International Journal of Dairy Technology

SDT Society of Dairy Technology

doi: 10.1111/1471-0307.12493

ORIGINAL RESEARCH

Temporal microbiota and biochemical profiles during production and ripening of Divle Cave cheese

SEBNEM OZTURKOGLU-BUDAK,[1,2]* DIDEM P AYKAS,[3,4] CELALETTIN KOCAK,[1] SEDAT DÖNMEZ,[5] AYSE GURSOY,[1] RONALD P DE VRIES[2] and PETER A BRON[6]

[1]*Department of Dairy Technology, Faculty of Agriculture, Ankara University, Ankara 06110, Turkey,* [2]*Fungal Physiology, Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology, Utrecht University, Utrecht 3584 CT, The Netherlands,* [3]*Department of Food Science and Technology, The Ohio State University, 110 Parker Food Science and Technology Building, 2015 Fyffe Road, Columbus, OH, USA,* [4]*Department of Food Engineering, Faculty of Engineering, Adnan Menderes University, Aydin 09100, Turkey,* [5]*Department of Food Engineering, Faculty of Engineering, University of Ankara, Ankara 06110, Turkey, and* [6]*NIZO Food Research, Kernhemseweg 2, Ede 6718 ZB, The Netherlands*

The aim of this study was to evaluate the proteolysis and lipolysis profiles of a raw ewes' milk cheese during production and ripening. The microbial community of this cheese was quantified, and their roles during proteolysis and lipolysis were studied. We observed an accumulation of substantial amounts of peptides during ripening, which might be attributed to the abundant presence of lactic acid bacteria, as well as to secondary microbiota including fungi, yeasts and actinobacteria. Similarly, high lipolysis levels were obtained, which can be explained by the indigenous lipase in milk and microbial lipases. These results should be of great interest to industrial manufacturers of this cheese.

Keywords Raw sheep milk, Cheese, Proteolysis, Lipolysis, Microbial, Divle.

INTRODUCTION

Cheese ripening is a complex process and involves the biochemical conversion of milk components such as its lactose, proteins and fat (McSweeney and Sousa 2000). In industrial Gouda-type cheeses, the starter lactic acid bacterium (LAB), *Lactococcus lactis,* is added to the milk and largely responsible for casein degradation (Visser 1993). Following the primary biochemical events of proteolysis and lipolysis, secondary events occur, which include the metabolism of fatty acids and amino acids. Cheese flavour is mainly caused by these secondary reactions. *Lactococcus lactis* cells import the small peptides as a result of extracellular proteolysis. They are subsequently converted intracellularly into amino acids by the activity of intracellular peptidases. Subsequently, these amino acids are converted to flavour compounds by both enzymatic and other chemical actions (Savijoki *et al.* 2006).

When compared to industrial cheeses, the ripening of artisanally made raw milk cheeses is typically a spontaneous process in which flavour formation is established by the combined action of nonstarter LAB as well as other bacteria, filamentous fungi and yeast that are either derived from raw milk or introduced from the environment during the ripening period (Ozturkoglu-Budak *et al.* 2016a). These complex communities of microorganisms contribute to cheese ripening through proteolysis and peptidolysis (Cavanagh *et al.* 2014), by similar mechanisms as described for starter LAB above (Collins *et al.* 2003). Similarly, the microbiota in traditional cheeses has the capacity to degrade milk fat, a process termed lipolysis, resulting in free fatty acids (FFAs) of a wide range of sizes, of which the short-chain and medium-chain FFAs contribute directly to the flavour of cheese (Cavanagh *et al.* 2014). Hence, these microorganisms collectively establish the complex sensory properties of cheese through their diverse enzymatic systems (Mullan 2014). In addition, the fungal species, their corresponding enzymes and the dairy animals from which the milk is

*Author for correspondence. E-mail: budak@ankara.edu.tr

derived from affect the proteolysis and lipolysis processes in cheese, further diversifying the combinations of possible cheese flavours.

Divle Cave cheese is a traditional cheese derived from the spontaneous fermentation of raw ewes' milk. It is produced in May and June in Karaman, a rural region in the middle of Turkey. The ripening occurs in Divle Cave, which is located in the southern part of this region. The average temperature in this cave is 5–10 °C with a humidity of 85–90%. The cheeses are ripened for 4 months in goatskin bags at a depth of 70 m. The surface of the goatskin bags colour changes from green to red during ripening, and this red colour is considered the distinguishing characteristic of Divle Cave cheese (Ozturkoglu-Budak *et al.* 2016b). It is a semi-hard cheese, which has a crumbly texture and an intense flavour. The cheese has unique organoleptic characteristics caused by the production practices and environmental conditions during ripening in the cave.

Studies on the proteolysis and lipolysis levels of Divle Cave cheese have so far focused on final cheese product types (Hayaloglu and Karabulut 2013a; 2013b). Here, we set out to establish the temporal development of proteolysis and lipolysis profiles during production and ripening. Moreover, to identify the microbial taxa that probably play a role in proteolysis and lipolysis, the microbial groups present throughout the production and ripening process were quantified.

