ± 0.8[a]	4.9 ± 0.1[a]
LC	Curd	6.75 ± 0.3[a]	69.8 ± 2.3[a]	11.5 ± 0.6[e]	12.9 ± 0.3[d]	0.3 ± 0.0[f]
	1	5.27 ± 0.2[bc]	57.3 ± 1.7[b]	15.2 ± 1.0[d]	17.9 ± 1.1[c]	1.9 ± 0.0[e]
	15	5.12 ± 0.1[c]	48.8 ± 1.5[c]	18.6 ± 1.0[c]	23.4 ± 1.1[ab]	3.3 ± 0.1[d]
	30	5.07 ± 0.1[c]	43.1 ± 1.6[d]	20.7 ± 1.2[b]	24.5 ± 1.2[a]	3.5 ± 0.2[c]
	45	5.20 ± 0.2[bc]	39.6 ± 1.3[e]	23.1 ± 1.1[ab]	25.9 ± 1.1[a]	4.0 ± 0.2[b]
	60	5.52 ± 0.2[b]	36.8 ± 0.8[f]	24.6 ± 0.9[a]	26.8 ± 1.0[a]	5.0 ± 0.1[a]
WC	Curd	6.78 ± 0.3[a]	70.5 ± 2.4[a]	11.6 ± 0.5[e]	12.8 ± 0.6[d]	0.3 ± 0.0[f]
	1	5.23 ± 0.2[bc]	56.9 ± 2.1[b]	15.3 ± 1.2[d]	18.6 ± 0.9[c]	1.8 ± 0.1[e]
	15	5.18 ± 0.2[bc]	48.7 ± 1.5[c]	18.9 ± 0.9[c]	23.1 ± 1.1[ab]	3.3 ± 0.0[d]
	30	5.09 ± 0.1[c]	42.8 ± 1.4[d]	20.2 ± 1.5[b]	24.2 ± 1.2[a]	3.7 ± 0.1[c]
	45	5.15 ± 0.1[bc]	40.3 ± 0.7[e]	22.7 ± 1.4[ab]	25.2 ± 1.3[a]	4.1 ± 0.1[b]
	60	5.38 ± 0.2[b]	37.9 ± 0.9[f]	24.2 ± 1.1[a]	26.1 ± 1.2[a]	4.9 ± 0.1[a]
DH	Curd	6.75 ± 0.3[a]	69.4 ± 2.6[a]	11.6 ± 0.7[e]	13.2 ± 0.6[d]	0.3 ± 0.0[f]
	1	5.23 ± 0.2[bc]	57.4 ± 1.3[b]	14.9 ± 0.8[d]	17.7 ± 1.0[c]	1.9 ± 0.1[e]
	15	5.20 ± 0.1[bc]	48.3 ± 1.7[c]	19.1 ± 0.7[c]	23.6 ± 1.1[ab]	3.2 ± 0.1[d]
	30	5.08 ± 0.2[c]	42.2 ± 1.2[d]	21.0 ± 1.1[b]	24.3 ± 1.2[ab]	3.6 ± 0.1[c]
	45	5.24 ± 0.2[bc]	39.7 ± 1.3[e]	23.2 ± 1.4[ab]	25.1 ± 0.9[a]	4.0 ± 0.1[b]
	60	5.45 ± 0.2[b]	36.5 ± 0.9[f]	25.0 ± 1.3[a]	26.2 ± 1.04[a]	4.9 ± 0.1[a]
HA	Curd	6.73 ± 0.3[a]	69.5 ± 2.1[a]	11.3 ± 0.6[e]	12.8 ± 0.8[d]	0.3 ± 0.0[f]
	1	5.29 ± 0.2[bc]	57.6 ± 2.2[b]	15.5 ± 0.8[d]	16.9 ± 1.0[c]	1.9 ± 0.1[e]
	15	5.15 ± 0.1[bc]	48.5 ± 1.7[c]	19.4 ± 0.7[c]	23.5 ± 1.1[ab]	3.3 ± 0.1[d]
	30	5.05 ± 0.1[c]	42.9 ± 1.5[d]	21.1 ± 0.8[b]	24.9 ± 1.2[a]	3.7 ± 0.1[c]
	45	5.25 ± 0.2[bc]	40.5 ± 0.6[e]	22.9 ± 1.1[ab]	25.6 ± 0.9[a]	4.1 ± 0.1[b]
	60	5.43 ± 0.2[b]	37.7 ± 0.7[f]	24.5 ± 1.4[a]	26.6 ± 1.0[a]	5.1 ± 0.2[a]

[a–f]Data in the same column with different letters are significantly different ($P < 0.05$).

[1]Mean values ± SD for 3 batches of each variant of cheese analyzed in triplicate.

[2]Curd after molding and addition of cell-free extracts.

[3]Cheese after dry salting.

manufacture (average value of 6.76) to 1 d of ripening (average value of 5.25), then it slightly varied. During the last 30 d of ripening, the values of pH slightly increased (average value of 5.45). Moisture and salt values were within the ranges previously reported for Pecorino cheeses (Coda et al., 2006). The level of fat and protein inversely followed the trend of moisture. After 60 d, the cheeses had average percentages of 24.5 and 26.4% for fat and protein, respectively.

Compared with CC, the addition of CFE also did not affect the cell numbers of the main microbial groups (Table 3). Cell numbers of CC during ripening were similar to those reported previously for the same variety of cheese (De Pasquale et al., 2014). High numbers of presumptive thermophilic cocci and lactobacilli were found after 1 d of ripening (average value of ~9.1 ± 0.3 and 8.9 ± 0.3 log cfu/g, respectively), which decreased throughout ripening to ~7.8 ± 0.2 and 7.7 ± 0.3 log cfu/g, respectively. This finding was consistent with the use of thermophilic primary starters. Presumptive mesophilic lactobacilli were found in the curd at an average value of ~4.5 ± 0.2 log cfu/g, which increased to ~8.2 ± 0.4 log cfu/g after 60 d of ripening. Initially, yeasts and molds were present at similar levels (~3.3 ± 0.1 and 3.1 ± 0.2 log cfu/g), with a common tendency to decrease (~2.5 ± 0.3 and 2.4 ± 0.2 log cfu/g, respectively). Total coliforms were counted into the curd (~4.3 ± 0.2 log cfu/g). The number significantly ($P < 0.05$) increased after 1 d, but they progressively disappeared at the end of ripening.

Proteolysis

Urea-PAGE electrophoresis of the pH 4.6-insoluble nitrogen fraction showed a few differences for primary proteolysis among cheeses (Figure 1). α_{S1}-Casein persisted to the end of ripening, and its main degradation began from 30 d onwards in the DH and HA cheese. At the end of ripening, a large amount of nonhydrolyzed β-CN also persisted in all cheeses. Characteristic polypeptide bands appeared in the WC cheese, which showed the most complex profile. The contribution of *W. cibaria* to proteolysis in cheeses or other fermented foods (e.g., sourdough, table olives) was previously described (Capuani et al., 2013; Fhoula et al., 2013). As previously shown (Coda et al., 2006), primary proteolysis of Italian Pecorino cheeses was characterized by the intense or complete hydrolysis of α_{S1}-CN, which indicated considerable chymosin activity. Chymosin activity on β-CN is lower than that toward α_{S1}-CN, mainly because of hydrophobic interactions between salt and proteins (Fox, 1989). The same primary proteolysis was recently described during ripening of ewe milk Canestrato Pugliese cheese (De Pasquale al., 2014).

