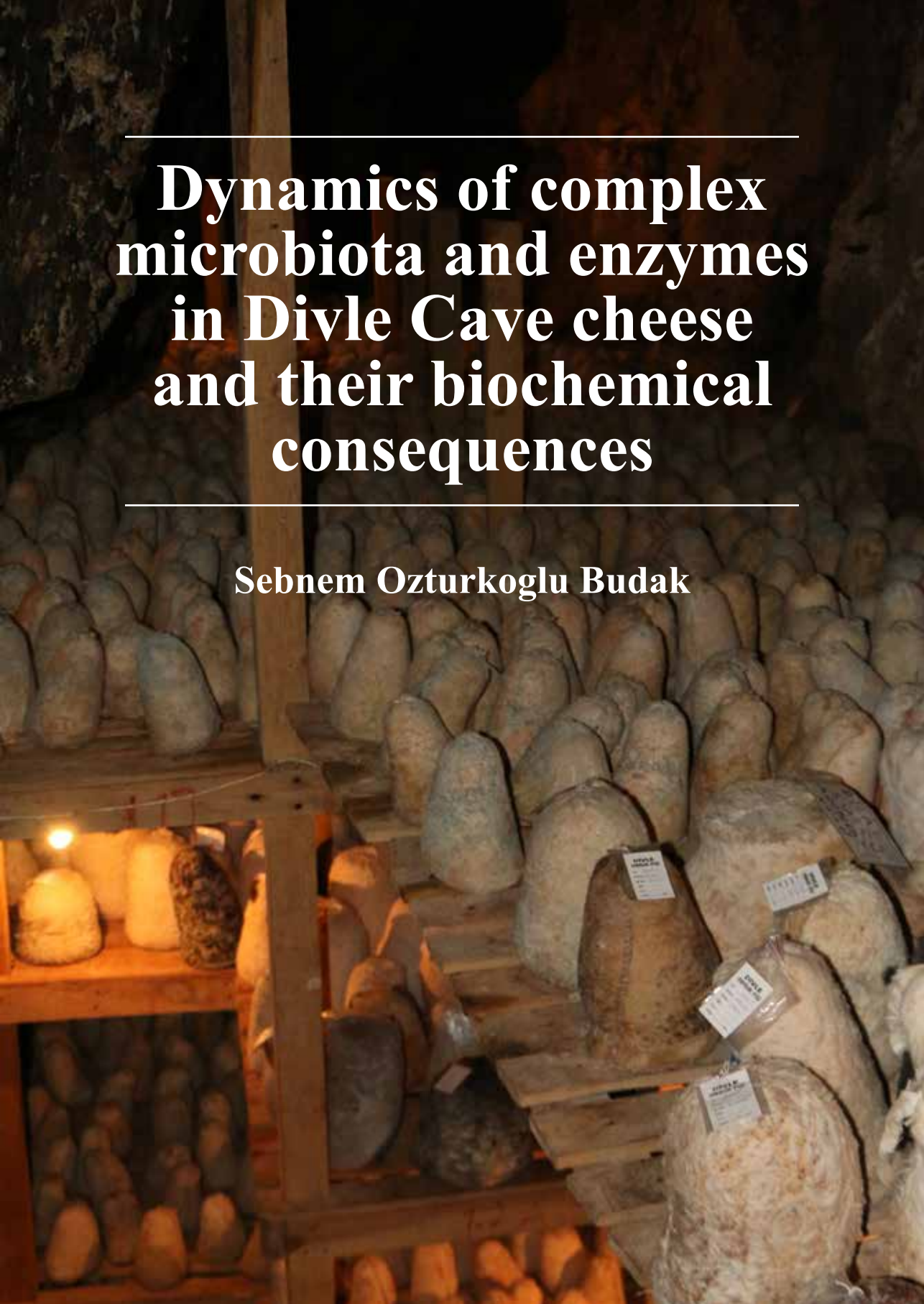

Dynamics of complex microbiota and enzymes in Divle Cave cheese and their biochemical consequences

Sebnem Ozturkoglu Budak



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**Dynamics of complex microbiota and
enzymes in Divle Cave cheese and their
biochemical consequences**

**De dynamiek van complexe microbiota
en enzymen in Divle Cave kaas en hun
biochemische consequenties**

(met een samenvatting in het Nederlands)

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For the memory of my lovely mother and father

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CHAPTER 1

General Introduction

Cheese Ripening

Cheese ripening is a complex process that influences the chemical, physical, microbiological, rheological and textural properties of cheese. The most eminent outcome of cheese ripening is the differential development of the flavor and textural characteristics of the huge variety of cheeses that are produced throughout the world. How the cheese ripens and how flavor/texture development progresses depends mainly on the starter bacteria inoculum and non-starter bacteria naturally occurring in milk (Beresford et al., 2001; Settanni & Moschetti, 2010). Several aspects are important in cheese production and ripening, and contribute to the specific properties of each cheese type (El Soda et al., 2000). These aspects are discussed below.

Role of microorganisms in cheese ripening

There are two different types of starter cultures used in cheese productions. One type is the defined starter culture, while the others are complex undefined starter cultures. Defined starter cultures are usually composed of one or more strains with known characteristics. The individual strains in defined starter cultures are usually isolated from undefined complex starter cultures to obtain single strain cultures (Smid et al., 2014). Several genera of LAB including *Lactobacillus delbrueckii* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc* spp., *Streptococcus thermophilus* and *Lactobacillus helveticus* (Cogan, 2014) can be considered as defined starter LAB, whereas the undefined starter cultures usually originated from cheese production plants, environments or farms (Smid et al., 2014). During artisanal production runs, undefined cheese starter LAB are typically added by the transfer of an inoculum from an old batch of cheese to the new batch (back-slopping) to initiate the fermentation with the activity of the indigenous microbiota present in the inoculum. However, during industrial production, consistent and controlled processes are required in order to ensure a safe and standardized final product quality (Erkuş et al., 2013). A significant difference between defined starter cultures and undefined complex starters is their sensitivity to bacteriophages. During cheese production, defined starter cultures are generally more vulnerable to bacteriophage attack than undefined complex starters (Stadhouders, 1986). This property as well as their function as a source for the isolation of new dairy strains, explains the interest in undefined complex starter cultures (Erkuş et al., 2013).

The starter cultures involved in cheese production and ripening can originate from milk or they can be intentionally or unintentionally added to cheese production.

Intentionally added microorganisms

These are microorganisms that are carefully selected and deliberately added to milk or cheese during production. They can be divided into two groups:

Primary cultures

These microorganisms are also called as ‘starter bacteria’, ‘lactic cultures’ or starter lactic acid bacteria (LAB) because of their properties to start (initiate) the production of lactic acid from lactose (Parente & Cogan, 2004). In this respect, the primary role of starter bacteria during cheese production is to swiftly acidify the milk and decrease its pH by the production of lactate from lactose. This pH decrease is also required for rennet activity during coagulation. pH also influences the cell lysis, and this release of a vast range of enzymatic activities is the principal factor enabling starter cultures to accelerate the cheese ripening. Intracellular enzymes of LAB, such as proteases and lipases that are released into the cheese matrix due to cell lysis by mechanical, physical and chemical factors, have been shown to participate in the breakdown of peptides, amino acids and fatty acids (Parente & Cogan, 2004). During cheese ripening, these biochemical reactions influence the textural and sensory properties of cheese (Collins et al., 2003; Ong & Shah, 2008; Singh et al., 2003).

Secondary (Adjunct) cultures

These microorganisms are deliberately added as adjuncts to blends of the primary acid-producing starter cultures (Parente & Cogan, 2004). They have no function in lactic acid production and are only involved in ripening. Their major role is to contribute to the biochemical changes through their diverse enzymatic systems. Secondary cultures can be bacteria, yeast or fungi (El-Soda et al., 2000). They can be incorporated into milk or applied deliberately on to the surface of cheese (Fox et al., 2000; Cogan, 2014). *Brevibacterium linens*, *Arthrobacter* spp. and coryneform bacteria in surface ripened cheeses such as Tilsit, Munster and Limburger, *Penicillium roqueforti* in Blue-veined cheeses, *Penicillium camemberti* in Camembert cheese, and *Propionibacterium freudenreichii* subsp. *shermanii* in Swiss-type cheese are the most employed secondary strains. Moreover, *Geotrichum candidum*, *Debaryomyces*

hansenii, *Kluyveromyces* spp. are other examples of strains that are deliberately inoculated on the surface of cheeses.

Unintentionally added (adventitious) microorganisms

Non-starter lactic acid bacteria (NSLAB) are naturally occurring microorganisms present in milk or beneficial contaminants originated from cheese production environments. They display high tolerance to the hostile environment and strongly influence the biochemistry of curd maturation, contributing to development of the final characteristics of cheese (Settanni & Moschetti, 2010). The presence of NSLAB in cheese was reported to lead to an increase in the level of small peptides and amino acids in cheese (El Soda et al., 1999) and consequently they were reported to play a role in secondary events, such as flavor formation (Bhowmik & Marth, 1990) in certain types of cheese varieties, particularly artisanal cheeses (Beresford et al., 2001). Among NSLAB, *Lactobacillus* strains are the most common and can be found in relatively high numbers. *L. casei*, *L. paracasei*, *L. plantarum* and *L. curvatus* are the predominant species. In addition, non-*Lactobacillus* species of NSLAB include *Pediococci* (*Pediococcus acidilactici*, *Pediococcus pentosaceus*) and *Enterococci* (*Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*), but they are typically present in relatively low numbers (Chamba & Irlinger, 2004; Fox et al., 1998).

Other than the favourable effects, NSLAB also introduce variability into the ripening process that can result in fluctuations in the final characteristics (Franciosi et al., 2008). Due to this properties, significant differences were reported between cheeses produced at the same factory on different days and between cheeses from different vats of the same day (Fitzsimons et al., 1999). To minimize microbial variability during the ripening process, the dominance of desired NSLAB could be selected and used as a secondary culture (Settanni & Moschetti et al., 2010). However, their health properties should also be considered when selecting strains as secondary cultures (Settanni & Moschetti et al., 2010).

Apart from NSLAB, secondary microbiota might also be adventitious in some cases, depending on the selective conditions such as pH, humidity, temperature and water activity, although they are described as selected microorganisms that are inoculated to milk or curd (Fox et al., 2000).

Role of enzymes during cheese ripening

In general, the main flavor forming pathways in cheese are catalyzed by enzymes originating from: (i) milk (ii) coagulant, (iii) starter LAB, (iv) secondary/adjunct culture, (v) NSLAB, and (vi) exogenous enzymes. These enzymes play a principal role in the formation of amino acids and fatty acids that contribute directly to the basic taste of cheese and indirectly to cheese flavor because of their role as precursors for aroma components (McSweeney, 2004).

Indigenous (native) milk enzymes

These enzymes originate mainly from leakage of blood or immune system components into the milk. The key indigenous protease enzymes of milk include plasmin (milk alkaline protease) and cathepsin D (milk acid proteinase). The hydrolysis of milk proteins by proteases affects the texture and flavor of dairy products. This can have either beneficial or detrimental effects, depending on the extent of hydrolysis and type of dairy product (Fox et al., 1993). Plasmin and cathepsin D act on β - and α_{s1} -casein, respectively. Plasmin occurs in milk along with its precursor plasminogen (inactive form of plasmin) and is associated with the casein micelles (Bastian & Brown, 1996). As for cathepsin D, it originates from the lysosomes of somatic cells (Wilkinson & Kilcawley, 2005). Similar to indigenous proteases, lipoprotein lipase (LPL) is the indigenous milk lipase (Fox & Stepaniak, 1993) and originates from blood where it participates in the lipolysis and off-flavors in cheese. LPL is rather nonspecific for the type of fatty acid but is specific for sn1 and sn3 positions of mono-, di- and triglycerides. Therefore, lipolysis in milk leads to preferential release of short- and medium-chain fatty acids, which in milk triglycerides are esterified predominantly at the sn3 position.

Coagulant

Addition of milk clotting enzymes such as chymosin and pepsin are able to hydrolyze peptide bonds and they are responsible for the coagulation during cheese production (Visser, 1993) by the specific cleavage of the Phe105-Met106 bond of κ -casein, which destabilizes the casein micelle structure (Yegin et al., 2011). Enzymatic milk coagulation is a two-phase process. In the first phase, the enzyme hydrolyzes the κ -casein molecule and splits the protein into two fragments: 1. hydrophobic para- κ -casein, 2. hydrophilic macropeptide. The second phase consists of the coagulation

of the casein micelles that have been destabilized by proteolytic attack (Claverie-Martin & Vega-Hernández, 2007).

Other than chymosin from mammalian species (mainly bovine), there are also commercially available ones produced by *Rhizomucor* spp. have gained wide industrial acceptance as substitutes for the bovine chymosin (Claverie-Martin & Vega-Hernández, 2007). However, it is known that fungal rennet preparations may present a degree of residual proteolytic activity that subsequently participates in proteolysis of the caseins during ripening and results in the production of bitter peptides during cheese ripening (Yegin et al., 2011)

Indigenous enzymes and coagulants are not stable against heat treatments. Inactivation of indigenous protease (Farkye & Imafidon, 1995) and lipase (Deeth & Fitz-Gerald, 1983) was observed during heat treatments of milk or curd. It was reported that heating above 65°C resulted in reduced activity in plasmin (Metwalli et al., 1998). Another study performed by (Deeth & Fitz-Gerald, 1983) demonstrated that activity of LPL in raw milk cheeses was extensively inactivated at 72°C for 15 seconds. Rennet enzymes can also be inactivated at the curdling stage and are inhibited by added salt. Hayes et al., (2002) reported that chymosin activity showed minimal inactivation during the production of Swiss-type cheese, whereas after cooking residual chymosin activity in whey decreased to approximately 10%. It was also reported that residual chymosin activity decreased during ripening (Hayes et al., 2002). Therefore, especially for ageing cheeses, it might be considered that the most significant factor during ripening is the presence of microbial enzymes released by primary (starter LAB) and secondary cultures added by cheese makers, as well as NSLAB present in milk or contaminants from environment (McSweeney, 2004; Upadhyay et al., 2004)

Proteases

Proteases can be divided into four classes: cysteine proteases, serine proteases, aspartic proteases and metalloproteases. Each class has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site. Proteases can also be subdivided into two major groups based on their ability to cleave N- or C-terminal peptide bonds (exopeptidase) or internal peptide bonds (endopeptidase) (Yegin et al., 2011).

Aspartic (acid) protease, having an endopeptidase property with two aspartic acid residues, is the most important protease group in cheese technology because of its milk clotting effect during cheese production (Claverie-Martin & Vega-Hernández, 2007). The commercial milk-clotting enzyme (rennet mainly in the form of chymosin) also belongs to the group of aspartic proteases. Most aspartic proteases show maximal activity at low pH values (~3–5) and are inhibited by pepstatin A (Sumantha et al., 2006). However, the cell wall associated proteases of LAB are serine proteases having a molecular weight of 145kDa and a pH optimum of 5.5–6.2 (Fox et al., 2000).

Enzymes from microbial sources

Enzymes of starter LAB and NSLAB

The starter LAB reach maximum numbers of life cells shortly after manufacture, followed by a gradual decrease during ripening as a consequence of lysis. In contrast, NSLAB are typically present at low numbers in the beginning of the cheese production process, but increase within the following months of ripening and ultimately dominate the microbiota of long-ripened cheeses (Fox et al., 2000; Beuvier & Buchin, 2004). The proteases in LAB are anchored to the cell membrane and protrudes through the cell wall (Fox et al., 2000). The proteolytic system of *Lactococcus* was described as cell envelope-associated proteases (CEPs) and are reported in the formation of many small peptides in cheese and are responsible for the release of amino acids (Fox & McSweeney, 1996). In most cheese varieties, LAB are the major contributors to flavor development through the action of their intracellular enzyme systems (Wilkinson & Kilcawley, 2005).