MATERIALS AND METHODS

Cheese production and sampling

The cheeses were produced as previously reported (Ozturkoglu-Budak *et al.* 2016b). Two independent batches were produced at each farm with an interval of 15 days. Samples were taken from the stages where the raw milk was obtained, the whey and curd were produced, and during cheese ripening at days 1, 30, 60, 90 and 120. In both independent batches produced, 30 different goatskin bags were analysed (five timepoints at three farms with technical duplicates), resulting in a total of 60 goatskin bags of cheese. Each cheese sample was made up of one goatskin bag (3–5 kg). Cheese samples obtained as a whole goatskin bag were taken to the laboratory at 4 °C and divided into different aliquots which were stored at −20 °C prior to analysis except for microbiological analyses which were analysed immediately after taken to the laboratory. Microbiological analyses were performed on the samples taken from the inside and outside of the cheese individually. However, the other analyses were performed on the mixture of the different parts of cheeses within goatskin bags in triplicate.

Microbial counts

Cheese samples were taken from inside (mid-points in interior part of cheese in goatskin bags) and outside (from the boundary part of cheese and goatskin bag again in the interior of goatskin bags). 10 mL milk and whey, and 10 g curd and cheese (inside and outside) were homogenised with 90 mL Ringer solution (Merck, Darmstadt, Germany) in a Stomacher (Bag Mixer 400 VW; Interscience, St Nom, France) for 3 × 1.5 min. Serial 10-fold dilutions were made and plated in duplicate.

Total aerobic mesophilic bacteria were determined on plate count agar (Merck) including 1% skimmed milk at 35 °C for 48 h. Filamentous fungi and yeasts were grown on potato dextrose agar (Merck) at 28 °C for 5–7 days. Presumptive lactobacilli were grown on de Man–Rogosa–Sharpe Agar (pH 5.4; Merck) and incubated for 72 h at 30 °C under anaerobic conditions. Presumptive lactococci were anaerobically grown on M17 agar (Merck) and incubated for 48 h at 30 °C. Enterobacteriaceae and total coliforms were grown on violet red bile dextrose agar (Merck) and violet red bile agar (Merck), respectively, using the pour-plate and overlay technique, and quantified after 48 h of incubation at 30 °C. Staphylococci were grown on Baird-Parker agar (Merck) supplemented with egg yolk tellurite solution (Merck), and black colonies were quantified after 24 h of incubation at 37 °C. Results were calculated in $\log_{10}$ cfu/mL for milk and whey and $\log_{10}$ cfu/g for curd and cheese.

Analysis of proteolysis

The cheese samples were treated to obtain pH 4.6-soluble nitrogen (pH 4.6-SN) extracts according to Kuchroo and Fox (1982). The peptides of the pH 4.6-soluble fraction of cheeses were determined using reversed-phase high-performance liquid chromatography (RP-HPLC, 1100 series; Agilent Technology, CA, USA) equipped with a UV detector (HP, 1100 series; Agilent Technology, CA, USA) at 214 nm and a C18 (4.6 cm × 250 mm × 5 μm particle size, 300 Å pore size) column. The pH 4.6-soluble fraction was dissolved in 0.2% trifluoroacetic acid (TFA; Sigma, St Louis, MO, USA) and deionised water (1:1) mixture, and 40 μL of sample was injected to the HPLC after being filtered through 0.45-μm Millex-HV filter with a flow rate of 0.75 mL/min.

Analysis of lipolysis

To determine the lipolysis products, FFAs of cheese samples were extracted following the method described by De Jong and Badings (1990). Analyses were carried out with a gas chromatograph (6890 Series; Agilent Technology) equipped with a flame ionisation detector (FID) at 260 °C and using a capillary TR-FFAP column (30 m × 0.25 mm × 0.25 μm; Agilent Technology). The output signal from the detector was integrated using HP 6890 ChemStation software. For the calculation of the FFA levels, calibration curves were determined at five concentrations using a mixture of individual FFA standards, from butyric (C4:0) to

linolenic (C18:3) fatty acids, with fixed concentrations of internal standards of valeric acid (C5:0), heptanoic acid (C7:0) and heptadecanoic acid (C17:0; Sigma-Aldrich). The FFA concentrations were calculated in mg/kg.

Statistical analysis

Analysis of variance (ANOVA) was performed on all data obtained from three different dairy farms (DF) and two batches at each stage of production and ripening using SPSS Statistics software version 22.0 (International Business Machines Corp., Armonk, NY, USA). One-way ANOVA, followed by Duncan's multiple comparison test, was used for statistical analysis for the comparison of data, and evaluations were based on a significance level of $P < 0.05$. Principal component analysis (PCA) was performed using a covariance matrix and varimax rotation with Kaiser normalisation.