The pH 4.6-soluble nitrogen fractions were analyzed by both urea-PAGE electrophoresis and RP-HPLC. Almost the same results were found and only those by RP-HPLC were reported (data not shown). The number of peaks, which were recognized and matched visually with the Unicorn program (Amersham Biosciences, Piscataway, NJ), varied among cheeses manufactured with CFE and between them and CC throughout ripening. Quantitative and, especially, qualitative differences were mainly evident after 60 d of ripening. In particular, DH and LC cheeses showed the highest number of peptide peaks (21 ± 1.0 and 17 ± 1.0, respectively) distributed throughout the acetonitrile gradient, followed by HA and WC cheeses (15 ± 1.0 and 14 ± 1.0, respectively). Fourteen peptide peaks were also detected for CC. The peptide profiles were consistent with the level of enzyme activities referred to secondary proteolysis, which were preliminarily estimated for each CFE (Table 1). Overall, the addition of CFE was responsible for the accumulation of small-sized peptides and FAA, without any effect on the primary proteolysis (Wilkinson, 1999). At the beginning of ripening, no significant ($P > 0.05$) differences were found among cheeses for the concentration of total FAA (range = 408.9 ± 13.5 to 469.6 ± 21.7 mg/kg of cheese; Table 4), with exception of that made with the addition of CFE from *H. alvei* ATCC 51815 (503.2 ± 14.0 mg/kg of cheese). At 30 d of ripening, the DH and HA cheeses showed the highest values (2,407.1 ± 72.2 and 2,244.1 ± 67.3 mg/kg of cheese, respectively). These concentrations were approximately 2 times higher than that found in CC (1,045.2 ± 31.3 mg/kg of cheese). At the end of ripening, the concentrations of FAA further increased for all the cheeses made with CFE, and the values remained markedly higher than that of CC (2,890.7 ± 91.7 mg/kg of cheese). The highest concentration of FAA was found for the LC cheese (5,029.8 ± 151.0 mg/kg of cheese), which contained the highest level of Phe, Lys, and Pro. Several studies already reported accelerated or increased liberation of FAA when commercial enzymes were used for cheesemaking (Yarlagadda et al., 2014). The concentrations of total FAA attained with cheeses added of CFE approached those found for other Italian ewe milk cheeses ripened for longer times (Coda et al., 2006).

Regardless of the the cheese variant, Glu, Ala, Val, Met, Ile, Leu, Phe, Lys, and Pro were the FAA found at the highest concentrations (>100 mg/kg of cheese). These FAA are typically released during ripening of several Italian semi-hard and extra-hard cheese varieties (Gobbetti et al., 1999; Coda et al., 2006; De Pasquale et al., 2014).

The enzyme activities were determined in the water-soluble extracts of the cheeses during ripening (Figure

Table 3. Cell numbers[1] of microbial groups during manufacture and ripening of control cheese without addition of freeze-dried cell-free extract (CFE) (CC) and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA)

Item	Day of ripening	Mesophilic lactobacilli	Thermophilic lactobacilli	Mesophilic cocci	Thermophilic cocci	Enterococci	Yeasts	Molds	Total coliforms
CC	Curd[2]	4.54 ± 0.15^{c}	7.22 ± 0.26^{c}	5.64 ± 0.22^{d}	7.44 ± 0.20^{c}	5.25 ± 0.16^{c}	3.38 ± 0.12^{b}	3.14 ± 0.12^{b}	4.33 ± 0.21^{b}
	1[3]	8.52 ± 0.39^{ab}	8.91 ± 0.40^{a}	7.42 ± 0.30^{c}	9.18 ± 0.35^{a}	6.07 ± 0.22^{b}	4.03 ± 0.13^{a}	3.65 ± 0.17^{a}	5.66 ± 0.23^{a}
	30	9.05 ± 0.43^{a}	8.15 ± 0.35^{b}	8.66 ± 0.34^{a}	8.43 ± 0.39^{ab}	6.81 ± 0.18^{a}	3.75 ± 0.10^{ab}	3.48 ± 0.10^{a}	1.77 ± 0.05^{c}
	60	8.24 ± 0.32^{b}	7.77 ± 0.25^{bc}	8.34 ± 0.31^{ab}	7.85 ± 0.24^{b}	5.79 ± 0.21^{b}	2.37 ± 0.09^{c}	2.41 ± 0.06^{c}	$<1^{d}$
LC	Curd	4.47 ± 0.12^{c}	7.21 ± 0.27^{c}	5.82 ± 0.26^{d}	7.62 ± 0.27^{b}	5.22 ± 0.24^{c}	3.35 ± 0.12^{v}	3.13 ± 0.07^{b}	4.41 ± 0.14^{b}
	1	8.63 ± 0.36^{ab}	8.84 ± 0.41^{a}	7.61 ± 0.37^{c}	9.19 ± 0.42^{a}	5.87 ± 0.26^{b}	4.08 ± 0.07^{a}	3.44 ± 0.16^{ab}	5.69 ± 0.20^{a}
	30	9.12 ± 0.37^{a}	8.23 ± 0.22^{b}	$8.62 \pm 0.35a$	8.57 ± 0.38^{ab}	6.96 ± 0.28^{a}	3.62 ± 0.17^{ab}	3.33 ± 0.15^{ab}	1.65 ± 0.06^{c}
	60	8.33 ± 0.41^{b}	7.64 ± 0.18^{bc}	8.44 ± 0.33^{ab}	7.75 ± 0.19^{b}	5.99 ± 0.22^{b}	2.54 ± 0.06^{c}	2.43 ± 0.08^{c}	$<1^{d}$
WC	Curd	4.49 ± 0.19^{c}	7.11 ± 0.20^{c}	5.78 ± 0.23^{d}	7.63 ± 0.26^{b}	5.23 ± 0.18^{c}	3.31 ± 0.14^{b}	3.16 ± 0.09^{b}	4.31 ± 0.16^{b}
	1	8.65 ± 0.37^{ab}	8.81 ± 0.37^{a}	7.45 ± 0.26^{c}	9.11 ± 0.42^{a}	6.17 ± 0.23^{b}	3.84 ± 0.11^{a}	3.48 ± 0.14^{a}	5.68 ± 0.12^{a}
	30	9.08 ± 0.24^{a}	8.26 ± 0.31^{b}	8.68 ± 0.33^{a}	8.38 ± 0.34^{ab}	6.81 ± 0.21^{a}	3.57 ± 0.15^{ab}	3.44 ± 0.15^{ab}	1.52 ± 0.07^{c}
	60	8.35 ± 0.26^{b}	7.69 ± 0.22^{bc}	8.31 ± 0.35^{ab}	7.83 ± 0.31^{b}	5.84 ± 0.24^{b}	2.55 ± 0.06^{c}	2.53 ± 0.07^{c}	$<1^{d}$
DH	Curd	4.64 ± 0.19^{c}	7.02 ± 0.26^{c}	5.81 ± 0.17^{d}	7.48 ± 0.20^{c}	5.34 ± 0.16^{c}	3.41 ± 0.13^{b}	3.22 ± 0.09^{ab}	4.35 ± 0.19^{b}
	1	8.72 ± 0.31^{ab}	8.97 ± 0.39^{a}	7.72 ± 0.24^{c}	9.05 ± 0.44^{a}	6.04 ± 0.23^{b}	3.87 ± 0.18^{a}	3.32 ± 0.10^{ab}	5.71 ± 0.21^{a}
	30	9.17 ± 0.35^{a}	8.15 ± 0.36^{b}	8.87 ± 0.39^{a}	8.31 ± 0.34^{ab}	6.65 ± 0.20^{a}	3.76 ± 0.14^{ab}	3.32 ± 0.13^{ab}	1.38 ± 0.04^{c}
	60	8.29 ± 0.27^{b}	7.68 ± 0.27^{bc}	8.26 ± 0.36^{ab}	7.73 ± 0.19^{b}	6.13 ± 0.27^{b}	2.57 ± 0.08^{c}	2.48 ± 0.04^{c}	$<1^{d}$
HA	Curd	4.52 ± 0.11^{c}	7.28 ± 0.24^{c}	5.79 ± 0.15^{d}	7.54 ± 0.28^{b}	5.21 ± 0.13^{c}	3.48 ± 0.15^{b}	3.03 ± 0.09^{b}	4.28 ± 0.05^{b}
	1	8.44 ± 0.36^{ab}	8.95 ± 0.33^{a}	7.46 ± 0.28^{c}	8.96 ± 0.37^{a}	5.92 ± 0.22^{b}	4.34 ± 0.19^{a}	3.54 ± 0.11^{a}	5.52 ± 0.09^{a}
	30	9.14 ± 0.43^{a}	8.19 ± 0.31^{b}	8.84 ± 0.32^{a}	8.55 ± 0.29^{ab}	6.73 ± 0.22^{a}	3.48 ± 0.16^{b}	3.42 ± 0.14^{ab}	1.49 ± 0.03^{c}
	60	8.28 ± 0.28^{b}	7.75 ± 0.24^{bc}	8.25 ± 0.30^{ab}	7.71 ± 0.22^{b}	6.01 ± 0.26^{b}	2.48 ± 0.07^{c}	2.41 ± 0.05^{c}	$<1^{d}$

[a–d]Data in the same columns with different letters are significantly different ($P < 0.05$).