Similar to starter LAB, NSLAB strains were also shown to contain an active proteolytic system that contributes the cheese ripening (Broome et al., 1990). They appear to contribute little to primary proteolysis in Cheddar cheese but to contribute to the release of free amino acids (Fox et al., 1998). Therefore, studies performed on the proteolytic system of NSLAB showed the possibility of their application as adjunct cultures to enhance proteolysis or to generate specific flavor characteristics in cheese (Klein & Lortal, 1999).

Enzymes of secondary culture

Most cheese varieties, particularly traditional cheeses made from raw milk, have a secondary part in their microbiota which is important for functionalities other than acid production. (Fox et al., 2000; Gupta et al., 2004). Among fungi, *Penicillia* are good producers of extracellular enzymes, such as lipases and proteases (Hamlyn et al., 1981). *P. roqueforti* and *P. camemberti* are reported to secrete aspartyl and metalloproteases and specifically hydrolyze α_{s1} - and β - caseins. Similarly, extracellular and intracellular aminopeptidases purified from *Brevibacterium linens* are particularly active on α_{s1} - and β - caseins (Rattray & Fox, 1997, 1999). *Pseudomonas* spp. is an other bacterial strain that was reported to have proteolytic activity (Schokker & van Boekel, 1997). *Propionibacterium* spp. are weakly proteolytic but strongly peptidolytic.

Exogenous (adjunct) enzymes

The main aim of employing exogenous enzymes in cheese industry is to accelerate cheese ripening. Furthermore, the use of these enzymes provides high organoleptic properties (El Soda et al., 2000). Commercial protease and lipase preparations (Wilkinson & Kilcawley, 2005) or selected strains can be added to cheese to produce the relevant enzymes (El Soda et al., 2000). In contrast to NSLAB, adjuncts are specifically selected and intentionally added to cheese or milk to balance the textural and flavor profiles that are otherwise hard to attain (Wilkinson & Kilcawley, 2005). Despite their positive effects, addition of these enzymes can also stimulate the bitter taste in cheese (Wilkinson & Kilcawley, 2005).

Biochemical events during cheese ripening

The biochemical alterations in cheese during ripening may be grouped into primary and secondary events. Primary events include the metabolism of residual lactose (glycolysis) and of lactate and citrate, breakdown of fat (lipolysis) and protein (proteolysis). Lactose is broken down to glucose and galactose. The glucose is efficiently used for the production of ATP and lactate, whereas galactose accumulates in dairy products due to partial lactose fermentation by lactic acid bacteria (Neves et al., 2010). Following the primary events, secondary events occur including the catabolism of fatty acids and amino acids. Products of secondary biochemical events

directly influence the development of many volatile flavor compounds (McSweeney & Sousa, 2000). Primary and secondary biochemical events are presented in Figure 1.

Glycolysis

Glycolysis includes the fermentation of lactose to lactic acid by lactic acid bacteria and it is an essential primary reaction in the manufacture of all cheese varieties. Glycolysis is the fastest reaction that occurs during ripening of cheese. In many cheese varieties glycolysis is terminated with the transition of lactose to lactic acid, by the inhibition effect of salt. However, in some cheese varieties it partially continues during cheese ripening (Fox et al., 1993; Fox & McSweeney, 1996).

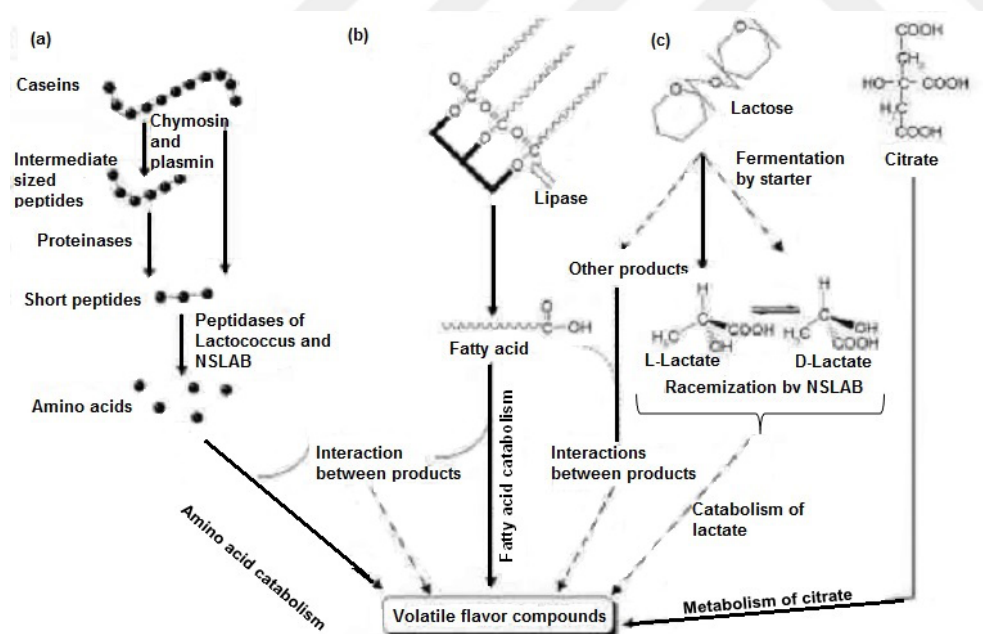


Figure 1. Principal biochemical mechanisms occurred during cheese ripening (Reproduced from Fox et al., 2004)

The three main metabolic changes that occur during glycolysis are lactose metabolism, lactic acid metabolism and citrate metabolism. During the lactose metabolism, the largest proportion of lactose (98%) in milk is removed with separated whey during cheese production. However, the residual lactose in cheese curd is rapidly metabolized to lactate and/or lactic acid during the early stages of ripening by the

activity of LAB or NSLAB, which decreases the pH from pH 6.5-6.6 to 6.2-6.4. The extent of lactate metabolism depends mainly on the salt-in-moisture (S/M) content in the curd. At low S/M content on low populations of NSLAB, residual lactose is converted mainly to L-lactate by LAB. Whereas, at high populations of NSLAB, considerable amounts of D-lactate are formed by conversion of L-lactate (Fox et al., 2000). Moreover, secondary microbiota contributes to lactate metabolism in some cheese varieties such as surface mold-ripened, surface smear-ripened and Blue cheese. The metabolism of lactate is reported to be very extensive in these varieties. The secondary microbiota that colonize and dominate the cheeses, rapidly metabolizes lactate to CO_2 and H_2O and causes an increase in pH. When lactate is exhausted, secondary cultures metabolize the proteins, producing NH_3 , which diffuses inside the cheese and increase the pH. The elevated pH stimulates the activity of native or microbial proteases which contributes to proteolysis in the further stages (McSweeney & Fox, 2004).

During glycolysis, moreover, citrate metabolism is performed by (Cit^+) microorganisms and mainly important for Dutch cheese with holes (eyes). The citrate found in milk is approximately 1.8 g/L and 94% of it is in soluble form, which is also lost with whey during production. The remaining 6% is in colloidal form, and is metabolized to metabolic products such as acetic acid, diacetyl and CO_2 by *Leuconostoc* spp. and (Cit^+) *Lactococcus* spp. (Fox & McSweeney, 1996; Parente & Cogan, 2004).

Lipolysis (Breakdown of fat)

Lipolysis is defined as the hydrolysis of ester bonds in triglycerides resulting in the generation of fatty acids and glycerol (Schweiger et al., 2014) (Figure 2). The release of free fatty acids (FFA) are catalyzed by lipases which could be residual native milk lipoprotein lipase (LPL), rennet preparations containing pregastric esterase (PGE), lipase and esterase of starter LAB and NSLAB, adjunct cultures or secondary fungal cultures and exogenous lipolytic enzymes (Wilkinson & Kilcawley, 2005). Lipolysis and modification of resultant FFAs are significant contributors to overall cheese aroma (McSweeney & Sousa, 2000). However, extensive lipolysis is also considered undesirable in most cheese varieties (Sousa & Malcata, 1997), as it can cause a

rancid taste due to an excessive accumulation of volatile FFAs (Collins et al., 2003; Wilkinson & Kilcawley, 2005).



Figure 2. Formation of FFAs from triglyceride during lipolysis (Based on Fox et al., 1993) Lipolysis is an important biochemical event that takes place during the ripening of many cheese varieties. Particularly, mold-ripened cheeses and cheeses from goat and sheep milk are characterized by extensive lipolysis (Molimard & Spinnler, 1996). The reason of this is that the triglycerides of ruminant (sheep, goat) milk fat are rich in short-chain fatty acids which have low thresholds and contribute significantly to the flavor of cheese, when liberated. Intensity of lipolysis in mold-ripened cheeses are often related to the lipolytic ability of the secondary microbiota or adjunct culture. The most common lipolytic organisms associated with cheese is *Penicillium* spp. which secrete very active extracellular lipases. The lipases of *P. roqueforti* was characterized previously, and found to secrete two lipases, one with a pH optimum at 6.0-6.5, the other at 7.5-8.0, belong to the acid and alkaline lipases, respectively. Similarly, smear ripened cheese varieties are often characterized by high levels of lipolysis (McSweeney, 2004).

Catabolism of fatty acids

FFAs released by lipolysis are used as the precursors for consecutive secondary reactions and are converted to other sapid and aromatic compounds like methyl ketones, lactones and alcohols. The aroma of Blue cheese is dominated by saturated n-methyl ketones including heptan-2-one, nonan-2-one and undecan-2-one. The concentrations of methyl ketones are proportional to the level of lipolysis (Fox et al., 2000).

The catabolism of fatty acids in cheese by *Penicillium* spp. involves 3 main stages:

1. oxidation of β -ketoacids,
2. decarboxylation to methyl ketones with one less carbon atom,
3. reduction of methyl ketones to the corresponding secondary alcohol.

A number of factors affect the rate of methyl ketone production, including temperature, pH, physiological state of the microorganism and the ratio of the fatty acids' concentration to the dry weight of spores. Both resting spores and fungal mycelium are capable of producing methyl ketones. The rate of production of methyl ketones does not directly depend on the concentrations of FFA precursors. In fact, high concentrations of FFA are toxic to *P. roqueforti* (Fox et al., 2000).

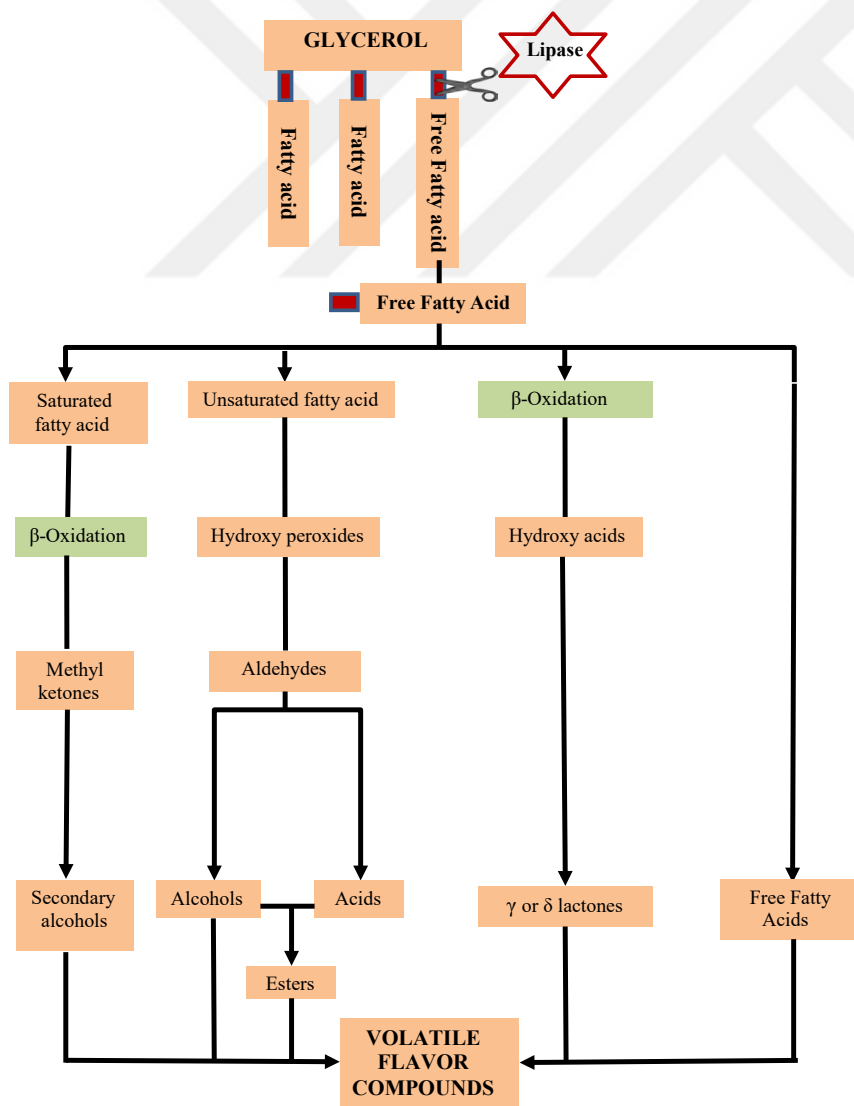


Figure 3. Catabolism of fatty acids and the transition of products to volatile flavor compounds in cheese (Reproduced from Molimard & Spinnler, 1996)

Lactones are cyclic esters resulting from the intramolecular esterification of a hydroxy acid through the loss of water to form a ring structure. Lactones possess a strong aroma, which are important for the overall aroma of cheese, despite not being specifically cheese-like. δ - and γ -lactones are found in cheese. They may have originated spontaneously from their corresponding hydroxy acids, which were released from triglycerides in a previous stage or produced from keto acids released by lipolysis, followed by reduction to hydroxy acids. δ -lactones have very low flavor thresholds and their concentrations correlate with age and flavor intensity. Moreover, they are reported to contribute to the aroma of Cheddar cheese (Fox et al., 2000).

Proteolysis (Breakdown of protein)

Proteolysis is the hydrolysis of cheese proteins leading to the formation of medium and small soluble peptides and free amino acids from large water-insoluble peptides (comparable in size to intact caseins). It is the most complex of the three primary biochemical events during cheese ripening. Furthermore proteolysis is widely considered the most important process for the development of sensory and textural properties of cheese (Fox, 1989; McSweeney, 2004), except for Blue cheese which is dominated by the mechanism of lipolysis (Fox et al., 2000). Unfortunately, it also contributes to off-flavors in cheeses (Upadhyay et al., 2004). Proteolysis contributes to cheese ripening through four pathways:

1. direct contribution to flavor via the formation of peptides and amino acids or indirect contribution via catabolism of aminoacids to amines, acids, thiols, thioesters etc.,
2. higher release of sapid compounds during mastication,
3. alterations in pH via the formation of NH_3 ,
4. Modifications of the texture of cheese due to the breakdown of the protein network, increase in pH and greater water binding by the newly-formed amino and carboxyl groups (Fox, 1989; McSweeney, 2004).

Proteolysis is catalyzed by proteases and peptidases from residual coagulant, native milk, (especially plasmin), starter LAB, NSLAB, and in certain varieties other secondary microbiota. Particularly, extensive proteolysis occurs in surface mold- and surface smear- ripened cheeses because of the very potent proteolytic system of their secondary cultures (Fox et al., 2000). *B. linens* are reported to have both

intracellular and extracellular proteases, but mostly extracellular proteases were purified and characterized, all of which are described as serine protease. It was also determined to catabolize amino acids actively (Hayashi et al., 1990).