RESULTS AND DISCUSSION

Assessment of microbial groups

Our earlier work has established which microbial species are present in matured Divle Cave cheese (Ozturkoglu-Budak *et al.* 2016a). However, these endpoint analyses were qualitative, and microbial species that are abundant earlier in the ripening process might also be more important for proteolysis and lipolysis. Therefore, we quantified the total number of aerobic mesophilic bacteria (TAMB), lactobacilli, lactococci, Enterobacteriaceae, total coliforms, staphylococci, filamentous fungi and yeast counts by a culture-dependent approach. The data obtained at various stages of the production (raw milk, whey and curd) and ripening (1st, 30th, 60th, 90th and 120th days) are shown in Figure 1. Results also indicated the levels of microbiota from the inside and outside of the cheese and their development during the ripening period. Although the absolute numbers are variable between samples from the inside and outside of the goatskin, the ratio of the seven microbial groups appears relatively stable, suggesting that microbiota members are predominantly introduced through the raw milk and as contaminants during the production rather than the ripening.

The TAMB decrease could have been caused by a decrease in pH and an increase in salt concentration during ripening (hence a decrease in the water activity), and the outgrowth of lactic acid bacteria as a competing member of the microbiota (Ozturkoglu-Budak *et al.* 2016a). Similar findings were reported in ewes' cheese (Galan *et al.* 2012), and some other Turkish cheeses ripened in skin (Hayaloglu and Kirbag 2007).

The numbers of lactobacilli demonstrated a rapid increase in the interior part of goatskin and reached their highest values on the 60th day of ripening, after which a decline until the end of ripening was observed. The samples taken from the outside showed lower *Lactobacillus* counts, probably due to competition for nutrients with fungi and yeasts.

Initial numbers of lactococci from milk plated on M17 medium were 5.2 log cfu/mL and attained the highest levels on the first day of ripening (8.4 log cfu/g). The numbers decreased until the end of ripening to a final value of 6.4 log cfu/g. The level of decrease was observed as 2.0 and 1.7 logs from the inside and outside samples, respectively. These results are in good agreement with an earlier study (Alegría *et al.* 2009) in which the lactococci were determined at the highest level in Spanish Casin cheese at day 7 of the ripening process.

Enterobacteriaceae, total coliforms and staphylococci are indicators of inadequate hygiene and sanitation (Alegría *et al.* 2009). These microbial groups were high at the beginning of ripening but showed a gradual decrease during

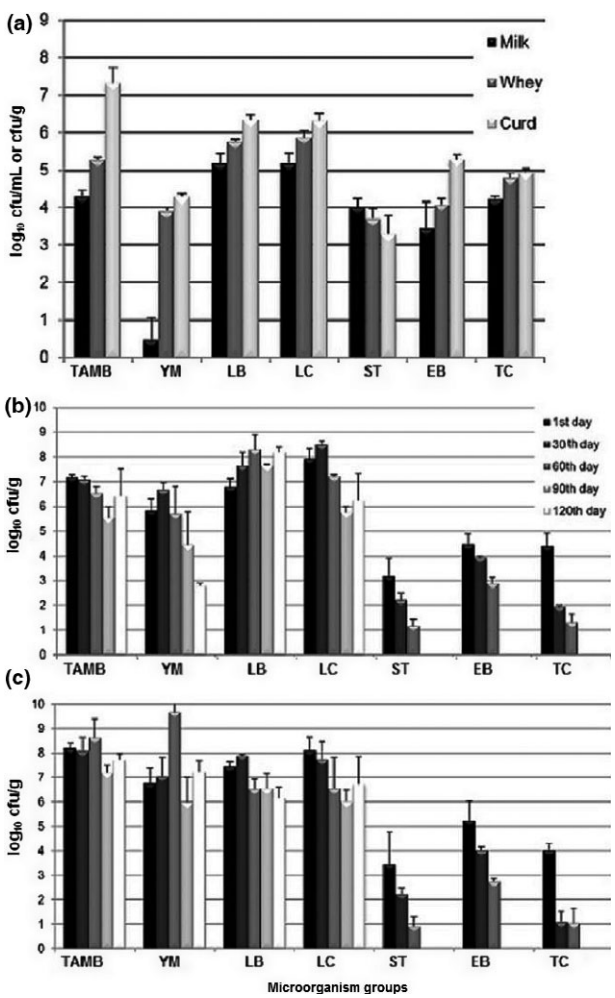

Figure 1 Average microbial counts ($\log_{10}$ cfu/mL or $\log_{10}$ cfu/g ±SD). (a) Raw milk, whey and curd during production; (b) cheese samples taken from inside of cheese in goatskin bags; (c) cheese samples taken from outside of cheese in goatskin bags in two replicate trials of three independent batches (TAMB, total aerobic mesophilic bacteria; YM, yeast and filamentous fungi; LB, lactobacilli; LC, lactococci; ST, staphylococci; EB, Enterobacteriaceae; TC, total coliforms).

ripening and were completely lost from the 90th day of ripening onwards. These results are similar with the ripening of other cheeses (Galan *et al.* 2012), in which Enterobacteriaceae and staphylococci were not detected after the 60th day of ripening in ewes' cheese. The reason for this loss of detection could be caused by growth of lactic acid bacteria, filamentous fungi and/or yeasts, which results in decreased pH and possibly the production of antibacterial compounds (Hayaloglu *et al.* 2007).