[1]Mean values ± SD for 3 batches of each variant of cheese analyzed in triplicate.

[2]Curd after molding and addition of cell-free extracts.

[3]Cheese after dry salting.

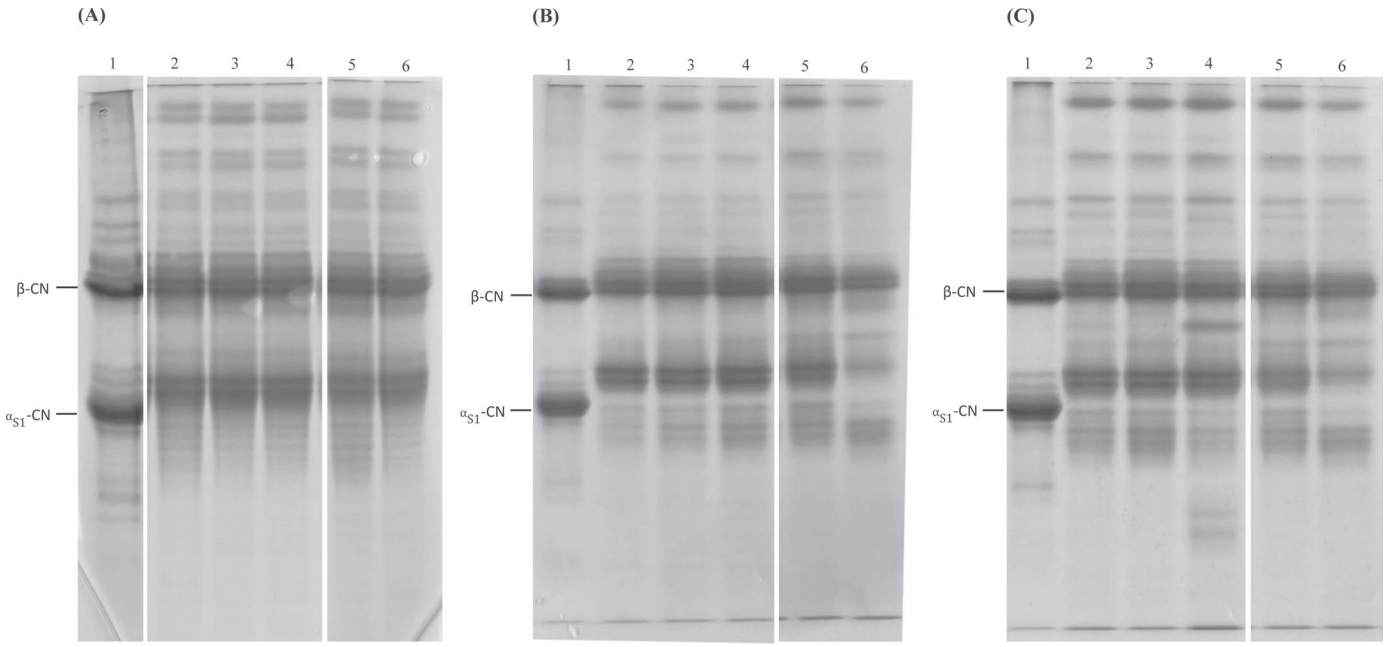

Figure 1. Urea-PAGE of pH 4.6-insoluble nitrogen fraction of ewe milk cheeses after 1 (A), 30 (B), and 60 (C) d of ripening. Lane 1 = ovine CN standard; lane 2 = control cheese without addition of freeze-dried cell-free extract (CFE); lanes 3 to 6 = cheeses with the additions of CFE from *Lactobacillus casei* LC01, *Weissella cibaria* 1XF5, *Debaryomyces hansenii* LCF-558, or *Hafnia alvei* ATCC 51815.

2A to F). Compared with CC, all the enzyme activities were higher in the cheeses made with CFE. The highest ($P < 0.05$) differences were found after 30 d. These findings were in contrast with those reported in other studies, where no significant differences were found when commercial enzymes or CFE were used for cheesemaking (Yarlagadda et al., 2014). Losses of enzymes in the drained whey (Wilkinson and Kilcawley, 2005) or the inherent instability of such enzymes under cheese ripening environmental conditions (Sheehan et al., 2009) may be the causes for these different behavior. The level of enzyme activities varied depending on the CFE added and mostly agreed with the in vitro characterization. The LC and DB cheeses showed the highest ($P < 0.05$) levels of most of the enzyme activities throughout ripening. Glutamate dehydrogenase and cystationine lyase activities were also positively affected by the addition of CFE from *H. alvei* ATCC 51815 and *W. cibaria* 1XF5. After 60 d of ripening, the highest esterase activity was found for LC and DB cheeses.

Based on the same method of determination and on the same cheese variety manufactured at the same dairy plant, it seemed that all the cheeses made with CFE contained levels of enzymatic activities markedly higher than those previously found when adjunct attenuated mesophilic lactobacilli were used (Di Cagno et al., 2011). Moreover, a contribution from autolyzed cells from primary starters and from endogenous mesophilic lactobacilli nonstarter found at high levels (8.2 ± 04 log cfu/g after 60 d of ripening) cannot be excluded. The differences found for enzyme activities in the water-soluble extracts of the cheeses reflected the trend observed for total FAA.

Volatile Components

Volatile components (**VOC**; 140 in total) were identified by PT-GC/MS. The VOC belonged to several chemical classes: aldehydes (20), alcohols (21), ketones (36), esters (39), sulfur compounds (13), furans (8), pyrazines (2), and nitrogen compound (1). Accelerated liberation of most AA in cheeses promotes an increased synthesis of volatile compounds, and it may influence the flavor of cheese (Fox and Wallace, 1997). Indeed, the level of 49 volatile compounds significantly ($P < 0.05$) differentiated CC from the cheeses made with CFE (Table 5). Overall, the level of some alcohols, ketones, sulfur compounds, and furans were the lowest in the CC, whereas most aldehydes (e.g., heptanal, octanal, decanal, 2-pentenal, 2,4-hexadienal, benzaldehyde) were the highest. Aldehydes are unstable compounds that are reduced to alcohols or oxidized to acids during cheese ripening (Carbonell et al., 2002). The highest level of these compounds may be considered an index of a not fully or optimal cheese ripening, which may cause off-flavors (Moio and Addeo, 1998). The levels of 3 aldehydes (e.g., acetaldehyde, 2-methyl-propanal), 8 alcohols (e.g., ethanol, 1-propanol), 9 ketones (e.g.,

Table 4. Mean values[1] for the level of free AA (mg/kg of cheese) found in control cheese (CC) without addition of freeze-dried cell-free extract (CFE) and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) after 1, 30, and 60 d of ripening

