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Multiple microbial cell-free extracts improve the microbiological, biochemical and sensory features of ewes' milk cheese

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ABSTRACT

This study used cell-free enzyme (CFE) extracts from *Lactobacillus casei*, *Hafnia alvei*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae* to condition or accelerate Pecorino-type cheese ripening. Compositional, microbiological, and biochemical analyses were performed, and volatile and sensory profiles were obtained. Lactobacilli and cocci increased during ripening, especially in cheeses containing CFE from *L. casei*, *H. alvei* and *D. hansenii* (LHD-C) and *L. casei*, *H. alvei* and *S. cerevisiae* (LHS-C). Compared to control cheese (CC), several enzymatic activities were higher ($P < 0.05$) in CFE-supplemented cheeses. Compared to the CC (1907 mg kg^{-1} of cheese), the free amino acid level increased ($P < 0.05$) in CFE-supplemented cheeses, ranging from approximately 2575 (LHS-C) to 5720 (LHD-C) mg kg^{-1} of cheese after 60 days of CFE-supplemented ripening. As shown by GC/MS analysis, the levels of several volatile organic compounds were significantly ($P < 0.05$) lower in CC than in CFE-supplemented cheeses. All cheeses manufactured by adding multiple CFEs exhibited higher scores ($P < 0.05$) for internal structure, acid taste and juiciness than CC samples. This study shows the possibility of producing ewes' milk cheese with standardized characteristics and improved flavor intensity in a relatively short time.

1. Introduction

The name Pecorino is commonly given to Italian cheeses made exclusively from pure ewes' milk, and Pecorino has in most cases a protected designation of origin or PDO status. Generally, this type of cheese is produced in the middle and south of Italy using a traditional procedure characterized by a different ripening times ranging from 8 to 12 months (Pecorino Romano, Fiore Sardo, and others) (Di Cagno et al., 2003). In addition, there are other Pecorino varieties produced throughout the Italian territory according to local or regional customs that are also characterized by a shorter ripening time (1–2 months) and semi-hard consistency but a mild flavor and aroma (Caridi et al., 2003). For this reason, increased flavor and aroma as well as a reduction of ripening time can positively influence the production and consumption of these products (Gobbetti, 2004). Acceleration of cheese ripening is of major interest in the cheese industry, mainly due to the high costs associated with long storage periods. Shortened ripening times provide benefits such as net savings in refrigeration, labor and inventory costs as well as increased cheese production when storage facilities are limited (Azarnia et al., 2006).

Research to accelerate ripening focuses on two enzyme-based approaches: (1) indirect process-based operations to enhance the existing enzymatic activity in cheese, and (2) augmentation of the enzyme complement in cheese using microbial/enzyme technology, e.g., addition of exogenous enzymes, use of starter strains, and lactic acid bacteria (LAB) adjuncts that have been selected and modified (genetically engineered) for elevated enzyme levels or enhanced enzyme release (Calasso et al., 2015; Corsetti et al., 1998; Wilkinson and Kilcawley, 2005).

Because the characteristic aroma, flavor and texture of a cheese results from the action of numerous enzymes, the use of a single enzyme to accelerate ripening will likely disturb flavor component equilibrium and cause flavor defects (Wilkinson and Kilcawley,

Abbreviations: CFEs, cell-free enzyme extracts; FAAs, free amino acids; VOCs, volatile organic compounds; VFFAs, volatile free fatty acids; LAB, lactic acid bacteria; NSLAB, non starter lactic acid bacteria; pepN, aminopeptidase type N; pepI, proline iminopeptidase; pepO, endopeptidase type O; GDH, glutamate dehydrogenase; CGL, cystathionine gamma-lyase.

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2005). The use of enzyme cocktails has been proposed to maintain balance during accelerated ripening. Crude cell-free extracts prepared from bacteria, yeasts or molds have been extensively studied (El Soda and Pandian, 1991), and blends of protease/peptidase, protease/lipase or protease/peptidase/lipase have also been evaluated to accelerate the maturation of several types of cheeses (Kheadr et al., 2003). All these studies were performed using free proteolytic and lipolytic enzymes added to cheese milk. The direct addition of enzymes to cheese milk was not successful due to enzyme loss in the whey, poor enzyme distribution, reduced yield and poor-quality cheese (Kailasapathy and Lam, 2005). The addition of free exogenous enzymes to the curd before molding is an effective strategy that guarantees little enzyme loss and increased homogeneity of distribution (Calasso et al., 2015). Although sheep and goat milk products are very important economically in certain areas, the literature remains scarce (Schirone et al., 2012). To date, only a few studies have examined the conditioning or acceleration of ewes' milk cheese ripening compared to that of cows' milk cheese.

Recently, cell-free extracts (CFEs) from *Lactobacillus casei*, *Hafnia alvei* or *Debaryomyces hansenii* have been used as diverse enzyme sources to condition/accelerate the ripening of Pecorino-type cheese (Calasso et al., 2015). CFEs from mesophilic lactic acid bacteria (LAB) and microorganisms not conventionally used in cheesemaking represent potential diverse enzyme sources that would not modify the main compositional features of the cheese. The effects on cheese sensory attributes vary depending on the microorganism used. *L. casei* LC01 treatment led to high levels of free amino acids (FAAs) and some volatile free fatty acids (VFFAs). However, the use of cell-free *H. alvei* extracts affected the synthesis of sulfur-containing compounds. Based on previous results, acceleration or conditioning of cheese ripening would be expected using CFEs as sources of tailored enzymatic activity.