The count of filamentous fungi and yeasts decreased in the inside (1st day: 5.2–5.8 log cfu/g, 120th day: 2.5–3.2 log cfu/g) of the cheese during the ripening period. In contrast, the concentration on the outer side of goatskin increased as ripening proceeds. At the end of ripening, the outer part counts (7.6 log cfu/g) were approximately 10^3-fold higher than those in the inside (3.3 log cfu/g). The decreased counts in the inside of the cheese can be explained by the anaerobic condition occurring inside the cheese, decreased humidity, increased salt in dry matter and the changing of pH that we determined in our previous study (Ozturkoglu-Budak *et al.* 2016b). Moreover, it was previously determined that some *Lactobacillus* species such as *Lactobacillus coryniformis* (Magnusson *et al.* 2003) and *Lactobacillus plantarum* (Ryu *et al.* 2014) produce antifungal compounds. The increase in the outside of samples results from the growth of natural fungi on the surface of the goatskin originating from the cave and the nonhomogenous transition of these fungi into the underlying layers.

Assessment of proteolysis

Soluble nitrogen at pH 4.6 (pH 4.6-SN) was the parameter used as an index for the extent of proteolysis (Diezhandino *et al.* 2015). The RP-HPLC peptide profiles of the peptide fractions of curd and cheeses at days 1, 30, 60, 90 and 120 of ripening are shown in Figure 2. In this study, total peptide analyses were performed in 85 min. Peptides that eluted in the early retention time (from 5 to 30 min) corresponded to hydrophilic peptides, while peptides that eluted in the late retention times (from 55 to 85 min) corresponded to hydrophobic peptides. At the intermediate time (from 30 to 55 min), low molecular weight hydrophobic peptides eluted (Hayaloglu and Karabulut 2013a). Fragmentation of macropeptides into micropeptides and amino acids is represented by a decrease in peak height and area in the hydrophobic zone.

In the intermediate region of the chromatogram, many peaks occurred during the course of ripening, which indicated that hydrophobic peptides are most dominant in matured Divle Cave cheese. New peptides were produced, while others decreased or disappeared at different ripening stages (Figure 2). The peptide eluted at 38th minute was determined as being the most prominent in all the cheese samples, followed by peptides that eluted at 26th, 37th, 64th and 84th minutes. The number and the level of peptides progressively increased during ripening, as already existing peaks increased in area and new ones appeared. A clear increase was observed at the 60th day of ripening. In our previous work detecting the microbiota of Divle Cave cheese, we found that on day 60, fungal strains began to increase both in number and in diversity (Ozturkoglu-Budak *et al.* 2016a), which may account for the additional proteolysis.

The changes in the hydrophilic region are an indicator of the hydrolysis of proteins to peptides and amino acids. According to the chromatogram that we obtained in our study (Figure 2), peaks eluted in this region at retention times of 17, 26 and 29 min showed an apparent increase from days 1 to 120. Moreover, a peak that eluted at 19 min first appeared on day 60 and continued to increase until the end of ripening. In the intermediate region, the most significant increase was observed in peak that eluted at min 37. The peaks that eluted at 36 and 38 min showed an increase during ripening. Also in this region, a peak that eluted at 49.5 first appeared on day 60 and increased until the end of ripening. In the hydrophobic region, the most prominent peaks were determined at 81 and 84 min.

Amino acid catabolism is particularly significant in mould- and smear-ripened cheese varieties and an indicator of extensive proteolysis (Fox *et al.* 2000). The enzymatic conversion of amino acids such as leucine, isoleucine and valine is catalysed by aminotransferase enzymes, and they form the carboxylic acids of 3-methyl butanoic (isovaleric acid), 2-methyl butanoic and 2-methyl propanoic acid (isobutyric acid), respectively (Smit *et al.* 2000). Further aldehydes can be formed from these acids and then can be reduced to the corresponding alcohols (McSweeney 2004). The presence of 3-methyl butanoic acid, 2-methyl propanoic acid, 3-methyl butanal, 2-methyl propanal, 2-butanol and 2-propanol in our analysis (Ozturkoglu-Budak *et al.* 2016b) indicated that amino acid catabolism is developing as a result of progressed proteolysis.

Principal component analysis of the peptide profiles

Principal component analysis of the peptide profiles revealed that the samples clearly separated according to their age (Figure 3). The variables used for PCAs were areas of the peaks obtained from chromatogram. Curd and cheese samples on days 1 and 30 had similarities in their peptide profiles, whereas cheese samples obtained on days 60, 90 and 120 had higher peak areas/levels compared to previous stages of ripening. Moreover, specific subtle differences were seen among the cheeses produced in different DF that might be due to variations in production techniques and possible diversity in microbial communities and their corresponding enzyme pallet during the uncontrolled ripening periods. Samples obtained early in the ripening period (1 and 30 days) were characterised by more hydrophobic peptides. In contrast, for the samples obtained later on