	Day of ripening														
	1					30					60				
AA	CC	LC	WC	DH	HA	CC	LC	WC	DH	HA	CC	LC	WC	DH	HA
Asp	6.4	6.8	8.1	5.5	18.1	26.9	38.7	31.8	45.5	70.3	110.0	123.3	95.8	84.7	125.1
Thr	1.5	5.7	5.8	4.3	3.8	8.0	14.8	13.3	9.6	14.2	49.9	73.5	52.0	20.6	58.7
Ser	17.8	29.4	35.8	17.4	6.5	71.1	91.5	112.0	41.5	24.2	111.1	137.1	267.1	73.5	60.3
Glu	28.0	46.8	53.6	40.2	55.0	107.4	112.8	158.7	221.4	229.5	328.0	465.3	426.1	401.9	568.8
Gly	9.0	12.5	4.1	4.8	7.6	8.9	10.3	14.7	12.2	19.1	51.7	33.3	36.4	27.5	63.1
Ala	53.4	35.5	49.3	38.6	24.3	65.0	70.4	73.7	66.0	64.4	141.7	122.2	115.1	97.2	293.5
Cys	7.1	8.1	7.7	7.8	7.6	14.7	30.7	21.5	43.6	20.5	33.9	45.7	36.2	26.7	31.9
Val	46.5	46.9	48.3	59.9	61.1	158.9	196.6	229.2	424.7	411.4	306.1	490.5	409.4	493.9	411.7
Met	8.8	20.4	16.3	5.2	16.4	35.2	64.7	70.4	65.0	73.3	109.7	301.9	116.6	105.6	123.0
Ile	4.2	9.9	7.5	4.2	10.8	21.2	48.5	49.7	53.2	59.0	120.5	247.7	130.1	103.5	162.2
Leu	51.2	55.3	54.3	56.5	72.1	165.9	237.1	258.41	480.0	449.7	416.3	538.4	491.5	630.9	564.6
Tyr	34.4	16.6	24.7	26.0	18.2	37.4	96.6	108.2	95.4	63.7	89.2	333.5	88.4	88.5	85.0
Phe	44.1	34.1	40.6	51.8	47.0	113.5	156.5	178.7	393.7	320.2	313.1	574.2	332.8	458.0	387.5
His	6.7	8.4	6.3	9.3	12.3	6.1	11.2	2.9	104.9	27.0	66.6	65.5	191.9	140.1	214.6
Trp	8.8	11.5	11.8	22.6	5.7	30.4	45.1	69.4	46.3	55.1	58.9	169.3	32.2	66.7	49.7
Orn	15.2	12.5	13.2	22.7	35.5	13.3	19.5	18.9	52.7	82.5	255.6	19.3	19.1	62.9	84.6
Lys	16.1	25.3	25.8	19.8	25.7	62.6	100.3	116.8	86.0	94.0	264.7	702.4	352.8	175.9	250.6
Arg	24.5	18.1	28.4	13.9	16.9	56.4	72.4	88.3	58.2	72.1	68.0	146.8	224.6	86.8	24.7
Pro	25.5	32.0	28.9	46.2	58.9	41.8	92.9	46,1	107.4	93.7	195.5	439.6	128.0	156.2	282.3
Total	408.9 ± 13.5^{h}	435.7 ± 19.8^{h}	469.6 ± 21.7^{h}	457.7 ± 16.4^{h}	503.2 ± 14.0^{h}	1,045.2 ± 31.3^{g}	1,511.2 ± 39.4^{f}	1,662.9 ± 38.9^{f}	2,407.1 ± 72.2^{e}	2,244.1 ± 67.3^{e}	2,890.7 ± 91.7^{d}	5,029.8 ± 151.0^{a}	3,514.3 ± 66.3^{bc}	3,301.4 ± 99.0^{c}	3,792.3 ± 113.7^{b}

$^{a–h}$Data in the same row with different letters are significantly different ($P < 0.05$).

[1]Mean values ± SD for 3 batches of each variant of cheese analyzed in triplicate.

2-butanone, 2-heptanone), 11 esters (e.g., ethyl acetate, ethyl butanoate), and 10 sulfur compounds (e.g., dimethyl-trisulfide, 2.4-dithiapentane) significantly ($P < 0.05$) differed among cheeses manufactured with CFE (Table 5). The levels of ketones were the highest in the LC cheese, mainly branched-chain aldehydes were the highest in WC cheese, secondary alcohols and branched alcohols distinguished DB cheese, and esters and sulfur compounds were the highest in HA cheese. Ketones are formed by enzymatic oxidation of free FA (McSweeney and Sousa, 2000), which, along with 2-heptanone, are responsible for the characteristic aroma of blue-veined cheeses (Rothe et al., 1982). Overall, ketones characterized the VOC profile of raw ewe milk cheeses (e.g., Fiore Sardo and Manchego), where mesophilic nonstarter lactic acid bacteria had an important role for ripening (Coda et al., 2006). Branched-chain aldehydes mainly result from the catabolism of FAA (McSweeney and Sousa, 2000) and are associated with pungent, almond, and chemical aromas (Tunick, 2007).Valine, Leu, Ile, Phe, Tyr, Trp, and Met are well known precursors of branched-chain aldehydes, and their levels were particularly abundant when CFE from *W. cibaria* 1XF5 was used. Secondary alcohols derive from the reduction