Catabolism of amino acids

Amino acid catabolism, which is particularly significant in mold- and smear ripened cheese varieties, is an indicator of extensive proteolysis, (Fox et al., 2000). As shown in Figure 4, catabolism of amino acids involves transamination, decarboxylation, desulfuration, deamination and hydrolysis of amino acid side chains, leading to the production of a wide array of compounds, including carboxylic acids, amines, NH_3 , CO_2 , aldehydes, alcohols, thiols and other sulphur compounds, phenols and hydrocarbons. All of these are particularly important in many cheese varieties. Decarboxylation involves the conversion of amino acid to the corresponding amine with loss of CO_2 . Deamination results in the formation of NH_3 and α -ketoacids. Further, transamination results in the formation of other amino acids by the action of transaminases. Aldehydes formed by the above processes can then be oxidized to acids or reduced to the corresponding alcohols. Sulphur containing compounds are another volatile group produced from amino acids (Yvon & Rijnen, 2001; Fox et al., 2000).

Aminotransferases secreted from microorganisms are specific to different groups of amino acids, such as branched-chain (Leu, Ile, Val), aromatic (Phe, Tyr, Trp), sulphuric (Cys, Met) or acidic (Asp). Further, a specific α -keto acid is produced from each amino acid by the help of aminotransferases (Yvon and Rijnen, 2001). Starter LAB have a limited capacity for synthesis of amino acids. However, as mentioned above, the number of essential amino acids is strain-dependent (Ayad et al., 1999). Therefore, it can be considered that both NSLAB and secondary cultures need fewer amino acids to grow and produce α -keto acid decarboxylase enzymes than the industrial starter LAB strains (van Kranenburg et al., 2002). Wild isolates of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains are reported to require only 1-3 amino acids. The absence of some amino acid biosynthetic pathways in NSLAB and secondary cultures might be a consequence of their adaptation to dairy products. In milk, amino acids are readily available by the proteolysis of caseins. Wild strains are not naturally associated with a rich environment such as milk which makes them more dependent on their own synthesis of amino acids compared to industrial strains (van Kranenburg et al., 2002).

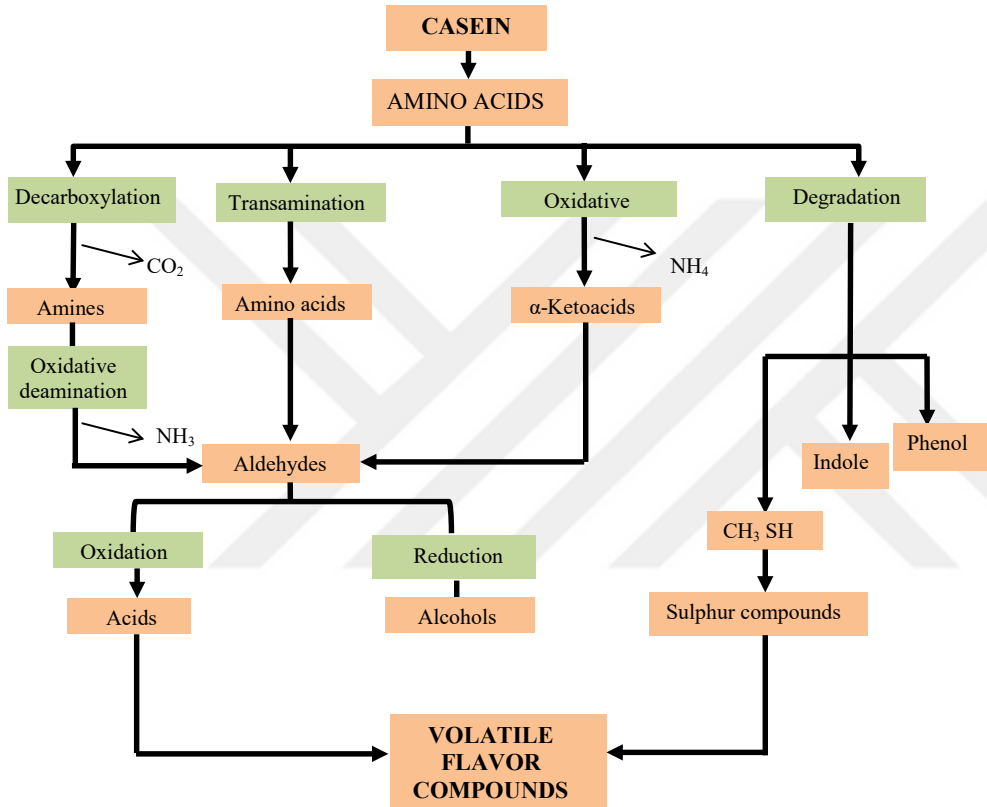


Figure 4. Amino acid catabolism and formation of cheese flavor (Reproduced from Molimard & Spinnler, 1996)

Raw milk cheeses vs. industrial cheeses

Individual or mixed *Lactococcus lactis* strains are the most widely used starter cultures in the production of industrial cheeses (Ayad et al., 1999; Limsowtin et al., 1995; Madrau et al., 2006; Smit et al., 2005). Other than *Lactococcus lactis*, a few specific species such as *Lactobacillus casei* and *Lactobacillus plantarum* are used as primary starters due to their technological attributes (Marshall, 1991). In industrial cheeses, incorporation of a secondary or adjunct culture is often preferred in addition to the starter LAB to develop the sensory properties of cheeses (Johnson, 2001).

Raw milk cheeses have a rich microbiota which is responsible for a more intense and diverse flavor development as compared to industrial cheeses (Parente & Cogan, 2004), due to their higher proteolytic and lipolytic activities (Buffa et al., 2001;

Chávez et al., 2010) compared to that of heat-treated industrial cheeses (Casalta et al., 2009). The indigenous and secondary microbiota present in raw milk cheeses were reported to have improved metabolic capabilities as compared to industrial dairy cultures, such as more active amino acid converting enzymes (Cavanagh et al., 2014; Centeno et al., 2002). Sensory characteristics (Murtaza et al., 2013) and textural properties (Yoon et al., 2016) of cheese were also reported to be better in raw milk cheeses. Therefore, these microbiota are used in different kind of cheese types as adjunct culture to improve the organoleptic properties and to accelerate the cheese ripening (Poveda et al., 2015).

The increased health risk of raw milk cheeses compared to industrial milk cheeses has been questioned. However, it was reported that in some cases, pasteurized milk cheese caused a higher incidence of outbreaks of food-borne illness, than observed for raw milk cheese (Koch et al., 2010). The advantageous microbiota in raw milk play a relevant role in the safety of the product, by preventing the accumulation of undesirable substances (Poveda et al., 2015). They also prevent the growth of contaminating food-borne pathogens during cheese making. In this respect, raw milk cheese can be considered as a microbiologically safe food (Brooks et al., 2012; Masoud et al., 2012).

In recent years there is an increasing interest in isolating new wild strains of lactic acid bacteria and other secondary cultures with novel properties from raw milk and raw milk cheeses (Cogan, 2014).

Mold-ripened cheeses

Mold-ripened cheeses are usually made from raw cow's milk, such as Brie, Camembert, Saint-Nectaire, Neufchatel, or raw ewe's milk, such as Roquefort. A minor number of mold-ripened cheeses are produced from pasteurized milk like Stilton and Gorgonzola. In general, they are dry-salted and ripened in cellars at temperature from 8 to 18 °C. Most of them are cheeses with Protected Designation of Origin (PDO).

Mold-ripened cheeses are categorized in two major groups:

Externally mold-ripened (surface mold-ripened) cheeses

Camembert and Brie cheeses are the most famous surface mold-ripened cheeses which are generally ripened by *Penicillium camemberti* and in most cases by *Geotrichum candidum* (Desmaures, 2014). During the production of this type of cheese, milk is coagulated at 32-35°C which favors the growth of LAB. A significant acidification occurs, mainly after the curds are placed into blocks, as well as slow whey draining. Ripening occurs in an environment with low temperature (8-15 °C) and high humidity (80-85% relative humidity).

Internally mold-ripened (Blue-veined) cheeses

The best known internally mold-ripened cheeses are Blue-veined (Roquefort), Danablu, Stilton, and Gorgonzola. A substantial part of Blue-veined cheeses are made from ewe's milk, but cow and buffalo milk are also used to a lesser extent. These cheeses are characterized by pronounced gradients of pH, salt, and water activity. Milk coagulation is performed at 28-30°C (strong flavored) or at 35–40°C (mild flavored). Coagulation time is between 30 and 75 min. The coagulum is cut into strips or cubes. After stirring, when the grains of curd are firm enough, it is put into blocks. During this application, no pressure is applied during draining, but blocks are inverted frequently. At the end of draining step, curd is salted in brine or with dry salt to obtain a generally high salt concentration. To create and maintain openings, piercing of the curd is realized to allow further gas exchange. Ripening occurs in caves or in temperature- and humidity- controlled cellars (low temperature and high humidity) for at least 3 months. Mainly in Blue-veined cheeses, *Penicillium roqueforti* is localized in openings in the veins (paste) of cheese to give a blue-green color (Desmaures, 2014).

Microbiota of mold-ripened cheeses

The production and ripening of mold-ripened cheeses involve LAB, yeasts and filamentous fungi. LAB are used as starter cultures for the production of different varieties of mold-ripened cheeses. Mesophilic cultures including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* are used during the production of mold-ripened cheeses. Thermophilic starters are also used in mold-ripened cheeses depending on the cheese variety. The thermophilic starters in blue-veined cheeses usually involve *Lactobacillus delbrueckii* subsp. *bulgaricus* and

Streptococcus thermophilus. Additionally, *Leuconostoc mesenteroides* is preferred in Blue cheeses which produce CO₂ and create an open structure in the cheese. Beside starter cultures, LAB found in cheese may also derived from cheese production and ripening environment or raw milk, leading to high diversity in strains. For example, Roquefort was reported to contain 94 strains of *Lactococcus* spp. and 49 strains of *Leuconostoc* spp. As an example, Roquefort was reported to contain 94 strains of *Lactococcus* spp. and 49 strains of *Leuconostoc* spp. (Desmaures, 2014).

Coryneform bacteria and various *Staphylococcus* spp. have been frequently isolated from mold-ripened cheeses. Other than commercially used *Brevibacterium* lines, *Corynebacterium* spp., *Brevibacterium aurantiacum*, *Arthrobacter* spp., *Brachybacterium* spp., and *Micrococcus* spp. were also reported. Gram-negative bacteria such as *Pseudomonas* spp. and *Psychrobacter* spp. have also been described on the surface or in the core of mold-ripened cheeses (Desmaures, 2014).

Yeasts are also significant components of mold-ripened cheeses. During production and at the beginning of ripening lactose fermenting yeasts, including *Debaryomyces hansenii*, *Candida* spp. and *Kluyveromyces marxianus*, play a role. Lactate resulting from the activity of LAB, can be used by these and various other yeasts (Desmaures, 2014). While, *P. roqueforti* and *P. camemberti* are the main fungal cultures, a great diversity of fungi can be present in mold-ripened cheeses. *P. brevicompactum*, *P. commune*, *P. bifforme*, *P. fuscoglaucum* and *P. palitans* are the main species isolated from mold-ripened cheeses (Desmaures, 2014).

Biochemical properties in mold-ripened cheeses

In mold-ripened cheeses, fungal strains are notably involved in the consumption of lactic acid as well as in proteolysis and lipolysis by their enzymatic systems. β -casein is hydrolyzed by mold-derived proteases, mainly from *Penicillium* spp. Several extracellular and intracellular peptidases of fungal strains have been described, but the latter play a much more limited role. In the early phases of *Penicillium* growth, the proteolysis of β -casein is largely due to extracellular proteases of *Penicillium* spp. Two extracellular proteases are produced: a metalloproteinase (optimum for casein hydrolysis at pH 5.5–6.0) and an aspartic proteinase (optimum for casein hydrolysis at pH 4.0 for *P. camemberti* and at 5.5 for *P. roqueforti*) (Desmaures, 2014).

In Camembert and Blue cheeses, the proteolytic activity increases after 6-14 days of ripening, depending on the cheese variety and strain, which is the period that correlates to the onset of growth of *P. camemberti* and *P. roqueforti*. *Geotrichum candidum* also produces proteolytic enzymes, with variable activity from one strain to another. However, extracellular proteolytic activity of these strains is low compared to the intracellular activity (Desmaures, 2014).

Mold-ripened cheeses are also characterized by an intense lipolysis. *P. camemberti*, *P. roqueforti*, and *G. candidum* produce lipases that degrade triglycerides and generate FFAs having between 6 and 20 carbons. In the secondary biochemical events during cheese ripening, beginning from FFA, many flavor compounds are produced. Methyl ketones produced by the oxidation of fatty acids play an important role in determining the flavor of mold-ripened cheeses. Primary and secondary alcohols, along with ketones, are considered to be the most important compounds in the aroma of mold-ripened cheeses (Desmaures, 2014).

Divle Cave cheese

Divle Cave cheese is a semi-hard cheese made from unpasteurized and semi-skimmed ewe's milk. It is traditionally produced in spring when milk is abundant, in Karaman, a rural region of Turkey. The south of this region consists of a big cavity among the mountains, named Divle cave, at a depth of 70 meters. Cheeses are produced without using any starter culture or fungal spores and are ripened in goatskin bags in Divle cave for 4 months. Divle Cave cheese has its own characteristics mainly from ripening period in the cave where the temperature is 8-10 °C and the humidity is 85-90 %. The goatskin bags containing cheese are blueish green at the beginning of ripening, but turn to a red color after 60th day of ripening. The secondary microbiota, including filamentous fungi, yeast, coryneform bacteria, originate from both the cheese production environment and the cave. In addition, NSLAB derived from raw milk play a role in the ripening process and contribute to the characteristic aroma of cheese. This semi-hard cheese has a distinctive taste, flavor and crumbly texture. The production flow chart of Divle Cave cheese is given in Figure 6.