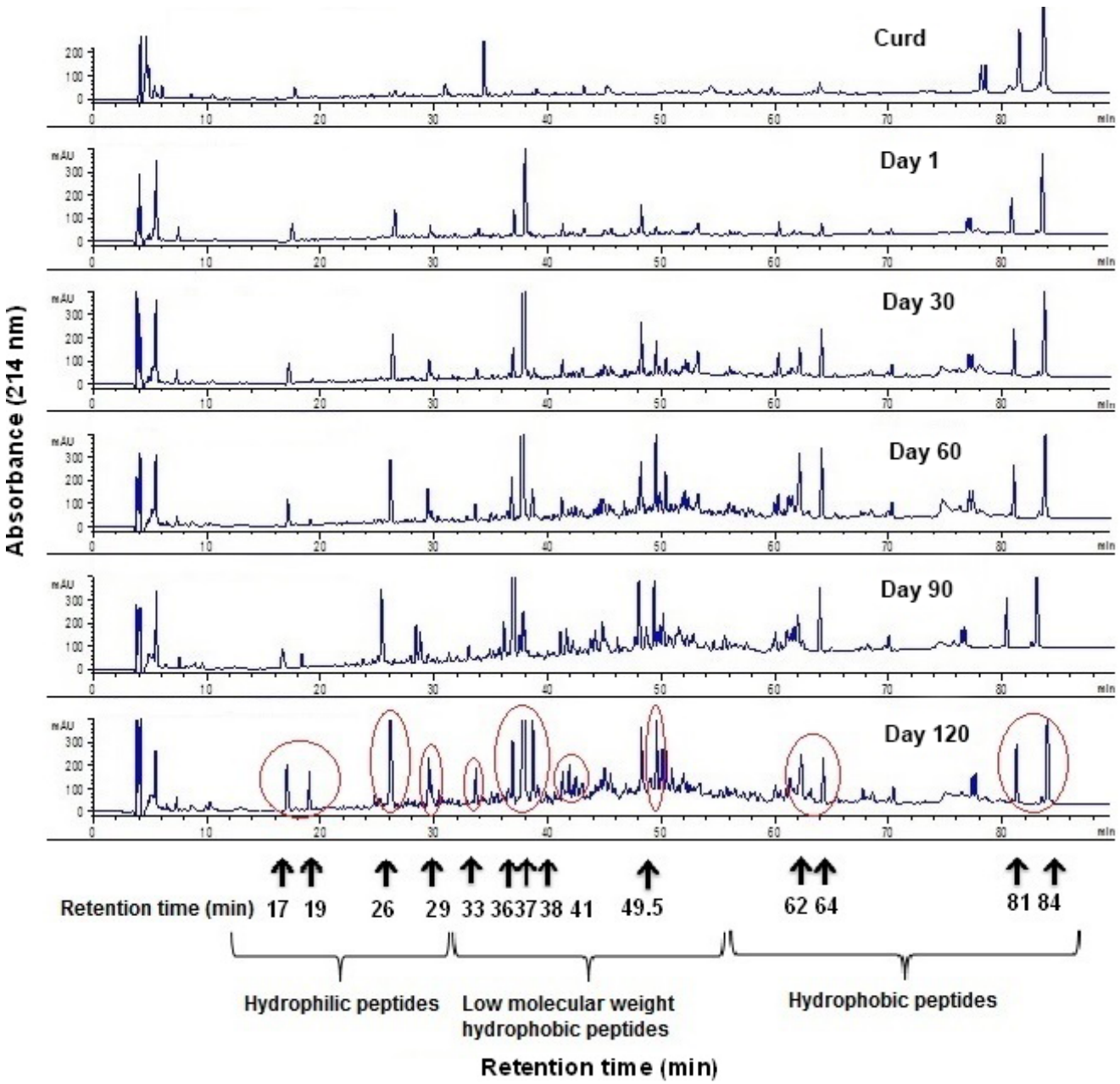

Figure 2 Peptide profiles resulting from proteolysis in Divle Cave cheese. [Colour figure can be viewed at wileyonlinelibrary.com]

ripening (60, 90 and 120 days), more hydrophilic peptides were observed.

Similarly, in studies of other fungal cheeses (Blue cheese), a decrease was observed in the ratio of hydrophobic and hydrophilic peptides during the ripening period (Diezhandino *et al.* 2015). The ratio of hydrophobic and hydrophilic peptides in blue-veined cheeses was determined lower than those observed in other varieties due to the action of exopeptidases, which degrade hydrophobic peptides and release low molecular weight peptides and amino acids (Gonzales De Llano *et al.* 1995).

Assessment of lipolysis

The FFA composition of different types of cheeses are highly variable and depend on the composition of milk, the production and ripening technology employed, and the degree of lipolytic activity during the ripening period. In particular, sheep milk cheeses and mould-ripened cheeses have higher values of total FFA and are characterised by an intense lipolysis (Svensson *et al.* 2006).