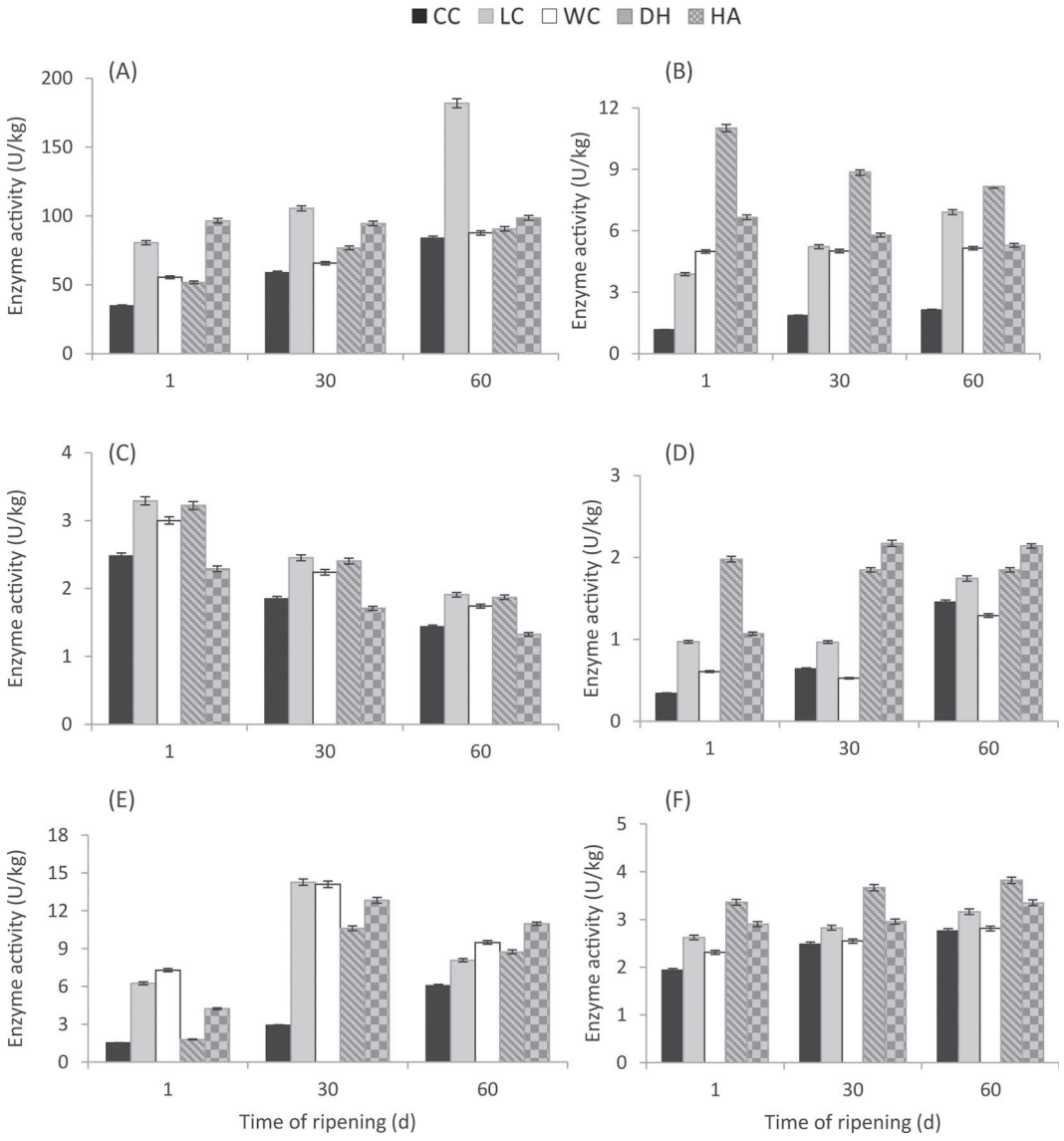

Figure 2. Enzymatic activities (arbitrary units/kg) of water-soluble extracts from control cheese without addition of freeze-dried cell-free extract (CFE; black bars), and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (solid gray bars), *Weissella cibaria* 1XF5 (white bars), *Debaryomyces hansenii* LCF-558 (checkered bars), or *Hafnia alvei* ATCC 51815 (striped bars) after 1, 30, and 60 d of ripening. Panels show activities of aminopeptidase type N (A), iminopeptidase (B), endopeptidase type O (C), glutamate dehydrogenase (D), cystathionine lyase (E), and esterase (F). Values represent the average of 3 triplicates (±SD) from 3 cheesemaking experiments.

Table 5. Concentration of volatile components (arbitrary units of area/100) that differentiated control cheese (CC) without addition of freeze-dried cell-free extract (CFE) and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) after 60 d of ripening

Chemical class[1, 2]	CC	LC	WC	DH	HA
Aldehydes					
Acetaldehyde	5.70E+05^{c}	1.34E+05^{d}	**2.70E+06**^{a}	8.80E+05^{b}	1.86E+06^{ab}
Heptanal	*7.26E+05*^{a}	4.08E+04^{d}	3.59E+05^{b}	3.68E+05^{b}	4.72E+05^{bc}
Octanal	*3.62E+05*^{a}	3.32E+04^{c}	2.48E+05^{ab}	1.69E+05^{ab}	2.09E+05^{b}
Decanal	*1.83E+04*^{a}	6.11E+03^{bc}	5.68E+03^{c}	4.58E+04^{b}	3.31E+03^{c}
2-Pentenal	*1.81E+05*^{a}	3.29E+03^{c}	8.83E+04^{b}	8.25E+04^{b}	7.36E+04^{b}
2-Methyl-propanal	2.53E+06^{c}	6.35E+06^{b}	**2.54E+07**^{a}	1.46E+06^{cd}	2.86E+06^{c}
2-Methyl-butanal	2.09E+06^{b}	**1.06E+07**^{a}	**1.30E+07**^{a}	8.40E+05^{c}	2.89E+06^{b}
2,4-Hexadienal	*3.66E+04*^{a}	4.14E+03^{c}	2.46E+04^{ab}	1.48E+04^{b}	0.00E+00^{d}
Benzaldehyde	*2.43E+06*^{a}	1.49E+06^{b}	1.30E+06^{b}	1.15E+06^{b}	2.04E+06^{ab}
Alcohols					
Ethanol	6.90E+07^{b}	2.21E+06^{c}	9.13E+07^{b}	**1.30E+08**^{a}	6.68E+07^{b}
1-Propanol	3.01E+06^{c}	8.29E+04^{e}	7.76E+05^{d}	**1.57E+07**^{a}	6.82E+06^{b}
1-Butanol	1.61E+06^{b}	1.27E+06^{b}	**4.64E+06**^{a}	**4.10E+06**^{a}	**4.45E+06**^{a}
2-Propanol	7.69E+06^{c}	1.33E+06^{d}	**2.85E+07**^{a}	1.07E+07^{b}	1.51E+07^{b}
2-Butanol	2.51E+07^{b}	3.82E+05^{d}	3.96E+06^{c}	**1.49E+08**^{a}	5.35E+07^{b}
2-Pentanol	9.92E+06^{b}	1.49E+05^{d}	3.14E+06^{c}	**7.08E+07**^{a}	3.08E+07^{ab}
2-Methyl-1-butanol	5.37E+06^{b}	2.11E+06^{c}	4.84E+06^{b}	2.99E+06^{c}	**2.10E+07**^{a}
3-Methyl-1-butanol	3.29E+07^{b}	3.37E+07^{b}	2.68E+07^{bc}	2.78E+07^{bc}	**1.31E+08**^{a}
Ketones					
2-Butanone	2.30E+07^{c}	3.83E+06^{d}	4.79E+07^{b}	**1.03E+08**^{a}	5.55E+07^{b}
3-Pentanone	0.00E+00^{c}	4.68E+04^{b}	**5.12E+05**^{a}	**5.39E+05**^{a}	0.00E+00^{c}
2-Heptanone	2.93E+07^{c}	**3.21E+08**^{a}	1.32E+08^{ab}	3.29E+07^{c}	9.26E+07^{b}
2-Octanone	3.29E+05^{e}	**6.15E+06**^{a}	3.90E+06^{b}	4.05E+05^{d}	9.16E+05^{c}
2-Nonanone	2.11E+06^{d}	**8.61E+07**^{a}	5.97E+07^{b}	2.93E+06^{cd}	3.69E+06^{c}
8-Nonen-2-one	2.63E+04^{c}	**2.34E+06**^{a}	1.64E+06^{ab}	3.29E+04^{b}	3.97E+04^{b}
2,3-Butanedione	2.88E+06^{c}	3.06E+06^{c}	**5.80E+06**^{a}	4.45E+06^{b}	4.59E+06^{b}
2,3-Pentanedione	5.81E+04^{c}	1.14E+05^{b}	4.10E+05^{ab}	2.13E+05^{b}	**5.85E+05**^{a}
3-Methyl-2-pentanone	1.15E+06^{b}	7.55E+05^{c}	**1.01E+07**^{a}	5.23E+05^{c}	3.20E+06^{b}
Esters					
Methyl butanoate	9.41E+04^{b}	7.72E+04^{c}	**2.78E+05**^{a}	**2.19E+05**^{a}	**2.82E+05**^{a}
Ethyl acetate	1.59E+06^{c}	2.98E+05^{d}	1.75E+06^{c}	3.08E+06^{b}	**5.44E+06**^{a}
Ethyl propanoate	4.48E+04^{c}	8.00E+02^{d}	5.00E+04^{c}	9.91E+04^{b}	**2.03E+05**^{a}
Ethyl butanoate	7.02E+06^{b}	1.12E+06^{c}	**2.46E+07**^{a}	**1.90E+07**^{a}	**2.68E+07**^{a}
Ethyl hexanoate	8.56E+06^{c}	1.10E+06^{d}	2.10E+07^{ab}	1.61E+07^{b}	**3.73E+07**^{a}
Propyl butanoate	2.35E+05^{b}	2.40E+04^{d}	6.47E+04^{c}	**6.60E+05**^{a}	**6.58E+05**^{a}
Propyl hexanoate	4.60E+04^{c}	6.10E+03^{e}	2.61E+04^{d}	**1.11E+05**^{a}	9.74E+04^{b}
1-Methyl-propyl butanoate	1.09E+05^{b}	2.80E+03^{d}	1.23E+04^{c}	**3.91E+05**^{a}	**3.52E+05**^{a}
1-Methyl-propyl hexanoate	1.19E+04^{ab}	0.00E+00^{c}	0.00E+00^{c}	**4.71E+04**^{a}	9.30E+03^{b}
2-Methyl-butyl acetate	1.06E+05^{b}	2.22E+04^{d}	4.84E+04^{c}	4.88E+04^{c}	**5.90E+05**^{a}
3-Methyl-butyl acetate	1.22E+06^{b}	1.24E+06^{b}	2.16E+05^{d}	6.55E+05^{c}	**6.29E+06**^{a}
Sulfur compounds					
Methanethiol	5.82E+04^{ab}	6.28E+03^{d}	3.16E+04^{b}	1.18E+04^{c}	**6.78E+04**^{a}
Dimethyl-sulfide	3.85E+05^{c}	3.20E+03^{d}	**3.03E+06**^{a}	4.12E+05^{c}	8.83E+05^{b}
Dimethyl-disulfide	**6.86E+06**^{a}	1.92E+06^{c}	4.39E+06^{b}	8.97E+05^{d}	**6.45E+06**^{a}
Dimethyl-trisulfide	5.40E+04^{b}	2.00E+04^{c}	2.04E+04^{c}	1.78E+03^{d}	**8.61E+04**^{a}
2.4-Dithiapentane	3.40E+06^{b}	6.55E+04^{e}	6.76E+05^{c}	3.81E+05^{d}	**9.89E+06**^{a}
3-Methyl-thio-1-propene	1.30E+05^{c}	2.99E+03^{d}	2.23E+05^{b}	1.46E+05^{c}	**5.48E+05**^{a}
Methional	0.00E+00^{c}	0.00E+00^{c}	**8.01E+02**^{a}	0.00E+00^{c}	2.00E+02^{b}
S-Methyl thioacetate	**1.24E+05**^{a}	1.60E+04^{c}	8.26E+03^{d}	3.90E+04^{b}	**2.74E+05**^{a}
1-Methyl-thiopropane	2.41E+04^{c}	0.00E+00^{d}	4.87E+04^{b}	2.66E+04^{c}	**9.19E+04**^{a}
S-Methyl thiopropanoate	3.25E+03^{b}	0.00E+00^{c}	0.00E+00^{c}	0.00E+00^{c}	**1.98E+04**^{a}
Furans					
2,5-Dimethyl-furan	6.70E+03^{d}	1.19E+04^{c}	**1.16E+05**^{a}	3.11E+04^{c}	7.46E+04^{b}
2,5-Dimethylpyrazine	2.44E+04^{c}	**5.19E+05**^{a}	1.85E+04^{c}	8.00E+02^{d}	1.25E+05^{b}