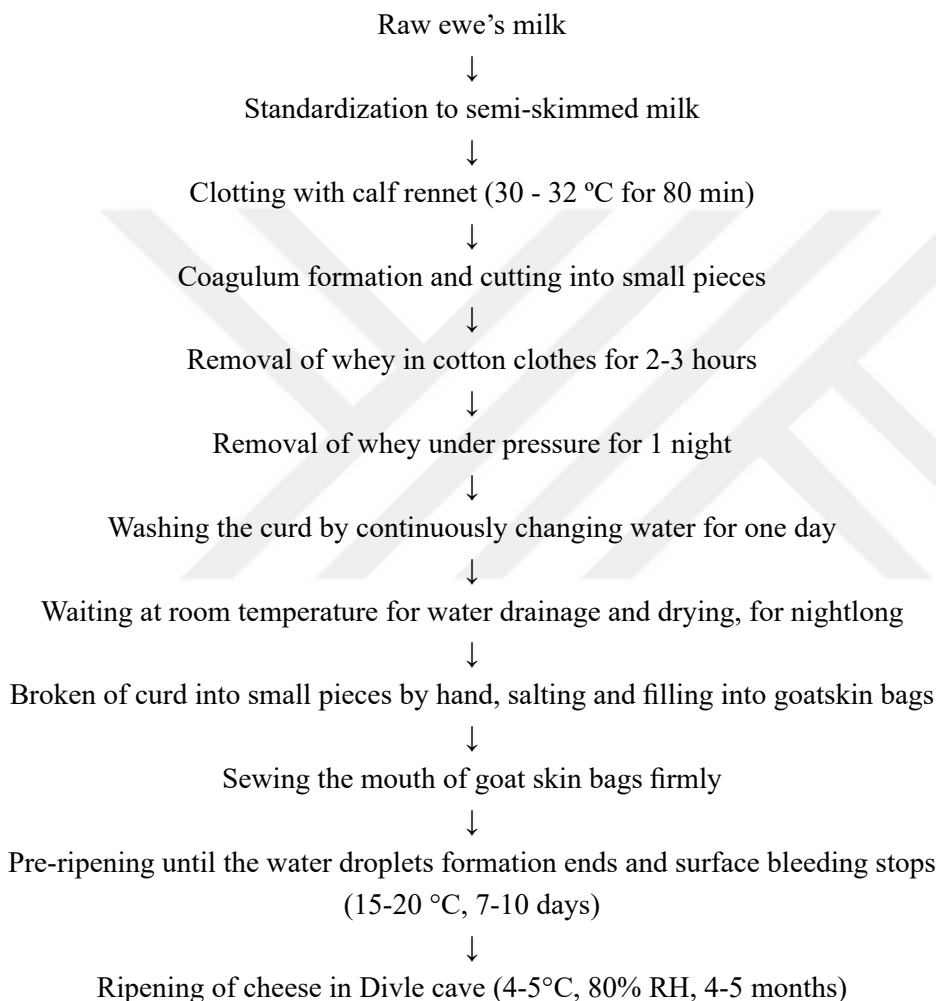


Figure 6. Production flow chart of Divle Cave cheese

Outline of Thesis

The study presented in this thesis aims to describe:

- the diversity and evolution of the complex microbiota of a raw ewe's milk cheese during ripening in a cave
- the protease and lipase activities of the determined strains to select the dominant contributors to ripening
- the volatile, proteolysis and lipolysis profiles of cheese during the production and ripening processes in comparison with the previous two issues.

These approaches will ultimately enable accurate prediction of the dominant strains and support the control of the cheese production process, using these strains in a controlled manner by their deliberate addition. In this way, a standardized and controlled Divle Cave cheese production and ripening can be achieved while preserving its traditional character. The obtained results may also allow the development of new starter/adjunct cultures for industrial cheeses. Moreover, this thesis presents data that contributes to a better characterization of Divle Cave cheese which will aid in determining the geographical origin of this artisanal cheese in the future. The obtained data can also be useful for comparative purposes both on mold-ripened and raw ewe's milk cheeses.

In **Chapter 1**, an introduction to raw milk cheeses and related microorganisms including starter LAB, NSLAB and secondary microbiota are given and an overview of their effects on cheese ripening via their enzymatic systems is presented. Furthermore, the main biochemical events including glycolysis, lipolysis and proteolysis during cheese ripening are explained by emphasizing their end products which contribute to the development of the overall aroma of cheese.

Chapter 2 describes the identification of bacteria, filamentous fungi and yeasts present in the middle and at the end of ripening of Divle Cave cheese by molecular techniques. A broad diversity including some unique and intrinsic microorganisms was determined both in the interior and exterior part of the cheese.

Chapter 3 focuses on the quantification of microbial groups present throughout the production and ripening process of Divle Cave cheese, and establishes the role of this microbial community in the temporal development of proteolysis and lipolysis profiles during production and ripening. The lipolysis and proteolysis degree generally increased during the whole ripening period and relatively high levels were established at the end of ripening which could be explained by the indigenous and secondary microbiota as well as native enzymes derived from raw milk.

Chapter 4 describes the temporal development of the chemical properties and volatile compounds during production and ripening of Divle Cave cheese. 110 volatile compounds including mainly carboxylic acids, alcohols, ketones, esters and

terpenes were determined during the whole period. The presence and concentration of these volatile compounds varied between specific phases of the production and at different stages of 120 days of ripening. These variations indicated that a variable cheese medium and dynamic conditions were available in the structure of cheese. The results of this study also identified the most abundant or key flavor compounds, likely contributing to the characteristic aroma and unique taste of this artisanal raw ewe's milk cheese.

Chapter 5 describes protease and lipase production by strains of yeasts, filamentous fungi and bacteria that were isolated from Divle Cave cheese in Chapter 2. Quantification of activity was performed in submerged cultivations of the isolates in medium made from irradiated cheese at conditions adapted from cheese ripening. The detection of the activities of strains isolated from Divle Cave cheese is crucial to estimate their contribution to the ripening process. The results obtained from this study confirmed the high frequency of enzymatically active strains in Divle Cave cheese by identifying the best protease and lipase producer strains. The effects of protease inhibitors were also monitored in terms of the inhibition of the proteolytic activities of strains to determine the class of proteases.

Chapter 6 demonstrates the value of genomic analyses for the description of proteolytic profiles of fungal strains. As a test case a combined analysis of comparative genomics, proteomics and enzymology tests on seven *Aspergillus* species grown on wheat bran and sugar beet pulp, was performed. Putative proteases were identified by homology search and Pfam domains. These genes were then clustered based on orthology and extracellular proteases were identified by protein subcellular localization prediction. Proteomics was used to identify the secreted enzymes in the cultures, while protease assays with and without inhibitors were performed to determine the overall protease activity per protease class. All this data was then integrated to compare the protease productivities in these *Aspergilli*.

Chapter 7 summarizes the main results obtained in this thesis and gives some concluding remarks and future perspectives.

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CHAPTER 2

The diversity and evolution of microbiota in traditional Turkish Divle Cave cheese during ripening

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Abstract

The microbial diversity of traditional Turkish Divle Cave cheese was evaluated in three independent batches. Using molecular techniques, twenty three bacterial species were identified in the interior and outer part of the cheese on days 60 and 120. Bacilli and Gammaproteobacteria classes were predominant during early stages of ripening and Actinobacteria at later stages. Nineteen species of filamentous fungi and five yeast species were identified and the most frequently isolated species were *Penicillium polonicum*, *P. biforme*, *P. roqueforti*, *P. chrysogenum* and *Debaryomyces hansenii*. The microbiota displayed similar communities among batches but high diversity in different parts of the cheese during ripening. Novel cheese starter cultures could be developed after the technological characterization of the isolated strains.

Introduction

The secondary microbiota contributes to cheese ripening in raw milk cheeses. Growth of this microbiota depends on intrinsic and/or external factors, and is usually unique to specific cheese varieties. There are some studies interested in isolating new wild strains of secondary cultures with novel properties from raw milk cheeses (Beresford et al., 2001; Feutry et al., 2012).

Divle Cave cheese is a semi-hard cheese made from raw ewe's milk, which has a crumbly texture and a strong flavor. It is produced in May and June in Karaman, a rural region in the middle of Turkey, with an estimated annual production of 70-80 tones (District Governor Erkan İsa Erat, personal communication). Ripening takes place in Divle cave, in the south of this region, where the average temperature is 5-10°C with a humidity of 85-90%. The cheeses are produced without starter culture and ripened for 4 months in goatskin bags at a depth of 70 meters. The bag surface turns from green to red during ripening and this red color is the distinguishing characteristic of Divle Cave cheese (Figure 1). Studies on Divle Cave cheese so far focused on chemical composition and microbial counts (Gönç, 1974; Keleş & Atasever, 1996; Morul & İşleyici, 2012).



Figure 1. Growth of microorganisms on the surface of goatskin bags during the ripening period of Divle Cave cheese, **A:** A view from cave on day 30 during ripening, **B:** Appearance of the goatskin bag at the end of the ripening (on day 120)

There is a lack of standardization in production of Divle Cave cheese and it can be overcome by identification of the dynamics of microorganism communities and the

determination of the predominant contributors to cheese ripening. If these strains can be used in productions under controlled conditions, standard and high quality cheeses can be produced. In the present study we examined the microbial community of Divle Cave cheese to provide insight into the species diversity and evolution from mid- to late ripening. The resulting data can be used to develop specific starter or adjunct cultures to accelerate the cheese ripening, obtain high diversity in cheese flavor and improve the organoleptic properties.

Materials and methods

Cheese production and sampling

The batches used in this study were produced by farmers at three different dairy farms in Karaman province. Two independent batches were produced per farm with an interval of 15 days. During production, semi-skimmed raw ewe's milk (4.5 % fat content) was coagulated with calf rennet. The curd was broken into small pieces, dry-salted and 3-5 kg salted curd was tightly pressed into salted and dried goatskin bags. The goatskin bags were sewed firmly and kept under cool storage (14-15 °C) until whey drain off stops (7 to 10 days). Finally, they were left in Divle cave for 4-5 months for ripening.

Each sample was taken from cheese stuffed into goatskin bags. Eighteen cheeses coming from three different farms were analyzed at each ripening stage. Analyses were performed in triplicate on the inner part (white core) and on outer crust of cheese inside the goatskin (subsurface).

Isolation of strains

Total aerobic mesophilic bacteria were determined on Plate Count Agar (Merck, Darmstadt, Germany) including 1% skim-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. Lactobacilli were grown on de Man-Rogosa-Sharpe Agar (pH 5.4; Merck) incubated for 72 h at 30°C under anaerobic conditions. Lactococci were grown on M17 agar (Merck) 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 and incubated at 30°C for 48 h. Staphylococci were grown on Baird-Parker Agar (Merck) supplemented with egg yolk tellurite solution (Merck), incubated at 37°C for 24 h.

For the isolation of bacteria, aerobic strains were sub-cultured on Tryptone Soya Agar (Oxoid, Basingstone, Hampshire, UK) at 30°C for 48 h and 15°C for 5 days. Lactobacilli were sub-cultured on MRS agar, incubated at 30°C for 72 h under anaerobic conditions containing a mixture of 90% CO₂ and 10% H₂ (Mart Anoxomat System, Drachten, The Netherlands). Lactococci and enterococci were sub-cultured on M17 agar. Filamentous fungi and yeasts were isolated on malt extract agar (Merck). Colonies were restreaked for purification and used for DNA extraction. Each strain was stored in their respective broth mixed with 60% glycerol at -80°C.

Molecular identification of bacteria and mycobiota

DNA extraction and PCR amplification of bacteria

Extraction of DNA was performed by boiling the loopful culture in 500 µL in sterile demineralized water. PCR amplification of the partial 16S rDNA was performed in 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with PCR GoTaq Green Master Mix (Promega, Madison, WI, USA). The conditions were: denaturation at 94°C for 2 min and 36 cycles at 94°C for 1 min, annealing 54°C for 1 min, amplification at 72°C for 2 min and final amplification at 72°C for 5 min.

DNA extraction and PCR amplification of filamentous fungi and yeasts

Isolates of filamentous fungi and yeast strains were sub-cultured on Malt Extract Agar (Merck) and incubated for 5-7 days at 25°C. The extraction of DNA was performed using the Microbial DNA Isolation Kit (MoBio Inc., Solana Beach, CA, USA) according to the manufacturer's protocol. The ITS barcode and a part of the β-tubulin genes (BenA) were amplified according to the parameters mentioned for bacteria amplification.

Sequencing, data analysis and identification

Sequencing was performed with the BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction and analyzed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems). Sequences were edited and trimmed using SeqMan software in the Lasergene package (DNASTAR Inc., Madison, WI, USA). A homology search with the generated partial 16S rRNA, ITS and BenA gene sequences was performed on GenBank and/or internal databases of the CBS-KNAW Fungal Biodiversity Centre.

Results and Discussion

In total, 98 bacteria, 101 filamentous fungi and 27 yeast isolates were picked from plates. Among them, 23 bacterial, 19 filamentous fungi and 5 yeast species were identified (Tables 1 and 2). GenBank accession numbers and culture collection numbers of bacterial and fungal species are given in Supplementary Table 1. Both bacterial and fungal biota demonstrated low diversity in different dairy farms, however they showed high diversity between two ripening stages.

Table 1. Bacteria species identified from cheese samples obtained from 3 different batches in the middle (60th day) and at the end (120th day) of ripening

Class*	Species identification on the basis of the highest partial 16S rRNA similarity score	Number of isolates				Total
		60th day		120th day		
		inside	outside	inside	outside	
Bacilli						
	<i>Staphylococcus equorum subsp. equorum</i>	3	3	3	4	13
	<i>Lactobacillus paraplantarum</i>	5	1	2	0	8
	<i>Lactococcus lactis subs lactis</i>	6	0	0	0	6
	<i>Enterococcus faecium</i>	4	2	0	0	6
	<i>Staphylococcus warneri</i>	3	2	0	0	5
	<i>Lactobacillus brevis</i>	2	1	0	0	3
	<i>Lactobacillus coryniformis</i>	1	0	0	0	1
	<i>Bacillus stratosphericus</i>	0	1	0	0	1
	<i>Solibacillus silvestris</i>	0	1	0	0	1
Gammaproteobacteria						
	<i>Psychrobacter glacincola</i>	4	4	3	4	15
	<i>Pseudomonas proteolytica</i>	2	0	1	0	3
	<i>Halomonas titinacae</i>	0	1	0	0	1
Actinobacteria						
	<i>Brevibacterium antiquum</i>	0	0	3	8	11
	<i>Brachybacterium tyrofermentans</i>	0	0	3	4	7
	<i>Micrococcus luteus</i>	0	0	1	3	4
	<i>Brachybacterium species</i>	0	0	1	2	3
	<i>Microbacterium oryzae</i>	0	0	1	1	2
	<i>Kocuria salsicia</i>	1	1	0	0	2
	<i>Arthrobacter arilaitensis</i>	0	0	1	1	2
	<i>Microbacterium halotolerans</i>	0	0	1	0	1
	<i>Microbacterium gubbenense</i>	0	0	1	0	1
	<i>Brevibacterium species</i>	0	0	0	1	1
	<i>Brevibacterium species</i>	0	0	0	1	1
						98

*: Identification were done on the basis of unique similarity score of partial 16S rRNA species

Diversity among bacterial microbiota

Three different bacterial classes were identified, Bacilli, Gammaproteobacteria and Actinobacteria (Table 1). Bacteria were mostly detected from the interior part of cheese. Gammaproteobacteria and Bacilli were largely detected and predominant at day 60 in all batches. Bacilli were mainly represented by the following species: *Staphylococcus equorum* subsp. *equorum*, *Lactobacillus paraplantarum*, *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecium*, *Staphylococcus warneri* and *Lactobacillus brevis*. These species were also detected as predominant species in ewe's cheese (Pangallo et al., 2014), and traditional raw milk cheeses (Casalta et al., 2009; Fontana et al., 2010; Hantsis-Zacharov & Halpern, 2007).

With regard to Gammaproteobacteria, *Psychrobacter glacincola* demonstrated dominance both in the interior and outer layer of cheese at day 60. *Psychrobacter* species are described as aerobic, halotolerant, psychrophilic bacteria and particularly isolated from soil and moist habitats, as well in dairy products (Hantsis-Zacharov & Halpern, 2007; Pangallo et al., 2014).

The detected Bacilli and Gammaproteobacteria decreased drastically at day 120 and were replaced by Actinobacteria as the most abundant group on day 120. Until the 60th day of ripening, due to the growth of yeast strains, an increase was observed in pH, presumably because of the utilization of lactate and in some cases production of ammonia by yeasts (Gori et al., 2007). The increase of pH favors growth conditions of fungi and Actinobacteria in the following days of ripening until day 120. In this thesis, chemical composition data (Chapter 4, Ozturkoglu Budak et al., 2016b) confirmed these results. In the beginning of ripening pH of cheese was low, but a rise was seen collaterally with the rise in yeast and filamentous fungi number when ripening progressed (Chapter 3). The yeast species and *Brevibacterium* spp. among Actinobacteria contribute to the cheese flavor and cheese color (Arfi et al., 2005). Actinobacteria were represented by *Brevibacterium antiquum*, *Brachybacterium tyrofermentas*, *Micrococcus luteus*, *Kocuria salcia*, *Microbacterium oryzae*, *Arthrobacter arilaitensis*, *Microbacterium gubbenense*, *Microbacterium halotolerans*, *Brevibacterium* spp., and *Brachybacterium* spp. Similar results were obtained in Italian Taleggio cheese (Panelli et al., 2012). Actinobacteria are able to hydrolyze casein (Collins, 2006), so could be effective for proteolysis and ripening. (Beresford,

Fitzsimons et al. 2001) Some species of *Brevibacterium* were seen as white colonies when incubated at 30°C and produced orange pigment at 15°C.