In this study, the free fatty acid concentrations of Divle Cave cheese produced in three DF were determined at the stages of raw milk, whey, curd and cheeses after 1, 30, 60,

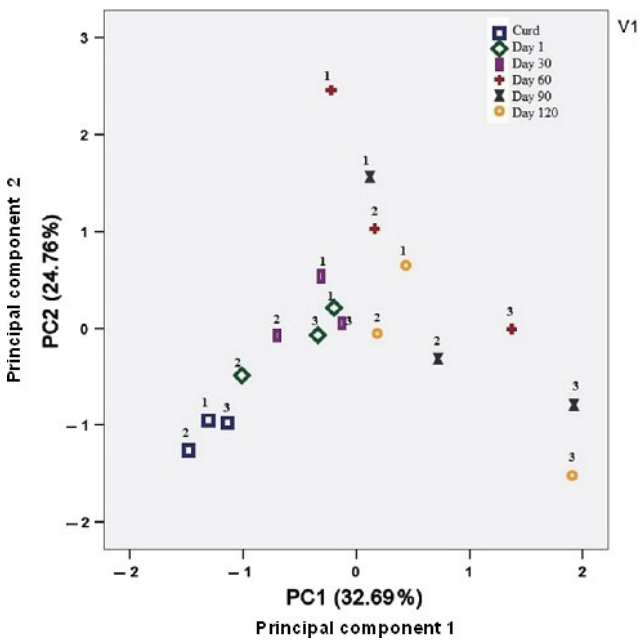

Figure 3 Score plot of principal component analysis based on peptide profile data (RP-HPLC) of curd and cheese on days 1, 30, 60, 90 and 120 during the ripening of Divle Cave cheese. [Colour figure can be viewed at wileyonlinelibrary.com]

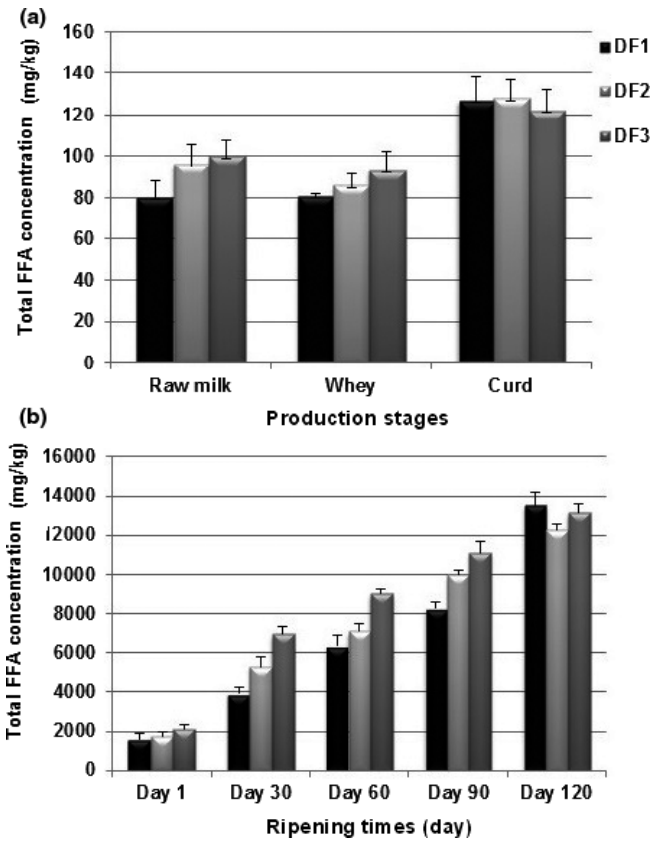

Figure 4 Total free fatty acid concentrations (a) during production, (b) during ripening of Divle Cave cheese.

90 and 120 days of ripening. Accumulated FFA compounds during the ripening period are considered representative of the degree of lipolysis (Svensson *et al.* 2006). The individual FFAs are classified into short- (C4:0–C8:0, SCFA), medium- (C10:0–C12:0, MCFA) and long-chain fatty acids (C14:0–C18:3, LCFA; Collins *et al.* 2003).

In Divle Cave cheese, the ratio of LCFA to total fatty acids determined at day 120 compared to day 1 was higher than the increase obtained for MCFA and SCFA (Table S1). In particular, high concentrations of fatty acids with carbon numbers greater than 16 were observed, including palmitic (C16:0), stearic (C18:0), oleic (C18:1) and myristic (C14:0) acids. Also the MCFA concentration changed significantly as the cheese aged. These results may have relevance for the characteristic taste of long-matured Divle Cave cheese, which includes 15 different strains of *Penicillium* spp. (Ozturkoglu-Budak *et al.* 2016a). Moreover, Atasoy and Türkoğlu (2009) determined that cheeses made without starter culture underwent significantly higher lipolysis than cheeses produced with mesophilic or thermophilic starter bacteria.

Figure 4 shows the total FFA concentrations of cheeses produced in three different DFs during production and ripening. Individual FFAs were determined at low levels in raw milk, curd and whey, and a progressive increase was observed in all FFAs during ripening (Table S1). This result could be associated with physical characteristics of fatty acids. Water solubility of fatty acids is associated with carbon number, and fatty acids with smaller carbon number are more soluble in water while solubility steadily declines as the carbon number increases and fatty acids with carbon number >C10 are less soluble in water (Kayahan 1998).

The progression of lipolysis throughout production and ripening was consistent with earlier studies of sheep milk cheese (Mallatou *et al.* 2003) and Blue cheese (Diezhandino *et al.* 2015). Similarly, a steady increase in FFAs content was determined in a range of cheeses such as Reggianito Argentino (Wolf *et al.* 2010) and Turkish cheeses (Hayaloglu and Karabulut 2013b) during ripening.