$^{a–e}$Data in the same row with different letters are significantly different ($P < 0.05$).

[1]Compounds that mainly ($P < 0.05$) differed among control cheese and cheeses manufactured with CFE are in italics.

[2]Compounds that mainly ($P < 0.05$) differed among cheeses manufactured with CFE are in boldface.

of methyl ketones, whereas methyl-branched alcohols are synthesized from aldehyde reduction, which are formed from branched FAA or free FA (Yarlagadda et al., 2014). The highest level of alcohols found in the cheese made with CFE from *D. hansenii* LCF-585 was consistent with the high esterase and iminopeptidase

Table 6. Concentrations (mg/kg)[1] of volatile free fatty acids (VFFA) in control cheese (CC) without addition of freeze-dried cell-free extract (CFE) and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) after 60 d of ripening

VFFA	CC	LC	WC	DH	HA
Acetic acid	700.0 ± 23.3^{b}	513.3 ± 15.2^{d}	550.3 ± 16.8^{c}	1,294.4 ± 34.5^{a}	686.7 ± 14.7^{b}
Propionic acid	17.5 ± 1.2^{b}	49.5 ± 11.4^{a}	17.2 ± 1.3^{b}	17.2 ± 1.0^{b}	13.5 ± 0.9^{c}
Isobutyric acid	46.6 ± 2.4^{b}	275.0 ± 8.5^{a}	23.4 ± 1.3^{b}	24.2 ± 1.2^{b}	42.1 ± 1.5^{b}
Butyric acid	256.1 ± 7.4^{b}	728.1 ± 22.6^{a}	193.7 ± 6.5^{bc}	135.7 ± 3.1^{c}	145.4 ± 5.9^{c}
Isovaleric acid	121.8 ± 4.8^{b}	700.9 ± 19.7^{a}	35.6 ± 1.0^{c}	32.0 ± 1.4^{c}	150.4 ± 6.2^{b}
Isocaproic acid	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.3 ± 0.0^{b}	0.4 ± 0.0^{ab}	0.4 ± 0.1^{ab}
Caproic acid	66.4 ± 2.5^{b}	155.5 ± 5.4^{a}	52.2 ± 2.3^{bc}	27.3 ± 1.3^{c}	48.7 ± 1.5^{bc}

[a–d]Data in the same row with different letters are significantly different ($P < 0.05$).

[1]Mean values ± SD for 3 batches of each variant of cheese analyzed in duplicate.

activities. The synthesis of such flavor compounds by this yeast species was previously shown (Leclercq-Perlat et al., 2004). During ripening, the microbial catabolism of Met and Cys may lead to the synthesis of hydrogen sulfide and methanethiol. After oxidation, such compounds generate methional, carbon sulfide, dimethyldisulfide, and trisulfide. The water-soluble extracts of the HA cheese showed the highest level of cystathionine lyase activity, which is consistent with the highest levels of sulfur compounds found in this cheese. These sulfur compounds are considered indispensable for the characteristic aroma (e.g., garlic or very ripe cheese notes) of several cheese varieties (Ortigosa et al., 2001) due to the very low perception thresholds. The contribution of *H. alvei* to the synthesis of sulfur compounds was also previously described (Irlinger et al., 2012). Esters were also the main VOC of the HA cheese. Enzyme esterification of FA with primary alcohols frequently occurs during cheese ripening. High esterase activity was found in HA cheese. Esters were the main volatile components of Italian and Spanish ewe milk cheeses ripened for longer times (e.g., Canestrato Pugliese, Pecorino Romano, and Roncal; Izco and Torre, 2000; Larràyoz et al., 2001; Coda et al., 2006).

Seven VFFA (C2 to C6) were also identified in ripened cheeses (Table 6). Except for acetic acid that was found at the highest concentration in DB cheese, all VFFA were found at the highest levels in LC cheese. This is consistent with the highest esterase activity found both in vitro analysis and in the water-soluble extracts of the LC and DH cheeses. The high level of the VFFA found in the LC cheese may explain the highest level of ketones found in the same cheeses (McSweeney and Sousa, 2000). The use of intracellular CFE from selected microorganisms, combined to the addition of a commercial proteinase, positively affected the level of VFFA in the Ras cheese (Ezzat, 1990).

Sensory Analysis

Table 7 shows the sensory scores of the 5 variants of cheese. Both DH and HA cheese obtained the highest score in terms of aroma (7.5 ± 0.2 and 7.5 ± 0.1, respectively), flavor (7.7 ± 0.1 and 7.6 ± 0.2, respectively), and aftertaste (7.4 ± 0.2 and 7.4 ± 0.1, respectively). The DH and LC cheeses were characterized by the highest score in terms of solubility (6.9 ± 0.2 and 6.6 ± 0.1, respectively); WC cheese was judged as the more friable (6.9 ± 0.3). Overall acceptability (7.8 ± 0.2) was the highest ($P < 0.05$) in the DH cheese followed by the HA, LC, CC, and WC cheeses. Using proteolysis data, the profiles of volatile compounds and free FA, and

Table 7. Sensory scores[1] of control cheese (CC) without addition of freeze-dried cell-free extract (CFE) and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) after 60 d of ripening

Attributes	CC	LC	WC	DH	HA
Aroma	7.2 ± 0.1^{ab}	7.0 ± 0.1^{b}	5.9 ± 0.3^{d}	7.5 ± 0.2^{a}	7.5 ± 0.1^{a}
Flavor	6.8 ± 0.2^{c}	7.5 ± 0.2^{a}	7.3 ± 0.1^{b}	7.7 ± 0.1^{a}	7.6 ± 0.2^{b}
Aftertaste	6.4 ± 0.1^{b}	5.9 ± 0.2^{c}	6.3 ± 0.2^{b}	7.4 ± 0.2^{a}	7.4 ± 0.1^{a}
Friability	5.8 ± 0.2^{b}	6.2 ± 0.1^{a}	6.9 ± 0.3^{a}	5.1 ± 0.3^{c}	5.2 ± 0.1^{c}
Solubility	6.2 ± 0.1^{b}	6.6 ± 0.1^{b}	5.9 ± 0.2^{c}	6.9 ± 0.2^{a}	6.3 ± 0.2^{ab}
Overall acceptability	6.3 ± 0.2^{bc}	6.3 ± 0.1^{bc}	5.8 ± 0.2^{c}	7.8 ± 0.3^{a}	6.8 ± 0.3^{b}

[a–d]Data in the same row with different letters are significantly different ($P < 0.05$).