The genus *Arthrobacter* produces a great variety of pigments e.g. yellow, red, green and is commonly found in soil, water, air, food and dairy products (Sutthiwong et al., 2014). *Arthrobacter arilaitensis* has been isolated from smear ripened cheeses (Mounier et al., 2009; Sutthiwong et al., 2014), but to our knowledge it is the first time that it is isolated from a semi-hard or hard cheese. The other Actinobacteria species found in this study were previously detected in raw milk cheese (Hantsis-Zacharov & Halpern, 2007), traditional Italian cheeses produced without using any starter culture (Fontana et al., 2010), matured cheeses (Dolci et al., 2010) and they are frequently reported as contaminants from cheese environment.

Diversity among mycobiota

Penicillium species were the most frequently isolated filamentous fungi including mainly *P. polonicum*, *P. biforme*, *P. roqueforti*, *P. chrysogenum*, *P. cyclopium*, *P. commune* and *P. cavernicola* (Table 2). These species were generally isolated from the outside of cheese but were occasionally detected in the inside of cheese as well. Less frequently isolated species were *Mucor racemosus*, *M. flavus*, *Scopulariopsis fusca* and *Trichoderma* spp. indicating that they are not characteristic for typical Divle Cave cheese.

P. biforme was predominantly present on Divle Cave cheese. This species was synonymous with *P. camemberti* (Frisvad & Samson, 2004), but a more recent study showed that this species is distinct (Giraud et al., 2010). *P. biforme* has a strong association with cheese and cheese environments and is also known as cheese contaminant. It was frequently found in Roquefort cheese factories in France (Giraud et al., 2010). *P. commune* and *P. roqueforti* have a strong association with cheeses. Although *P. commune* is known as a spoilage fungus (Kure & Skaar, 2000; Montagna et al., 2004), it is also reported to contribute to the ripening and aroma of cheese (Lund et al., 1995), and has been isolated from different cheeses such as Kuflu (Hayaloglu & Kirbag, 2007), goat and sheep milk cheeses (Montagna et al., 2004) and Camembert and Brie cheeses (Giraud et al., 2010). *P. fuscoglaucum* is previously synonymized with *P. commune* but later categorized separately with a close relationship. It was isolated from washing water, wood and feta cheese (Giraud et al., 2010).

Two of the commonly found *Penicillium* species (*P. polonicum* and *P. cyclopium*) belong to series *Viridicata*. Species belonging to this series are associated with seeds and cereal habitats. Their relation with cheese is not evident, although *P. polonicum* has been detected in Bryzdza cheese made from raw ewe's cheese (Pangallo et al., 2014). Their presence might be explained by the presence of high salt concentrations and low temperatures, resulting in a specific niche for those species. They are known as producers of mycotoxins such as penicillic acid, verrucosidin and viridic acid (Frisvad & Samson, 2004). Their exact role during the cheese ripening is unknown and needs to be elucidated. However, their presence must be avoided to ensure food safety.

P. cavernicola is a rare, psychrotolerant species which has been isolated from caves in USA and Venezuela, but also from butter and salami (Frisvad & Samson, 2004). This species is not associated with any kind of cheese and probably comes from the environment of the cave during ripening. *P. brevicompactum*, *P. chrysogenum*, *P. verrucosum* and *P. roqueforti* were previously detected in Kuflu cheese (Hayaloglu & Kirbag, 2007) and *P. crustosum* in cheese, animal feed and soil (Giraud et al., 2010). Some species such as *P. corylophilum*, *P. olsonii* and *P. cyclopium* have not been previously reported in cheese.

Yeasts are also reported as important contributors to the aroma and quality of Blue cheeses as well as fungi (Gkatzionis et al., 2013; Samson et al., 2004; van den Tempel & Jakobsen, 2000). *Debaryomyces hansenii* was the most predominant yeast species isolated from both the inside and outside of the cheeses, followed by *Yarrowia lipolytica* and *Kluyveromyces lactis*. These three yeast species were detected at day 60 and 120 from both the inside and outside of the samples, and therefore belong to the native mycobiota of the Divle Cave cheese. *Candida zeylenoides* was only detected in the inner part of the cheese at day 60.

After the identification of phenotypic and technological characteristics of bacterial and fungal strains, it will be possible to use the results of this work for future studies for selecting new suitable strains and also for innovative cheese starter and adjunct cultures in different types of cheeses.

Table 2. Fungal species identified from cheese samples obtained from 3 different batches in the middle (60th day) and at the end (120th day) of ripening

Species*	Number of isolates				Total
	60 th day		120 th day		
	inside	outside	inside	outside	
Yeast					
<i>Debaryomyces hansenii</i>	2	6	1	5	14
<i>Yarrowia lipolytica</i>	0	1	0	6	7
<i>Kluyveromyces lactis</i>	4	1	0	0	4
<i>Candida zeylenoides</i>	1	0	0	0	1
<i>Geothricum candidum</i>	0	1	0	0	1
					27
Filamentous Fungi					
<i>Penicillium polonicum</i>	1	9	0	8	18
<i>Penicillium biforme</i>	0	5	2	8	15
<i>Penicillium roqueforti</i>	0	5	0	5	10
<i>Penicillium chrysogenum</i>	0	8	0	2	10
<i>Penicillium cyclopium</i>	1	3	0	4	8
<i>Penicillium commune</i>	0	2	0	5	7
<i>Penicillium corylophilum</i>	0	1	3	1	6
<i>Penicillium cavernicola</i>	0	0	2	3	5
<i>Penicillium brevicompactum</i>	0	1	0	4	5
<i>Penicillium rubens</i>	0	1	0	3	4
<i>Mucor racemosus</i>	0	1	1	2	4
<i>Mucor flavus</i>	0	0	0	3	3
<i>Penicillium crustosum</i>	0	1	0	1	2
<i>Penicillium olsonii</i>	0	1	0	1	2
<i>Penicillium spinulosum</i>	0	0	0	2	2
<i>Penicillium fuscaglaucum</i>	0	0	0	2	2
<i>Penicillium verrucosum</i>	0	1	0	0	1
<i>Scopulariopsis fusca</i>	0	0	0	1	1
<i>Trichoderma</i> spp.	0	0	1	1	1
					101

*: Identification were done on the basis of unique similarity score of ITS for all fungi and additionally of β -tubulin genes for *Penicillium* species

Conclusion

This study is the first report characterizing the bacteria, filamentous fungi and yeasts present in Divle Cave cheese by molecular techniques. A broad bacterial and fungal community, including some unique microorganisms which constantly fluctuate and dominate the cheese consortia from mid- to late-ripening, was determined. Many microorganisms dominated in cheese in the middle of ripening but diminished in number at the later stage and could not be detected at the end of ripening. On 60th day of ripening, yeasts and Bacilli class of bacteria were present in cheese whereas afterwards Actinobacteria class including some pigment producer coryneform bacteria such as *Arthrobacter* spp., *Brevibacterium* spp. began to develop. The bacterial and fungal community in interior and exterior parts of cheeses demonstrated strong differentiation.

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Supplementary Table 1. GenBank accession and culture collection numbers of bacteria, yeast and filamentous fungi species

Bacteria				
Class	Species	GenBank Accession Number	Similarity (%)	Culture collection number of NCCB [#]
Bacilli				
	<i>Staphylococcus equorum</i> subsp. <i>equorum</i>	NR_027520	99	NCCB 100530
	<i>Lactobacillus paraplantarum</i>	NR_025447	100	NCCB 100523
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	NR_113960	99	NCCB 100539
	<i>Enterococcus faecium</i>	NR_114742	99	NCCB 100527
	<i>Staphylococcus warneri</i>	NR_025922	100	NCCB 100524
	<i>Lactobacillus brevis</i>	NR_116238	100	NCCB 100526
	<i>Lactobacillus coryniformis</i>	NR_029018	100	NCCB 100543
	<i>Bacillus stratosphericus</i>	NR_042339	100	NCCB 100528
	<i>Solibacillus silvestris</i>	NR_028865	99	NCCB 100535
Gammaproteobacteria				
	<i>Psychrobacter glacincola</i>	NR_042076	99	NCCB 100548
	<i>Pseudomonas proteolytica</i>	NR_025588	99	NCCB 100532
	<i>Halomonas titinacae</i>	NR_116997	99	NS
Actinobacteria				
	<i>Brevibacterium antiquum</i>	NR_029079	99	NCCB 100525
	<i>Brachybacterium tyrofermentans</i>	NR_026272	99	NCCB 100540
	<i>Micrococcus luteus</i>	NR_075062	100	NCCB 100533
	<i>Brachybacterium</i> spp.	FJ_795650	100	NCCB 100537
	<i>Microbacterium oryzae</i>	NR_117527	99	NCCB 100542
	<i>Kocuria salsicia</i>	NR_117299	100	NCCB 100531
	<i>Arthrobacter arilaitensis</i>	NR_074608	99	NCCB 100541
	<i>Microbacterium halotolerans</i>	NR_036935	99	NCCB 100538
	<i>Microbacterium gubbenense</i>	EU_863414	99	NS
	<i>Brevibacterium</i> spp.	AM_398220	100	NCCB 100449
	<i>Brevibacterium</i> spp.	JF_970576	97	NCCB 100561

[#]: NCCB is the Netherlands Culture Collection of Bacteria

NS: not saved

<i>Yeast and filamentous fungi</i>				
Class	Species*	Gen Bank Accession Number		Culture collection number of CBS [#]
		ITS	β-tubulin	
Yeast				
	<i>Debaryomyces hansenii</i>	AF_210327		CBS 13941
	<i>Yarrowia lipolytica</i>	AF_335977		CBS 13944
	<i>Kluyveromyces lactis</i>	NR_131273		CBS 13942
	<i>Candida zeylenoides</i>	NR_131278		CBS 13943
	<i>Geothricum candidum</i>	KF_112070		CBS 13940
Fungi				
	<i>Penicillium polonicum</i>	AF_033475	AF_001206	CBS 139104
	<i>Penicillium biforme</i>	KC_411731	FJ_930951	CBS 140173
	<i>Penicillium roqueforti</i>	EU_427296	AY_674381	CBS 139105
	<i>Penicillium chrysogenum</i>	EF_200090	AY_495981	CBS 140170
	<i>Penicillium cyclopium</i>	KC_427059	AY_674309	CBS 140175
	<i>Penicillium commune</i>	GQ_458026	AY_674361	CBS 140169
	<i>Penicillium corylophilum</i>	AF_033450	JX_141042	CBS 140171
	<i>Penicillium cavernicola</i>	KJ_834505	KJ_834439	CBS 140172
	<i>Penicillium brevicompactum</i>	DQ_249211	AY_674437	CBS 140177
	<i>Penicillium rubens</i>	KP_398883	JX_091546	CBS 140174
	<i>Penicillium crustosum</i>	HQ_225712	FJ_004401	CBS 139107
	<i>Penicillium olsonii</i>	DQ_645803	AY_674444	CBS 139106
	<i>Penicillium spinulosum</i>	KJ_834513	HM_103380	CBS 140176
	<i>Penicillium fuscoglaucum</i>	JQ_434691	FJ_930977	CBS 140178
	<i>Penicillium verrucosum</i>	KC_618446	FJ_004438	CBS 140179
	<i>Mucor racemosus</i>	FJ_345353		CBS 139103
	<i>Mucor flavus</i>	JN_206467		CBS 139101
	<i>Scopulariopsis fusca</i>		JQ_434463	NS
	<i>Trichoderma</i> spp.		GQ_221185	CBS 139102

#: CBS is the Fungal Biodiversity Center in Netherlands

NS: not saved

CHAPTER 3

Microbiota-driven proteolysis and lipolysis during production and ripening of Divle Cave cheese

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Abstract

In previous work we have established which microbial species are present in Divle Cave cheese after ripening. Furthermore, we investigated the temporal development of the chemical properties and volatile compounds during production and ripening of this artisanal cheese. This work focuses on the quantification of bacterial groups during production and ripening of this artisanal cheese, and establishes the role this microbial community plays in proteolysis and lipolysis.

Although the total aerobic bacteria decreased, the abundance of lactobacilli and lactococci increased rapidly during ripening. In contrast, *Enterobacteriaceae*, total coliforms and staphylococci were high at the beginning of ripening but showed a gradual decrease during ripening and were completely lost from the 90th day of ripening onwards. Filamentous fungi and yeasts increased until the 60th day of ripening whereas a decrease was observed after that time particularly in the inside of the cheese. Relatively high lipolysis levels were observed in this cheese which potentially can be explained by the indigenous lipase in milk and/or by microbial lipases notably present in yeast of which the abundance is high early in ripening. Similarly, we observed the accumulation of substantial amounts of peptides during ripening which might be attributed to the increasing abundance of lactic acid bacteria, as well as the secondary microbiota including filamentous fungi and yeasts and actinobacteria which represent species with established high protease activity. These results are of great interest for the eventual manufacture of this cheese on an industrial scale, while maintaining its authenticity and traditionality.

Introduction

Cheese ripening is a complex process which is the result of biochemical conversion of milk ingredients such as lactose, caseins, and milk fat (McSweeney & Sousa, 2000). In industrial Gouda-type cheeses, the starter lactic acid bacterium (LAB) *Lactococcus lactis* is actively added to the milk and largely responsible for casein degradation (Visser 1993; Smit et al., 1991). Following the primary biochemical events of proteolysis and lipolysis, secondary events occur which include the metabolism of fatty acids and of amino acids. Cheese flavor is mainly caused by these secondary reactions (Ortigosa et al., 2001). *L. lactis* cells import the small peptides resulting from extracellular proteolysis, where 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 chemical action (Kunji et al., 1996; Savijoki et al., 2006).

When compared to industrial cheeses, ripening in traditional raw milk cheeses is typically a spontaneous process in which flavour formation is established by the combined action of non-starter 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; Centeno et al., 2002), by similar mechanisms as described for starter LAB above (Beresford et al., 2001; Collins et al., 2003; Feutry et al., 2012). Similarly, the microbiota in traditional cheeses has the capacity to degrade milk fat, a process termed lipolysis, resulting in free fatty acids (FFA) of a wide range of sizes, of which the short and medium chain FFA contribute directly to the flavor of cheese (Cavanagh et al., 2014). Hence, these microorganisms collectively establish the complex sensorial properties of cheese through their diverse enzymatic systems (Mullan, 2014). In addition, the fungal strains, their corresponding enzymes and the dairy animal from which the milk is derived all affect the proteolysis and lipolysis process in cheese, further diversifying the complex pallet of possible (combinations of) cheese flavours.

Divle Cave cheese is a traditional cheese derived from the spontaneous fermentation of raw ewe's 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 meters. The surface of the goatskin bags color changes from green to red during ripening and this red color 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 flavor. The cheese has unique organoleptic characteristics caused by the production practices and environmental conditions during ripening in the cave.