The highest increase in total FFA value was observed in the cheese sample produced in DF3. The reasons for this difference are likely to be the inability to produce standardised traditional cheeses, the variability in fat content of milk and the variation in lipolytic activity in the milk of different sheep or originating from the microbiota introduced during production and ripening phases. Similar to our results, Wolf *et al.* (2010) detected significant differences in lipolysis levels among cheeses produced in different dairy factories.

PCA of free fatty acid profiles

The PCA of FFA demonstrated that cheeses separated mainly according to ripening time (Figure 5). Due to extremely low concentrations of FFAs in raw milk and curd,

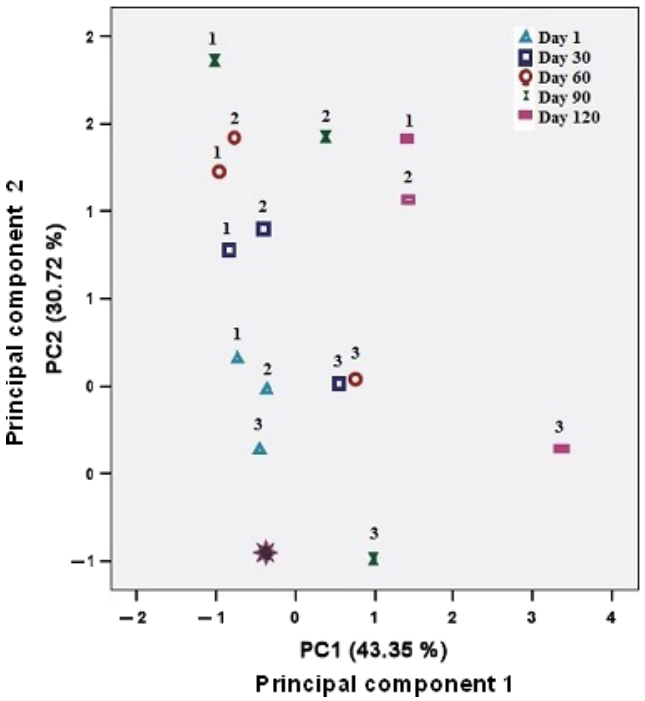

Figure 5 Score plot of principal component analysis based on free fatty acid data (GC) of cheese on days 1, 30, 60, 90 and 120 during the ripening of Divle Cave cheese. [Colour figure can be viewed at wileyonlinelibrary.com]

they were not included in the score plot. At day 1, cheeses produced in different DF grouped together, but when the concentrations of fatty acids increased in the processing stages, larger variations became visible. The PCA confirmed that cheeses produced in DF1 and DF2 were more similar to each other than to those from DF3.

Analyses of the total FFA amounts indicated that Divle Cave cheese undergoes a significant lipolysis during ripening. The intense lipolysis level determined in this cheese could be explained by the indigenous lipase of the milk and by microbial lipases especially present in yeast early in ripening (Ozturkoglu-Budak *et al.* 2016a) and fits the main contributors to lipolysis for raw milk cheeses previously described (McSweeney 1997). Moreover, during the protease and lipase assays of individual strains of microbiota isolated from this cheese indicated that yeast and fungal strains in particular have a high lipase activity (Ozturkoglu-Budak *et al.* 2016c).

CONCLUSION

This work provides the first characterisation of proteolysis and lipolysis of Divle Cave cheese during the production and ripening processes. Although the actual chemical components were not identified, the degree of lipolysis and proteolysis generally increased during the whole ripening period. The intense proteolysis and lipolysis at the end of ripening could be due to contributions of the secondary microbiota (particularly yeast, filamentous fungi and actinobacteria) that enters the product from the air or contaminated tools, but also from native proteases, lipases and esterases present in raw milk. With this study, we provide data for the characterisation of a raw milk artisanal cheese which is of great importance for the determination of geographical origin and authentication of traditional cheeses. Moreover, these results are of importance for the eventual standardised manufacture of this cheese under controlled conditions and on an industrial scale.

ACKNOWLEDGEMENTS

This work has been financially supported by Scientific Research Projects Unit of Ankara University, Turkey, and IDB Merit PhD-Scholarship Programme for High Technology.

REFERENCES

Alegría Á, Álvarez-Martín P, Sacristán N, Fernández E, Delgado S and Mayo B (2009) Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk. *International Journal of Food Microbiology* **136** 44–51.

Atasoy A F and Türkoğlu H (2009) Lipolysis in Urfa cheese produced from raw and pasteurized goats' and cows' milk with mesophilic or thermophilic cultures during ripening. *Food Chemistry* **115** 71–78.

Cavanagh D, Kilcawley K N, O'Sullivan M G, Fitzgerald G F and McAuliffe O (2014) Assessment of wild non-dairy lactococcal strains for flavour diversification in a mini-Gouda type cheese model. *Food Research International* **62** 432–440.

Collins Y F, McSweeney P L H and Wilkinson M G (2003) Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. *International Dairy Journal* **13** 841–866.

De Jong C and Badings H T (1990) Determination of free fatty-acids in milk and cheese- Procedures for extraction, clean-up, and capillary gas-chromatographic analysis. *Hrc-Journal of High Resolution Chromatography* **13** 94–98.