This study used multiple freeze-dried CFEs from selected cheese-related and non-related microorganisms as additional sources of diverse enzymes to condition or accelerate Pecorino-type cheese ripening. Compositional, microbiological, and biochemical analyses as well as volatile and sensory profiles were performed to characterize cheeses during manufacture and ripening.

2. Materials and methods

2.1. Microorganisms and culture conditions

L. casei LC01 and *Saccharomyces cerevisiae* A4 from the Culture Collection of the Department of Soil, Plant and Food Sciences (University of Bari, Italy), *H. alvei* Moller ATCC51815 (biosafety level 1), and *D. hansenii* DSM70590 were all used as CFE sources for cheesemaking. *L. casei* LC01 was propagated for 24 h at 30 °C on MRS broth (Oxoid, Basingstoke, UK). *H. alvei* ATCC51815 was grown for 24 h at 30 °C in Nutrient Broth (Oxoid). *D. hansenii* DSM70590 was propagated for 48 h at 25 °C in malt-yeast-peptone-glucose broth (MYGP), pH 6.5, containing yeast and malt extracts (0.3% wt/vol), bacteriological peptone (0.5% wt/vol), and glucose (1%, wt/vol). *S. cerevisiae* A4 was propagated for 24 h at 30 °C in Sabouraud Dextrose Liquid Medium (Oxoid).

2.2. Preparation of microbial cell-free extracts

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 mM potassium phosphate buffer, pH 7.0, resuspended in the same buffer at a cell density of ~11.0 ± 0.1 log CFU/mL, and sonicated in an ice bath. Sonication was performed using a VibraCell 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) (Calasso et al., 2015). The sonication treatment efficiency was estimated by plate count and Bradford assay (Bradford, 1976). At the end of the sonication, CFEs were recovered by centrifugation (15,000×g, 15 min, 4 °C). CFEs were freeze-dried, stored at −20 °C for 1 week, and used in cheese manufacturing.

2.3. Enzymatic activity of microbial CFE

Initially, the CFE enzymatic activity from *L. casei* LC01, *H. alvei* ATCC51815, *D. hansenii* DSM70590, and *S. cerevisiae* A4 were assayed as described by Gobbetti et al. (1999) for aminopeptidase (EC 3.4.11.11, pepN) type N activity on Leu-*p*-nitroanilide (Leu-*p*NA), proline iminopeptidase (EC 3.4.11.9, pepI) activity on Pro-*p*-nitroanilide (Pro-*p*NA), and endopeptidase type O (EC 3.4.23, pepO) activity on Z-Gly-Pro-NH-trifluoromethylcoumarin (Z-Gly-Pro-NA). 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 (pepN) and 0.1 (pepI and pepO) at 37 °C and pH 7.0. Glutamate dehydrogenase (EC 1.4.1.2, GDH) activity was assessed 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 gamma-lyase (EC 4.4.1.1, CGL) 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 CFE esterase activity was determined using α-naphthyl butyrate as the substrate (De Angelis et al., 1999). A unit of esterase activity was defined as the amount of enzyme that released 1 μmol of α-naphthol per minute at 37 °C and pH 7.0 (560 nm). CFEs were normalized for the initial cell density of each strain (11 log CFU/mL). In addition, different CFE batches were normalized by enzymatic activity.

2.4. Manufacture of cheese

The ewe's milk used for cheese manufacture had the following characteristics: protein 4.3% (wt/wt), fat 5.6% (wt/wt), salt 0.08% (wt/wt), and pH 6.62. The same batches of ewes' milk were used for making 5 cheese variants as follows: control cheese (CC), without CFE addition; cheese supplemented with (i) CFE from *L. casei* LC01 and *H. alvei* Moller ATCC51815 (LH-C); (ii) CFE from *L. casei* LC01 and *D. hansenii* DSM70590 (LD-C); (iii) CFE from *L. casei* LC01, *H. alvei* Moller ATCC51815 and *D. hansenii* DSM70590 (LHD-C); and (iv) CFE from *L. casei* LC01, *H. alvei* Moller ATCC51815 and *S. cerevisiae* A4 (LHS-C). Cheesemaking was performed in a pilot plant belonging to the Department of Agriculture Sciences, Food and Environmental of the University of Foggia (Italy) on 3 consecutive days (total of 3 batches for each cheese variant) using ewes' milk from 3 daily milkings. After heating (62 °C for 15 s), ewes' milk was cooled at 37 °C and inoculated with commercial primary starters for *Streptococcus thermophilus* and *Lactococcus lactis* (initial cell density ~7.0 ± 0.2 log CFU/mL; Mediterranea Biotecnologie, Srl, Termoli, Campobasso, Italy). The inoculated milk was incubated at 37 °C for 45 min, then liquid calf bovine chymosin (30 imcu/L milk, CHY-MAX Plus, Chr-hansen) was added, and coagulation occurred within 30 min. After cutting (size of ~0.5–1.0 cm), the curd-whey mixture was incubated at 40 °C for ~10 min. After whey drainage, multiple CFEs were added to the curds (0.3% wt/wt for each CFE).