Previously, we identified the microbiota of Divle Cave cheese in the middle and at the end of ripening, which revealed that a complex community of bacteria and fungi populate this artisanal cheese (Chapter 2, Ozturkoglu-Budak et al., 2016a). Moreover, we established temporal volatile compound profiles as well as the chemical properties during production and ripening. The pH of the cheese decreased during production, but increased during ripening. Fat, salt and dry matter content displayed a gradual increase while ripening progressed which might depend on removal of fragmentation products. A decrease in total nitrogen content was observed in the early stages of ripening followed by an increase after day 60 until the end of the ripening period. Water soluble nitrogen rates of cheese samples appeared to increase during the ripening period. In regards to volatile compounds, although minor differences were observed between farms and batches, carboxylic acids, alcohols, ketones and esters were consistently major classes that were abundantly present at the end of ripening, likely contributing to the characteristic aroma of the cheese.

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

Materials and Methods

Cheese production and sampling

The cheeses were produced as previously reported (Chapter 2, Ozturkoglu Budak et al., 2016a). Two independent batches were produced at each farm with an interval of 15 days. The cheeses were produced by the villagers and this proces was carefully monitored during the full production process, starting from the milking of the

animals up to the filling in goatskin bags. We personally took samples at the stages where the raw milk was obtained, the whey and curd were produced, and during cheese ripening at day 1, 30, 60, 90 and 120. In both independent batches produced, 30 different goatskin bags were analyzed (5 timepoints at 3 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. Analyses were performed in triplicate after mixing the different parts of cheeses within goatskin bags.

Analysis of proteolysis

pH 4.6 soluble nitrogen is a good indicator of proteolysis by residual coagulant (Barbano et al., 1993). The cheese samples were treated to obtain pH 4.6-soluble nitrogen (pH 4.6-SN) extracts according to Kuchroo and Fox (1982). 20 g of grated Divle Cave cheese was homogenized with 100 ml distilled water heated to 40°C with Ultra-Turrax (Heidolph, Schwabach, Germany). The mixture was incubated at 40°C for 1 h. The pH was adjusted to 4.6 with 1 M HCL followed by centrifugation at 5000 rpm for 15 min. The supernatant was filtered using Whatman No. 113 filter paper.

The filtrated 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 Dedector at 214 nm and a C18 (4.6 cm x 250 mm x 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, USA) and deionised water (1:1) mixture, and 40 µl of sample was injected to HPLC after being filtered through 0.45 µm Millex-HV filter with a flow rate of 0.75 ml/min.

The elution was conducted with a mobile phase consisting of two solvents; solvent A (0.1% TFA (v/v) in deionised HPLC-grade water) and solvent B (0.1% TFA (v/v) in UV-grade acetonitrile). The following gradient programme was used; 100% solvent A for 10 min, followed by a linear gradient to 50% B (v/v) over 80 min, increasing to 60% B (v/v) over 5 min and running at 60% B (v/v) for 5 min. Afterwards, the column was washed (95% B (v/v), 5 min) and equilibrated (100% A, 10 min) prior to injection of the next sample.

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, CA, USA) equipped with a flame ionization detector (FID) at 260°C and using a silica capillar TR-FFAP column (30 m x 0.25 mm x 0.25 µm), (Agilent, CA, USA). The carrier gas was high purity helium at flow rate of 2 ml/min with a split ratio of 40:1. The injector and detector temperatures were 250°C and 300°C, respectively. The oven temperature was initially held at 90°C for 1 min, then increased to 150°C with a rate of 7°C/min and held at 150°C for 3 min, followed by a temperature increase to 240°C (10°C/min) and held at this temperature for 15 min. The output signal from the detector was integrated using HP 6890 ChemStation software and the quantification of the FFA levels were performed using three different internal standards of valeric acid (C5:0), heptanoic acid (C7:0) and heptadecanoic acid (C17:0) (Sigma–Aldrich, St. Louis, USA).

For the calculation of the FFA levels, calibration curves were determined at five concentrations (100 mg/kg, 200 mg/kg, 300 mg/kg, 400 mg/kg, 500 mg/kg) by using a mixture of individual FFA standards, butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) fatty acids, with fixed concentrations of internal standards of valeric acid (C5:0), heptanoic acid (C7:0) and heptadecanoic acid (C17:0).

The formula below was used to calculate FFA concentrations (mg/kg)

$$Ci = \frac{Ai}{Ast} \times Cst \times RF \times SF$$

$$Ci = \frac{Ai}{Ast} \times Cst \times RF \times SF$$

$$RF = \frac{\text{Standard peak area}}{\text{Compound peak area}} \times \frac{\text{Compound concentration}}{\text{Internal standard concentration}}$$

Ci: Concentration of compound (mg/kg)

Ai: Peak area of compound

Ast: Peak area of internal standard

Cst: Concentration of internal standard (mg/kg)

RF: Response factor

SF: Dilution factor

Microbial counts

10 ml milk and whey, 10 g curd and cheese were homogenized with 90 ml Ringer solution in a Stomacher (Bag Mixer 400 VW, Interscience, France) for 3x1.5 min. Serial 10-fold dilutions were made in Ringer solution (Merck, Darmstadt, Germany) and plated in duplicate.

Total aerobic mesophilic bacteria were determined on Plate Count Agar (Merck, Darmstadt, Germany) 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. Lactobacilli were grown on de Man-Rogosa-Sharpe Agar (pH 5.4; Merck) and incubated for 72 h at 30°C under anaerobic conditions. Lactococci were anaerobically grown on M17 agar including lactose (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.

Statistical analysis

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

Results and Discussion

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). RP-HPLC peptide profiles of the peptide fractions of curd and cheeses at day 1, 30, 60, 90 and 120 days of ripening

are shown in Figure 1. In this study, total peptide analyses were performed in 85 minutes. Peptides that eluted in the early retention time (from 5 to 30 min) correspond to hydrophilic peptides, while peptides that eluted in the late retention times (from 55 to 85 min) correspond to hydrophobic peptides. At the intermediate time (from 30 to 55 min) low molecular weight hydrophobic peptides eluted (Hayaloglu & Karabulut, 2013a). Fragmentation of macro peptides into micro peptides and amino acids is represented by a decrease in peak height and area in the hydrophobic zone and an increase in peak height and area in hydrophilic zone. Formation of short peptides and aminoacids caused by further proteolysis are part of the development of cheese flavor. Towards the end of ripening, the breakdown of accumulated branched-chain amino-acids leads to the production of flavor compounds including aldehydes, alcohols and acids (McSweeney, 2004).

Qualitative and quantitative differences were observed in all regions over the course of ripening. Especially in the intermediate region of the chromatogram, many peaks occurred during the course of ripening which indicates 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 1). The peptide eluted at 38th minute was determined the most prominent in all 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. An apparent increase was observed at the 60th day of ripening coinciding with the increase in peptide-N content which multiplied as a result of the activity of peptidases, which were previously reported to be of fungal origin (Gobbetti et al., 1997; Prieto et al., 2002). This strong peptidase activity remained constant until the end 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). This could explain the reduction in the complexity of the chromatograms at day 90 and 120 of Divle Cave cheese ripening.

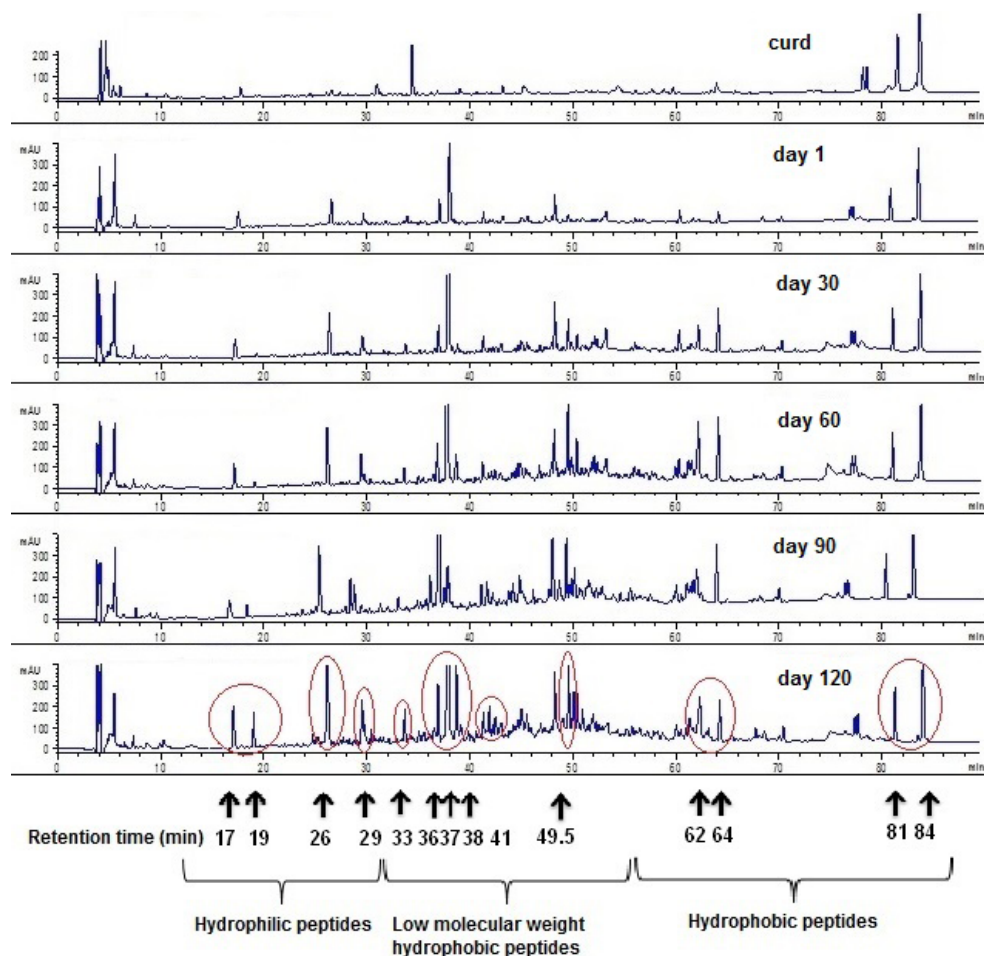


Figure 1. Peptide profiles resulting from proteolysis in Divle Cave cheese

In total, 23 peaks covering 90% of the total analysis period were detected in the chromatograms. During variance analyses, it was determined that 20 out of these 23 peaks significantly changed throughout the ripening period ($P < 0.05$). Six of these peaks (5.5., 7., 17., 19., 26., 29. min) occurred in hydrophilic zone, and also six peaks (33., 37., 38., 41., 48., 49.5. min) occurred in middle zone, while eight peaks (60., 62., 64., 68., 70., 77., 81., 84. min) occurred in hydrophobic zone.

The observed changes in the hydrophilic region is an indicator of the hydrolysis of proteins to peptides and amino acids. According to the chromatogram that we obtained in our study (Figure 1), peaks eluted in this region at retention times of 17, 26 and 29

min showed an apparent increase from day 1 to day 120. Moreover, a peak that eluted at 19 min first appeared on day 60 and continue to increase until the end of ripening. In the intermediate region, the most significant increase was observed in peak eluted at min 37. The peaks eluted at min 36 and 38 showed an increase during ripening. Also in this region, a peak that eluted at 49.5 first appeared on day 60 and increased through the end of ripening. In the hydrophobic region, the most prominent peaks were determined at 81 and 84 min. In this region peaks first tended to increase at day 30, whereas after this stage a decrease was observed for them. The fact that the vast majority of peptide levels still significantly alters between the last 2 measured stages of ripening suggests that shorter ripening times would lead to a distinctly different cheese, and even longer ripening periods could be considered to further mature the cheese.

Principal Component Analysis (PCA) of the peptide profiles

PCA of the peptide profiles revealed that the samples clearly separated according to their age (Figure 4). Curd and cheese samples on day 1 and 30 had similarities in their peptide profiles, whereas cheese samples obtained on day 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 dairy farms that might be due to variations in production techniques and possible diversity in microbial communities and their corresponding enzyme pallet during the un-controlled ripening periods. Samples obtained early in the ripening period (1 and 30 days) were characterized by more hydrophobic peptides. In contrast, for the samples obtained later on ripening (60, 90 and 120 days) more hydrophilic peptides were observed.

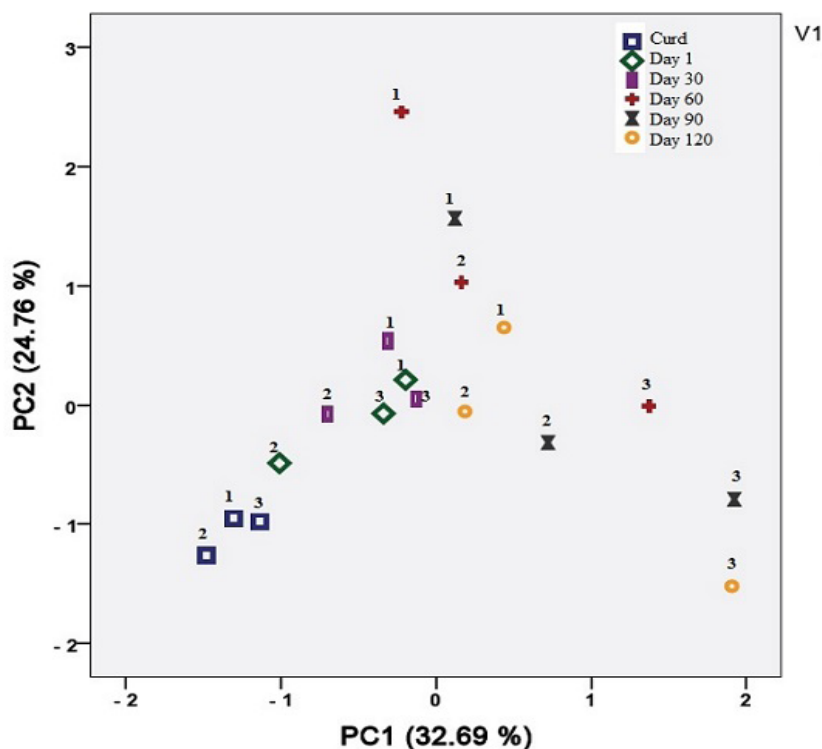


Figure 4. Score plot of principal component analysis based on peptide profile data (RP-HPLC) of curd and cheese on day 1, 30, 60, 90 and 120 during the ripening of Divle Cave cheese

Similarly, in the studies of other fungal cheeses (Blue cheese), a decrease was observed in the ratio between hydrophobic and hydrophilic peptides during the ripening period (Diezhandino et al., 2015; Gonzales De Llano et al., 1995). 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 aminoacids (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 (Larráyo et al., 1999). Especially sheep milk cheeses and mold-ripened cheeses have higher values of total FFA (Svensson et al., 2006).

FD1A (TUMPNR51.D)

Accumulated FFA compounds during the ripening period are considered representative of the degree of lipolysis (Woo & Lindsay, 1982; 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 increase ratio of MCFA and LCFA levels was relatively higher during ripening (Table 1). 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. Especially high values for fatty acids with carbon numbers greater than 16 were observed, including palmitic acid (C16:0), stearic (C18:0), oleic acid (C18:1) and myristic (C14:0) acids. The authors also reported that, MCFA was significantly changed by cheese age. These results may have relevance for the characteristics taste of long-matured Divle Cave cheese which includes 15 different strains of *Penicillium* spp. (Chapter 2, 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. Similar to Divle Cave cheese, it was also defined that cheese without starter culture had higher MCFA during ripening (Atasoy & Türkoğlu, 2009). However, despite the quantitative importance of MCFA and LCFA, they are not reported as the main contributors to cheese flavour (Freitas & Malcata, 1998).

Figure 3 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 1). 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).