[1]Mean values ± SD for 3 batches of each variant of cheese analyzed in triplicate.

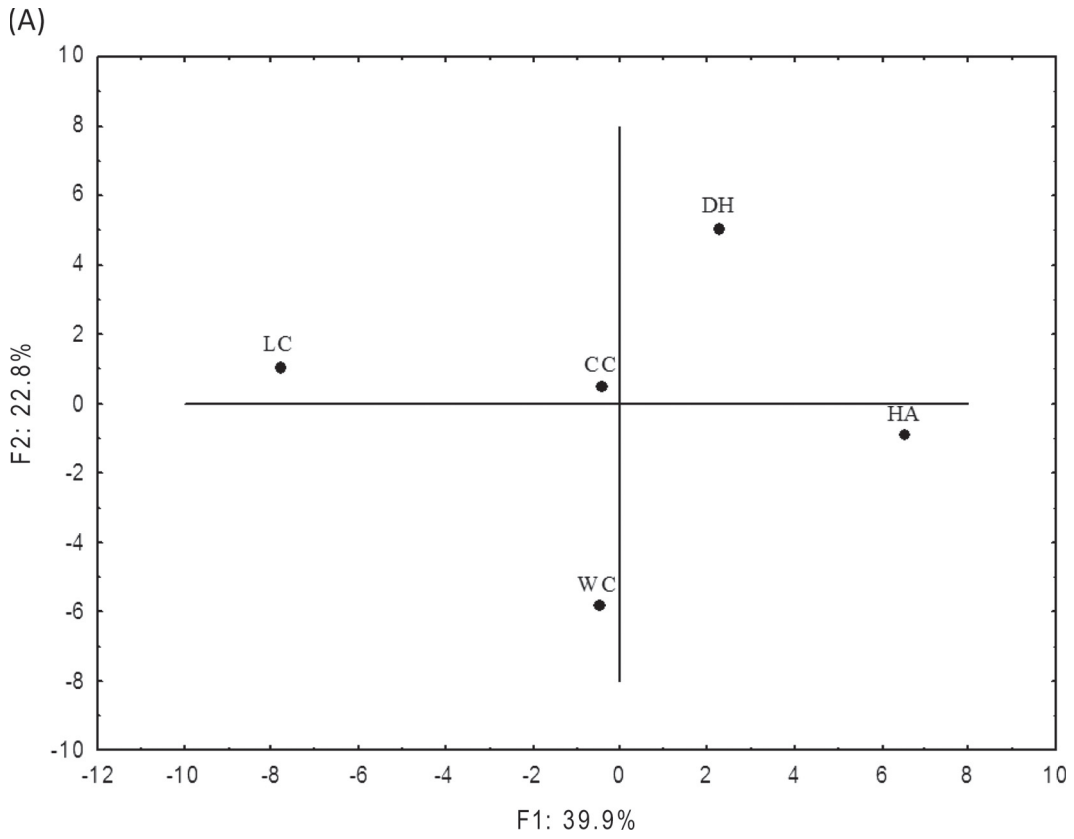

Figure 3. Score plot (A) and loading plot (B) of first and second principal components (PC) after PC analysis based on volatile components that mainly ($P < 0.05$) differentiated the 5 cheeses, free FA, number and area of peaks of the pH 4.6-soluble nitrogen fractions distributed throughout the acetonitrile gradient, overall acceptability score, free AA, enzyme activities in water-soluble extracts of the control cheese (CC) without addition of freeze-dried cell-free extract (CFE), and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) after 60 d of ripening. Acceptability = overall acceptability score; Acetald = acetaldehyde; C7 = heptanal; C8 = octanal; C10 = decanal; 2Pentenal = 2-pentenal; 2Mepropanal = 2-methyl-propanal; 2Mebutanal = 2-methyl-butanal; 2,4Hexadienal = 2,4-hexadienal; Benz = benzaldehyde; EtOH = ethanol; 1C3ol = 1-propanol; 1C4ol = 1-butanol; 2C3ol = 2-propanol; 2C4ol = 2-butanol; 2C5ol = 2-pentanol; 2MeC4ol = 2-methyl-1-butanol; 3MeC4ol = 3-methyl-1-butanol; MEK = 2-butanone; DEK = 3-pentanone; 2Heptanone = 2-heptanone; 2Octanone = 2-octanone; 2Nonanone = 2-nonanone; 8Nonen21 = 8-nonen-2-one; Diacetyl = 2,3-butanedione; 2,3Pentanedione = 2,3-pentanedione; 3Me2C5 = 3-methyl-2-pentanone; Me4C = methyl butanoate; E2C = ethyl acetate; E3C = ethyl propanoate; E4C = ethyl butanoate; E6C = ethyl hexanoate; P4C = propyl butanoate; P6C = propyl hexanoate; 1MeP4C = 1-methyl-propyl butanoate; 1MeP6C = 1-methyl-propyl hexanoate; 2MeBC4 = 2-methyl-butyl acetate; 3MeBC4 = 3-methyl-butyl acetate; Methanethiol = methanethiol; DMS = dimethyl-sulfide; DMDS = dimethyl-disulfide; DMTS = dimethyl-trisulfide; 2,4DSC5 = 2,4-dithiapentane; 3MeS1C3 = 3-methyl-thio-1-propene; Methional = methional; SMeSC2 = S-methyl thioacetate; 1MeSpropane = 1-methyl-thiopropane; SMeSpropanoate = S-methyl thiopropanoate; 2,5Mefuran = 2,5-dimethyl-furan; 2,5Mepyrazine = 2,5-dimethylpyrazine; PepN = aminopeptidase type N; PepI = proline iminopeptidase; PepO = endopeptidase type O; GDH = glutamate dehydrogenase; Cys = cystathionine lyase; Est = esterase; C2 = acetic acid; C3 = propionic acid; Ic4 = isobutyric acid; C4 = butyric acid; C5 = isovaleric acid; iC6 = isocaproic acid; C6 = caproic acid; FFA = free AA; 0–7 n = number of peaks distributed throughout the 0–7 acetonitrile gradient; 0–7 A = area of peaks distributed throughout the 0–7 acetonitrile gradient; 7–53 n = number of peaks distributed throughout the 7–53 acetonitrile gradient; 7–53 A = area of peaks distributed throughout the 7–53 acetonitrile gradient.

the overall acceptability score, the principal component analysis showed that cheeses made with CFE differed and were distinguished from the CC (without adjunct; Figure 3). The highest score for acceptability found for DH cheese might be related to the high area (0–7) and number (7–53) of peptide peaks, 1-methyl-propyl butanoate, 1-methyl-propyl hexanoate, 2-butanol, 1-propanol, glutamate dehydrogenase activity (Figure 3), as well as levels of Glu, Val, Leu, and Phe. Apart from the microbial source used, the cheeses with the addition of CFE showed higher acceptability than the control cheese.