Diezhandino I, Fernández D, González L, McSweeney P L H and Fresno J M (2015) Microbiological, physico-chemical and proteolytic changes in a Spanish blue cheese during ripening (Valdeón cheese). *Food Chemistry* **168** 134–141.

Fox P F, Guinee T P, Cogan T M and McSweeney P L H (2000) *Fundamentals of Cheese Science*, 1st edn. Gaithersburg, MD: Aspen Publishers Inc.

Galan E, Cabezas L and Fernandez-Salguero J (2012) Proteolysis, microbiology and sensory properties of ewes' milk cheese produced with plant coagulant from cardoon *Cynara cardunculus*, calf rennet or a mixture thereof. *International Dairy Journal* **25** 92–96.

Gonzales De Llano D, Ramos M, Polo C, Sanz J and Martinez-Castro I (1995) Evolution of the volatile components of an artisanal Blue cheese during ripening. *Journal of Dairy Science* **73** 1676–1683.

Hayaloglu A A and Karabulut I (2013a) Primary and secondary proteolysis in eleven Turkish cheese varieties. *International Journal of Food Properties* **16** 1663–1675.

Hayaloglu A A and Karabulut I (2013b) Characterization and comparison of free fatty acid profiles of eleven varieties of Turkish cheeses. *International Journal of Food Properties* **16** 1407–1416.

Hayaloglu A A and Kirbag S (2007) Microbial quality and presence of moulds in Kuflu cheese. *International Journal of Food Microbiology* **115** 376–380.

Hayaloglu A A, Cakmakci S, Brechany E Y, Deegan K C and McSweeney P L H (2007) Microbiology, biochemistry, and volatile composition of Tulum cheese ripened in goat's skin or plastic bags. *Journal of Dairy Science* **90** 1102–1121.

Kayahan M (1998) Lipids. In *Food Chemistry*, pp 107–194. Acar J, ed. Ankara: Hacettepe University, Sidas Press.

Kuchroo C N and Fox P F (1982) Fractionation of the water-soluble nitrogen from cheddar cheese-Chemical methods. *Milchwissenschaft-Milk Science International* **37** 651–653.

Magnusson J, Ström K, Roos S, Sjögren J and Schnürer J (2003) Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters* **219** 129–135.

Mallatou H, Pappa E C and Massouras T (2003) Changes in free fatty acids during ripening of Teleme cheese made with ewes', goats', cows' or a mixture of ewes' and goats' milk. *International Dairy Journal* **13** 211–219.

McSweeney P L H (1997) The flavour of milk and dairy products: III. Cheese: Taste. *International Journal of Dairy Technology* **50** 123–128.

McSweeney P L H (2004) Biochemistry of cheese ripening. *International Journal of Dairy Technology* **57** 127–144.

McSweeney P L H and Sousa M J (2000) Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait* **80** 293–324.

Mullan W M A (2014) Starter cultures: Importance of selected genera. In *Encyclopedia of Food Microbiology*, 2nd edn, pp 515–521. Tortorello C A B L, ed. Oxford: Academic Press.

Ozturkoglu-Budak S, Figge M J, Houbraken J and de Vries R P (2016a) The diversity and evolution of microbiota in traditional Turkish Divle Cave cheese during ripening. *International Dairy Journal* **58** 50–53.

Ozturkoglu-Budak S, Gursoy A, Aykas D P, Koçak C, Dönmez S, de Vries R P and Bron P A (2016b) Volatile compound profiling of Turkish Divle Cave cheese during production and ripening. *Journal of Dairy Science* **99** 1–12.

Ozturkoglu-Budak S, Wiebenga A, Bron P A and de Vries R P (2016c) Protease and lipase activities of fungal and bacterial strains derived from an artisanal raw ewe's milk cheese. *International Journal of Food Microbiology* **237** 17–27.

Ryu E H, Yang E J, Woo E R and Chang H C (2014) Purification and characterization of antifungal compounds from *Lactobacillus plantarum* HD1 isolated from kimchi. *Food Microbiology* **41** 19–26.

Savijoki K, Ingmer H and Varmanen P (2006) Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology* **71** 394–406.

Smit G, Verheul A, van Kranenburg R, Ayad E H E, Siezen R J and Engels W (2000) Cheese flavour development by enzymatic conversions of peptides and amino acids. *Food Research International* **33** 153–160.

Svensson I, Hernández I, Virto M and de Renobales M (2006) Determination of lipase activity in cheese using trivalerin as substrate. *International Dairy Journal* **16** 423–443.

Visser S (1993) Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. *Journal of Dairy Science* **76** 329–350.

Wolf I V, Perotti M C, Bernal S M and Zalazar C A (2010) Study of the chemical composition, proteolysis, lipolysis and volatile compounds profile of commercial Reggianito Argentino cheese: Characterization of Reggianito Argentino cheese. *Food Research International* **43** 1204–1211.

SUPPORTING INFORMATION

The following supporting information is available for this article:

Table S1 Free fatty acid concentrations (mg/kg) of Divle Cave cheese during production and ripening (mean ± SD).