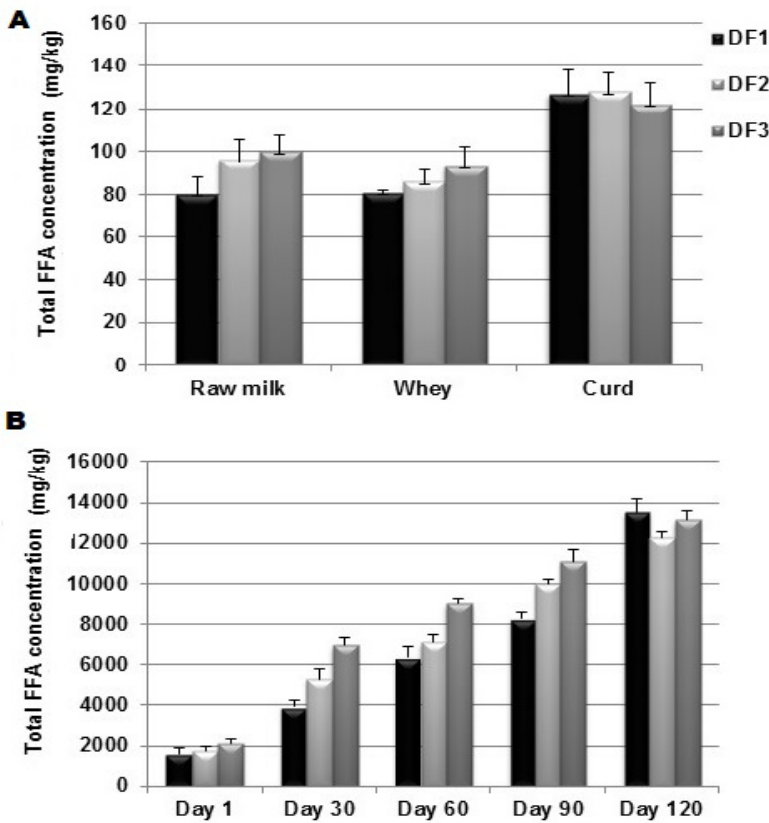


Figure 3. Total free fatty acid concentrations **A.** during production, **B.** during ripening of Divle Cave cheese

The progression of lipolysis throughout production and ripening is consistent with the earlier studies of ewes' milk cheese (Mallatou et al., 2003), blue cheese

(Diezhandino et al., 2015) and raw milk traditional cheese (Bontinis et al., 2012; Marrone et al., 2014). Similarly, a steady increase in FFAs content was determined in a wide range of cheeses such as Idiazabal (Chavarri et al., 1999), Turkish cheeses (Hayaloglu & Karabulut, 2013b) and Reggianito Argentino cheese (Wolf et al., 2010) 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 the inability to produce standardize cheeses, the variability in fat content of milk and for 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 for lipolysis levels among cheeses produced in different dairy factories.

Table 1. Free fatty acid concentrations (mg/kg) of Divle Cave cheese during production and ripening (mean $\pm$ standard deviation)

FFA	Cheese ^a	Production of cheese			Ripening of cheese					
		Raw milk	Whey	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
C4:0	DF1	4.38 $\pm$ 0.29 ^{Aa}	4.95 $\pm$ 0.09 ^{Aa}	5.11 $\pm$ 0.16 ^{Aa}	73.08 $\pm$ 0.58 ^{Bc}	75.96 $\pm$ 0.64 ^{Ca}	105.61 $\pm$ 0.57 ^{Da}	168.91 $\pm$ 0.47 ^{Eb}	222.8 $\pm$ 0.65 ^{Fb}	
	DF2	5.06 $\pm$ 0.04 ^{Ab}	5.05 $\pm$ 0.05 ^{Aa}	6.15 $\pm$ 0.05 ^{Bb}	71.09 $\pm$ 0.43 ^{Bb}	126.8 $\pm$ 0.55 ^{Db}	145.51 $\pm$ 0.54 ^{Eb}	158.39 $\pm$ 0.81 ^{Fa}	170.63 $\pm$ 0.34 ^{Ga}	
	DF3	7.86 $\pm$ 0.02 ^{Bbc}	5.36 $\pm$ 0.05 ^{Ab}	8.87 $\pm$ 0.05 ^{Cc}	68.81 $\pm$ 0.15 ^{Da}	139.1 $\pm$ 0.27 ^{Ec}	145.65 $\pm$ 0.38 ^{Fb}	171.24 $\pm$ 0.41 ^{Ge}	279.83 $\pm$ 0.93 ^{Hc}	
C6:0	DF1	3.27 $\pm$ 0.20 ^{Aa}	3.56 $\pm$ 0.07 ^{Ab}	3.65 $\pm$ 0.14 ^{Aa}	77.48 $\pm$ 0.47 ^{Bb}	85.7 $\pm$ 0.82 ^{Ca}	93.43 $\pm$ 0.45 ^{Da}	108.36 $\pm$ 0.30 ^{Ea}	192.08 $\pm$ 0.59 ^{Fa}	
	DF2	4.78 $\pm$ 0.08 ^{Bb}	3.4 $\pm$ 0.06 ^{Aa}	5.09 $\pm$ 0.12 ^{Bb}	90.76 $\pm$ 0.34 ^{Cc}	108.24 $\pm$ 0.73 ^{Db}	111.03 $\pm$ 0.44 ^{Eb}	158.24 $\pm$ 0.60 ^{Fb}	242.95 $\pm$ 0.44 ^{Gb}	
	DF3	5.16 $\pm$ 0.03 ^{Bc}	4.35 $\pm$ 0.07 ^{Ac}	5.01 $\pm$ 0.04 ^{Bb}	75.42 $\pm$ 0.26 ^{Ca}	209.06 $\pm$ 0.19 ^{Ec}	189.21 $\pm$ 0.36 ^{Dc}	317.94 $\pm$ 0.28 ^{Fe}	347.05 $\pm$ 0.20 ^{Ge}	
C8:0	DF1	2.55 $\pm$ 0.18 ^{Aa}	3.6 $\pm$ 0.06 ^{Ba}	4.07 $\pm$ 0.10 ^{Ba}	83.67 $\pm$ 0.44 ^{Bb}	100.41 $\pm$ 0.38 ^{Db}	114.7 $\pm$ 0.37 ^{Ea}	211.03 $\pm$ 0.58 ^{Fa}	463.77 $\pm$ 0.64 ^{Gb}	
	DF2	3.05 $\pm$ 0.05 ^{Ab}	3.71 $\pm$ 0.04 ^{Aa}	4.6 $\pm$ 0.05 ^{Bb}	74.28 $\pm$ 0.38 ^{Ca}	98.7 $\pm$ 0.37 ^{Da}	224.4 $\pm$ 0.46 ^{Eb}	240.09 $\pm$ 0.58 ^{Fb}	331.06 $\pm$ 0.53 ^{Ga}	
	DF3	4.08 $\pm$ 0.03 ^{Ac}	4.95 $\pm$ 0.10 ^{ABb}	4.47 $\pm$ 0.02 ^{Bb}	97.08 $\pm$ 0.12 ^{Cc}	163.17 $\pm$ 0.31 ^{Dc}	504.3 $\pm$ 0.30 ^{Ec}	780.84 $\pm$ 0.53 ^{Ge}	685.82 $\pm$ 0.80 ^{Fe}	
C10:0	DF1	6.01 $\pm$ 0.20 ^{Aa}	6.3 $\pm$ 0.04 ^{Aa}	6.75 $\pm$ 0.23 ^{Ab}	108.6 $\pm$ 0.50 ^{Ba}	134.08 $\pm$ 0.77 ^{Ca}	267.99 $\pm$ 0.45 ^{Da}	470.88 $\pm$ 1.33 ^{Ea}	688.15 $\pm$ 0.51 ^{Fa}	
	DF2	6.67 $\pm$ 0.07 ^{Ab}	6.96 $\pm$ 0.12 ^{Ab}	8.9 $\pm$ 0.17 ^{Bc}	119.42 $\pm$ 0.54 ^{Cc}	301.69 $\pm$ 0.91 ^{Db}	407.13 $\pm$ 0.49 ^{Eb}	510.86 $\pm$ 0.77 ^{Fb}	771.92 $\pm$ 0.41 ^{Gb}	
	DF3	5.85 $\pm$ 0.05 ^{Ba}	7.5 $\pm$ 0.03 ^{Cc}	4.65 $\pm$ 0.06 ^{Aa}	113.31 $\pm$ 0.36 ^{Db}	388.02 $\pm$ 0.55 ^{Ec}	473.12 $\pm$ 0.13 ^{Fe}	579.21 $\pm$ 0.71 ^{Ge}	797.68 $\pm$ 0.68 ^{Hc}	
C12:0	DF1	4.56 $\pm$ 0.19 ^{Ab}	4.77 $\pm$ 0.05 ^{Aa}	5 $\pm$ 0.15 ^{Aa}	70.58 $\pm$ 0.38 ^{Ba}	81.17 $\pm$ 0.71 ^{Ca}	282.49 $\pm$ 0.40 ^{Ea}	244.03 $\pm$ 0.47 ^{Da}	512.6 $\pm$ 0.61 ^{Fb}	
	DF2	3.68 $\pm$ 0.03 ^{Aa}	4.95 $\pm$ 0.06 ^{Bb}	5.66 $\pm$ 0.08 ^{Bb}	78.57 $\pm$ 0.71 ^{Bb}	245.83 $\pm$ 0.26 ^{Db}	298.68 $\pm$ 0.75 ^{Eb}	453.55 $\pm$ 0.56 ^{Fb}	479.87 $\pm$ 0.57 ^{Ga}	
	DF3	5.23 $\pm$ 0.05 ^{Ac}	5.56 $\pm$ 0.04 ^{Ac}	5.56 $\pm$ 0.06 ^{Ab}	85.6 $\pm$ 0.40 ^{Bc}	405.61 $\pm$ 0.25 ^{Cc}	492.16 $\pm$ 1.88 ^{Dc}	512.64 $\pm$ 0.38 ^{Ec}	575.33 $\pm$ 0.75 ^{Fe}	
C14:0	DF1	9.13 $\pm$ 0.12 ^{Aa}	9.01 $\pm$ 0.11 ^{Aa}	8.86 $\pm$ 0.35 ^{Aa}	72.45 $\pm$ 0.39 ^{Ba}	174.19 $\pm$ 0.53 ^{Ca}	544.31 $\pm$ 0.62 ^{Da}	631.09 $\pm$ 0.72 ^{Ea}	1354.82 $\pm$ 0.81 ^{Fb}	
	DF2	14.97 $\pm$ 0.31 ^{Bc}	11.05 $\pm$ 0.06 ^{Ac}	30.08 $\pm$ 0.19 ^{Cb}	194.08 $\pm$ 0.58 ^{Dc}	590.87 $\pm$ 0.44 ^{Eb}	727.87 $\pm$ 0.69 ^{Fb}	1128.78 $\pm$ 0.55 ^{Gb}	1311.81 $\pm$ 0.43 ^{Ha}	
	DF3	10.02 $\pm$ 0.08 ^{ABb}	10.66 $\pm$ 0.07 ^{Bb}	9.33 $\pm$ 0.04 ^{Aa}	162.8 $\pm$ 0.24 ^{Bb}	1220.09 $\pm$ 0.74 ^{De}	1516.9 $\pm$ 0.71 ^{Ec}	2020.74 $\pm$ 0.75 ^{Fe}	1543.13 $\pm$ 0.38 ^{Fe}	
C16:0	DF1	20.85 $\pm$ 0.51 ^{Aa}	22.46 $\pm$ 0.12 ^{Ba}	24.01 $\pm$ 0.37 ^{Ca}	396.19 $\pm$ 0.71 ^{Dc}	398.95 $\pm$ 0.65 ^{Ea}	1517.09 $\pm$ 0.98 ^{Fa}	1541.09 $\pm$ 0.48 ^{Ga}	4357.7 $\pm$ 0.43 ^{Hc}	
	DF2	26.8 $\pm$ 0.08 ^{Ac}	26.49 $\pm$ 0.15 ^{Ac}	35.83 $\pm$ 0.11 ^{Bc}	363.97 $\pm$ 0.53 ^{Bb}	1251.05 $\pm$ 0.33 ^{Db}	1542.16 $\pm$ 0.61 ^{Eb}	2477.83 $\pm$ 0.86 ^{Fb}	4046.34 $\pm$ 0.54 ^{Ga}	
	DF3	23.13 $\pm$ 0.04 ^{Ab}	25.89 $\pm$ 0.08 ^{Bb}	25.01 $\pm$ 0.12 ^{ABb}	357.3 $\pm$ 0.26 ^{Ca}	1546.2 $\pm$ 0.33 ^{Dc}	2547.01 $\pm$ 0.82 ^{Ec}	2900.65 $\pm$ 2.48 ^{Fe}	4200.88 $\pm$ 0.58 ^{Gb}	

Table 1. Free fatty acid concentrations (mg/kg) of Divile Cave cheese during production and ripening (mean $\pm$ standard deviation)