CONCLUSIONS

Cell-free extracts from mesophilic lactic acid bacteria and microorganisms unconventionally used in cheesemaking represented suitable sources of diverse enzyme activities to be used in cheesemaking, without modifying the main compositional features. The effect

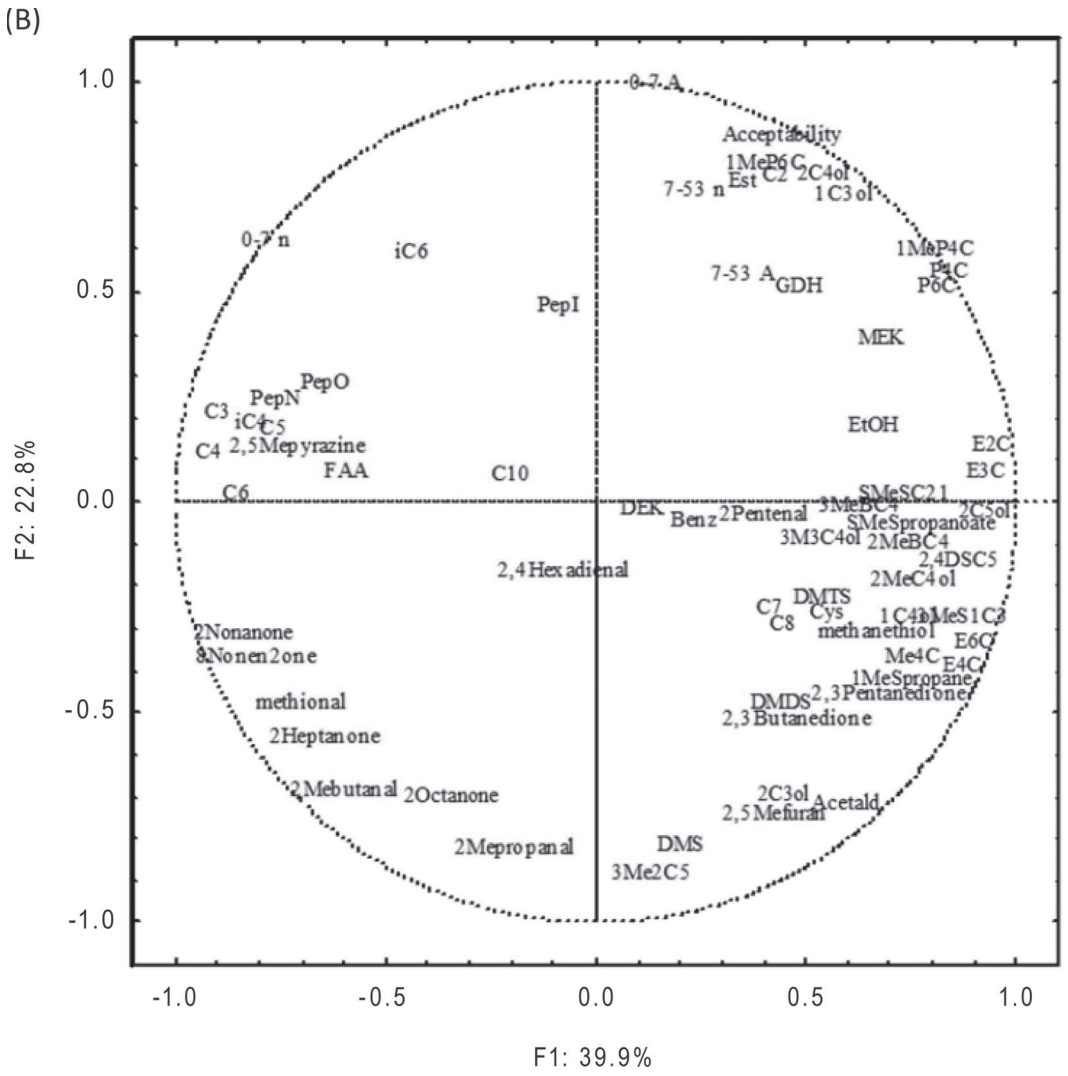

Figure 3 (Continued). Score plot (A) and loading plot (B) of first and second principal components (PC) after PC analysis based on volatile components that mainly ($P < 0.05$) differentiated the 5 cheeses, free FA, number and area of peaks of the pH 4.6-soluble nitrogen fractions distributed throughout the acetonitrile gradient, overall acceptability score, free AA, enzyme activities in water-soluble extracts of the control cheese (CC) without addition of freeze-dried cell-free extract (CFE), and cheeses with the additions of CFE from *Lactobacillus casei* LC01 (LC), *Weissella cibaria* 1XF5 (WC), *Debaryomyces hansenii* LCF-558 (DH), or *Hafnia alvei* ATCC 51815 (HA) after 60 d of ripening. Acceptability = overall acceptability score; Acetald = acetaldehyde; C7 = heptanal; C8 = octanal; C10 = decanal; 2Pentenal = 2-pentenal; 2Mepropanal = 2-methyl-propanal; 2Mebutanal = 2-methyl-butanal; 2,4Hexadienal = 2,4-hexadienal; Benz = benzaldehyde; EtOH = ethanol; 1C3ol = 1-propanol; 1C4ol = 1-butanol; 2C3ol = 2-propanol; 2C4ol = 2-butanol; 2C5ol = 2-pentanol; 2MeC4ol = 2-methyl-1-butanol; 3MeC4ol = 3-methyl-1-butanol; MEK = 2-butanone; DEK = 3-pentanone; 2Heptanone = 2-heptanone; 2Octanone = 2-octanone; 2Nonanone = 2-nonanone; 8Nonen21 = 8-nonen-2-one; Diacetyl = 2,3-butanedione; 2,3Pentanedione = 2,3-pentanedione; 3Me2C5 = 3-methyl-2-pentanone; Me4C = methyl butanoate; E2C = ethyl acetate; E3C = ethyl propanoate; E4C = ethyl butanoate; E6C = ethyl hexanoate; P4C = propyl butanoate; P6C = propyl hexanoate; 1MeP4C = 1-methyl-propyl butanoate; 1MeP6C = 1-methyl-propyl hexanoate; 2MeBC4 = 2-methyl-butyl acetate; 3MeBC4 = 3-methyl-butyl acetate; Methanethiol = methanethiol; DMS = dimethyl-sulfide; DMDS = dimethyl-disulfide; DMTS = dimethyl-trisulfide; 2,4DSC5 = 2,4-dithiapentane; 3MeS1C3 = 3-methyl-thio-1-propene; Methional = methional; SMeSC2 = S-methyl thioacetate; 1MeSpropane = 1-methyl-thiopropane; SMeSpropanoate = S-methyl thiopropanoate; 2,5Mefuran = 2,5-dimethyl-furan; 2,5Mepyrazine = 2,5-dimethylpyrazine; PepN = aminopeptidase type N; PepI = proline iminopeptidase; PepO = endopeptidase type O; GDH = glutamate dehydrogenase; Cys = cystathionine lyase; Est = esterase; C2 = acetic acid; C3 = propionic acid; Ic4 = isobutyric acid; C4 = butyric acid; C5 = isovaleric acid; iC6 = isocaproic acid; C6 = caproic acid; FFA = free AA; 0–7 n = number of peaks distributed throughout the 0–7 acetonitrile gradient; 0–7 A = area of peaks distributed throughout the 0–7 acetonitrile gradient; 7–53 n = number of peaks distributed throughout the 7–53 acetonitrile gradient; 7–53 A = area of peaks distributed throughout the 7–53 acetonitrile gradient.

on cheese sensory attributes varied depending on the microorganism used. As shown by principal component analysis, cheeses made with CFE differed and were distinguished from the CC (without adjunct). Each CFE seemed to specifically affect directly (e.g., *L. casei* LC01 was related to high levels of FAA and some VFFA, *H.*

alvei ATCC 51815 to the main synthesis of sulfur compounds) or indirectly (e.g., *L. casei* LC01 was related to high levels of branched AA as precursors of branched-chain aldehydes) by releasing precursor compounds an attribute of the cheese (e.g., *L. casei* LC01 was related to high levels of FFA, *H. alvei* ATCC 51815 to the main synthesis of sulfur compounds). An acceleration or conditioning of the cheese ripening would be expected using CFE as sources of tailored enzyme activities as well as their use in mixture should be exploited.

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