FFA	Cheese ^a	Production of cheese			Ripening of cheese					
		Raw milk	Whey	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
C18:0	DF1	8.77 $\pm$ 0.20 ^{Aa}	16.45 $\pm$ 0.05 ^{Ba}	28.46 $\pm$ 0.63 ^{Cc}	89.56 $\pm$ 0.51 ^{Db}	129.56 $\pm$ 0.37 ^{Ea}	656.6 $\pm$ 1.34 ^{Fb}	677.28 $\pm$ 0.61 ^{Ga}	1591.07 $\pm$ 0.71 ^{Hc}	
	DF2	13.1 $\pm$ 0.07 ^{Bbc}	17.81 $\pm$ 0.12 ^{Cb}	10.71 $\pm$ 0.07 ^{Aa}	83.15 $\pm$ 0.37 ^{Da}	368.52 $\pm$ 0.38 ^{Eb}	586.72 $\pm$ 0.38 ^{Fa}	1363.88 $\pm$ 1.09 ^{Gb}	1476.18 $\pm$ 0.93 ^{Hb}	
	DF3	11.52 $\pm$ 0.05 ^{Ab}	20.47 $\pm$ 0.07 ^{Bc}	25.99 $\pm$ 0.18 ^{Cb}	219.4 $\pm$ 0.12 ^{De}	1261.13 $\pm$ 0.21 ^{Ec}	1465.27 $\pm$ 0.33 ^{Gc}	3246.15 $\pm$ 0.76 ^{Hc}	1295.58 $\pm$ 0.55 ^{Fa}	
C18:1	DF1	17.45 $\pm$ 0.33 ^{Ab}	28.1 $\pm$ 0.02 ^{Bc}	33.31 $\pm$ 0.29 ^{Cc}	271.88 $\pm$ 0.43 ^{Da}	1614.07 $\pm$ 3.80 ^{Ea}	2151.62 $\pm$ 0.78 ^{Fb}	2980.84 $\pm$ 0.38 ^{Hc}	2565.55 $\pm$ 0.45 ^{Gb}	
	DF2	14.42 $\pm$ 0.08 ^{Aa}	25.46 $\pm$ 0.07 ^{Cb}	15.62 $\pm$ 0.29 ^{Ba}	409.34 $\pm$ 0.68 ^{Db}	1722.88 $\pm$ 0.43 ^{Eb}	2345.7 $\pm$ 0.35 ^{Fc}	2930.6 $\pm$ 0.67 ^{Hb}	2847.91 $\pm$ 0.94 ^{Gc}	
	DF3	21.79 $\pm$ 0.05 ^{Bc}	19.89 $\pm$ 0.10 ^{Aa}	28.46 $\pm$ 0.06 ^{Cb}	676.51 $\pm$ 0.27 ^{Dc}	1850.9 $\pm$ 0.33 ^{Ec}	1966.7 $\pm$ 0.80 ^{Fa}	2080.63 $\pm$ 0.58 ^{Ga}	2422.37 $\pm$ 2.03 ^{Ha}	
C18:2	DF1	2.67 $\pm$ 0.10 ^{Aa}	4.55 $\pm$ 0.05 ^{Bb}	6.61 $\pm$ 0.18 ^{Cc}	99.32 $\pm$ 0.49 ^{Eb}	46.29 $\pm$ 0.67 ^{Da}	254.05 $\pm$ 0.79 ^{Ga}	305.13 $\pm$ 0.33 ^{Hb}	124.58 $\pm$ 0.37 ^{Fa}	
	DF2	2.55 $\pm$ 0.08 ^{Aa}	3.9 $\pm$ 0.05 ^{Ba}	4.89 $\pm$ 0.06 ^{Cb}	30.87 $\pm$ 0.31 ^{Da}	145.24 $\pm$ 0.37 ^{Fb}	290.73 $\pm$ 0.38 ^{Hb}	108.54 $\pm$ 0.55 ^{Fa}	162.11 $\pm$ 0.96 ^{Gc}	
	DF3	4.75 $\pm$ 0.03 ^{Bb}	5.89 $\pm$ 0.04 ^{Cc}	3.79 $\pm$ 0.04 ^{Aa}	139.87 $\pm$ 0.54 ^{Dc}	234.04 $\pm$ 0.08 ^{Fc}	368.7 $\pm$ 0.37 ^{Gc}	755.69 $\pm$ 0.31 ^{Hc}	142.44 $\pm$ 0.61 ^{Eb}	
C18:3	DF1	0.35 $\pm$ 0.03 ^{Aa}	0.09 $\pm$ 0.00 ^{Aa}	1.08 $\pm$ 0.03 ^{Ac}	286.34 $\pm$ 0.99 ^{Bc}	306.69 $\pm$ 0.77 ^{Cb}	385.23 $\pm$ 0.75 ^{Db}	447.08 $\pm$ 0.25 ^{Eb}	589.01 $\pm$ 0.36 ^{Fc}	
	DF2	0.96 $\pm$ 0.07 ^{Ac}	1.1 $\pm$ 0.05 ^{Ac}	0.45 $\pm$ 0.02 ^{Aa}	235.95 $\pm$ 0.38 ^{Bb}	324.99 $\pm$ 0.27 ^{Cc}	431.69 $\pm$ 0.60 ^{Dc}	463.08 $\pm$ 0.77 ^{Fc}	444.63 $\pm$ 1.17 ^{Eb}	
	DF3	0.77 $\pm$ 0.02 ^{Ab}	0.56 $\pm$ 0.04 ^{Ab}	0.82 $\pm$ 0.02 ^{Ab}	114.75 $\pm$ 0.71 ^{Ba}	223.09 $\pm$ 0.17 ^{Ca}	350.65 $\pm$ 0.42 ^{Fa}	337 $\pm$ 0.37 ^{Ea}	237.2 $\pm$ 0.44 ^{Da}	
Total										
Fatty	DF1	79.99 ^{Aa}	103.85 ^{Ba}	126.91 ^{Cb}	1629.15 ^{Da}	3147.07 ^{Ea}	6373.12 ^{Fa}	7785.71 ^{Ga}	12662.13 ^{Hc}	
Acids	DF2	96.05 ^{Ab}	109.88 ^{Bb}	127.99 ^{Cb}	1751.48 ^{Db}	5284.82 ^{Eb}	7111.61 ^{Fb}	9992.82 ^{Gb}	12285.41 ^{Ha}	
	DF3	100.17 ^{Ac}	111.08 ^{Bc}	121.96 ^{Ca}	2110.84 ^{Dc}	7640.42 ^{Fc}	10019.67 ^{Fc}	13702.73 ^{Hc}	12527.32 ^{Gb}	

Mean $\pm$ SD values of the two replicate productions for each dairy farm analysed in triplicate, expressed as relative abundance to internal standard.

^a Cheese produced in DF1: Dairy Farm 1, DF2: Dairy Farm 2, DF3: Dairy Farm 3

^{A-D} Superscripted capital letters in the same row show the significant differences between ripening times $P < 0.05$

^{a-c} Subscripted lower case letters in the same column show the significant differences between different dairy farms $P < 0.05$

PCA of free fatty acid profiles

PCA of FFA demonstrated that cheeses separated mainly according to ripening time (Figure 4). Due to extremely low concentrations of FFAs in raw milk and curd, they were not included in the score plot. At day 1, cheeses produced in different dairy farms grouped together, but when the concentrations of fatty acids increased in the processing stages larger variations became visible. PCA confirmed that cheeses produced in DF1 and DF2 are more similar than those from DF3.

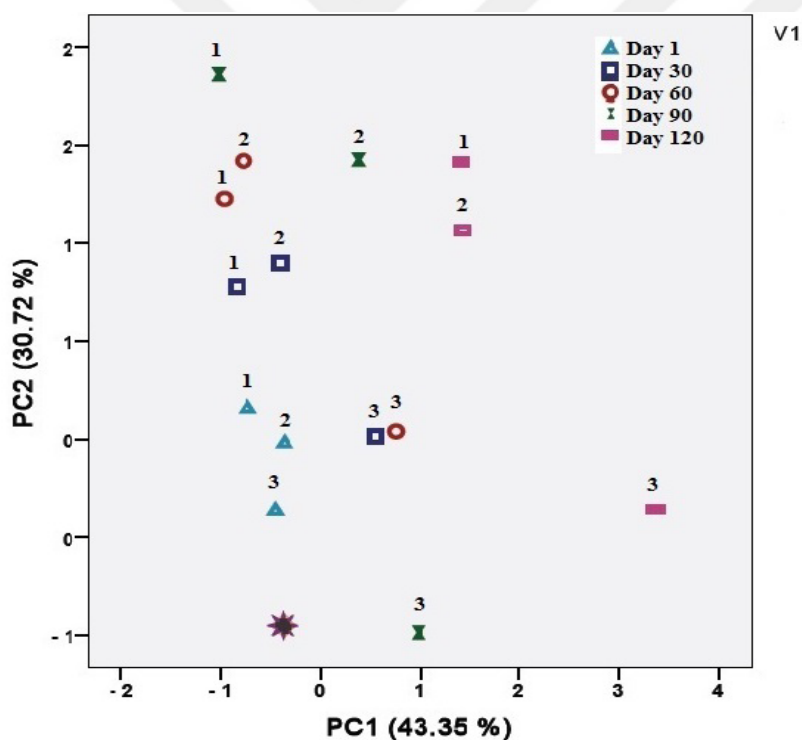


Figure 4. Score plot of principal component analysis based on free fatty acid data (GC) of cheese on day 1, 30, 60, 90 and 120 during the ripening of Divle Cave cheese

Analyses of the total FFA amounts indicates that Divle Cave cheese made from ewes' cheese undergoes a high 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) as reported by McSweeney (1997) about main contributors to lipolysis for raw milk cheeses. The other reasons of high lipolysis in mold-ripened

cheeses are lipase enzymes produced during the growth of the filamentous fungi and yeasts (Larsen and Jensen, 1999).

Assessment of microbial groups

Our earlier work has established which microbial species are present in matured Divle Cave cheese (Chapter 2). However, this end-point analyses was qualitative, and microbial species that are abundant earlier in the ripening process might also be more important for lipo- and proteolysis. 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, curd) and ripening (1st, 30rd, 60th, 90th and 120th day) are shown in Figure 5. 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 in- and outside of the goatskin, the ratio of the 7 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. Nevertheless, specific differences between the in- and outside microbiota of the goatskin bag cheeses were observed that are addressed below.

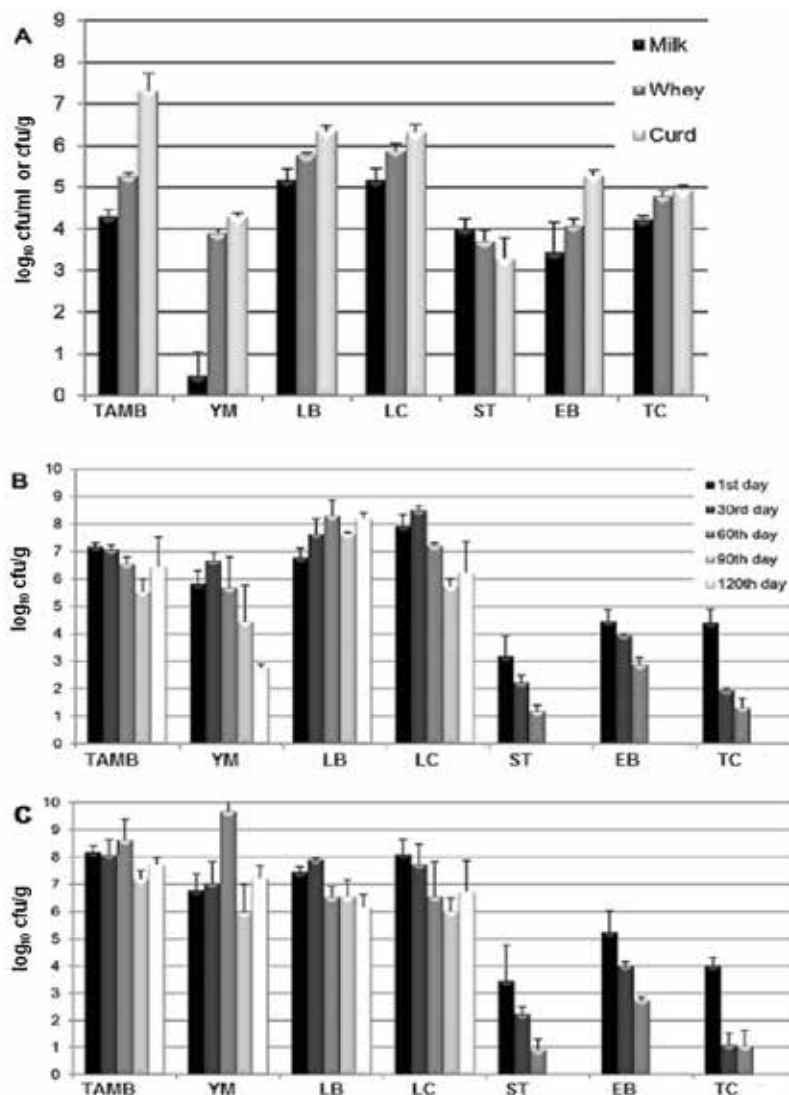


Figure 5. Average microbial counts ($\log_{10}$ cfu/ml or $\log_{10}$ cfu/g $\pm$ SD) **A.** raw milk, whey and curd during production, **B.** cheese samples taken from inside of cheese in goatskin bags **C:** cheese samples taken from outside of cheese in goatskin bags in two replicate trials of three independent batches (TAMB: Total Aerobic Mesophilic Bacteria, YM: Yeast and Filamentous fungi, LB: Lactobacilli, LC: Lactococci, ST: Staphylococci, EB: Enterobacteriaceae, TC: Total coliforms)

The TAMB decrease could be caused by a decrease in pH and an increase in salt concentration in the dry matter 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 Bryndza cheese (Pangallo et al., 2014), ewe's cheese (Galan et al., 2012), and some other Turkish cheeses ripened in skin (Arslaner, 2008; Hayaloglu & Kirbag, 2007).

The number of lactobacilli demonstrated a rapid increase in the interior part of goatskin and reached the highest value at 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.

Lactococci were highly abundant. Initial numbers from milk plated on M17 medium were 5.2 log cfu/ml and attained the highest level on the first day of ripening (8.4 cfu/g). The numbers decreased until the end of ripening to a final value of 6.4 log cfu/g. The number of lactococci was rather similar from the inside and outside. The level of lactococci showed a decrease in an average of 2.0 log and 1.7 log from the inside and outside samples, respectively. These results are in good agreement with an earlier study (Alegria 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 (Alegria et al., 2009). These microbial groups were high during production and at the beginning of ripening but showed a gradual decrease during ripening and were completely lost at the 90th day of ripening. 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 ewe's 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 10³-fold higher than those in the inside (3.3 log cfu/g). The decrease inside of the cheese can be explained by the anaerobic condition occurring inside the cheese, decreased humidity,

increased salt in dry matter and the pH alterations (Chapter 4; 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* (Gupta & Srivastava, 2014; Muhialdin et al., 2016; Ryu et al., 2014) produce antifungal compounds. The increase on the outside samples results from the growth of natural fungi on the surface of the goatskin originating from the cave and the non-homogenous transition of these fungi into the underlying layers.

Conclusions

Mold-ripened cheeses are particularly characterized by an intense lipolysis. The lipases secreted from *Penicillium camemberti*, *P. roqueforti*, and *G. candidum* degrade triglycerides and generate free fatty acids (FFAs) which are precursor for secondary biochemical events for the formation of flavor compounds. The FFA ratio to total fatty acids demonstrated the extend of lipolysis which is determined high for Camembert cheese made from raw milk (6–10%), very high for Roquefort cheese (7–12%) and diverse for Blue cheese (6–25%) (Desmaures, 2014). Among FFAs, particularly short chain fatty acids (SCFA) directly contribute to aroma development in many aged cheeses. The abundance of methyl ketones, produced by the oxidation of fatty acids, and related secondary alcohols produced from FFA play an important role in determining the characteristic flavor components in Blue cheese (Gallois & Langlois, 1990). The SCFA, or more specifically hexanoic and octanoic acids, are characteristic flavor components of aged cheeses (Curioni & Bosset, 2002). The MCFA are also key components in Cheddar, Roncal and probably other cheese varieties, because of their low perception thresholds. In contrast, LCFA have high perception thresholds, which limit their contribution to cheese flavor, in spite of the high concentrations commonly reached in many cheese types (Curioni and Bosset, 2002).

Similar to FFA catabolism, amino acid catabolism is also particularly significant in mold- 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 catalyzed by aminotransferase enzymes and they formed the carboxylic acids of 3-methyl butanoic (isovaleric acid), 2-methyl butanoic and 2-methyl propanoic acid (isobutyric acid), respectively (Ardö, 2006; Smit et al., 2000). Further aldehydes are formed from these acids and then can be reduced to the corresponding alcohols.

(McSweeney, 2004). According to our obtained data, the presence of 3-methylbutanoic acid, 2-methyl propanoic acid, 3-methyl butanal, 2-methyl propanal, 2-butanol and 2-propanol at a certain amount (Chapter 4) are indicating that the amino acid catabolism is developing as a result of progressed proteolysis.

This work provides the first characterization of proteolysis and lipolysis of Divle Cave cheese during the production and ripening processes. Although the actual chemical components were not identified, the lipolysis and proteolysis degree 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 that enters the product from the air or contaminated tools, particularly yeast, filamentous fungi and actinobacteria, as well as native proteases, lipases and esterases present in raw milk. With this work, we provide data for characterization of a raw milk artisanal cheese which is of great importance for determination of geographical origin and authentication of traditional cheeses. Moreover, these results are of importance for the eventual standardized manufacture of this cheese under controlled conditions and on an industrial scale. To definitely establish a link between specific microbial species and proteo- and lipolysis, we have employed a panel of microbes isolated from Divle Cave cheese (Chapter 2, Ozturkoglu Budak et al., 2016a) and assessed their lipase and protease activity (Chapter 5).

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CHAPTER 4

Volatile compound profiling of Turkish Divle Cave cheese during production and ripening

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Abstract

The formation of volatile compounds in Turkish Divle Cave cheese produced in three different dairy farms was determined during production and ripening, revealing 110 compounds including acids, alcohols, ketones, esters and terpenes. The presence and concentration of these volatile compounds varied between specific phases of the production and the 120 day ripening process. Smaller differences were also detected between cheeses produced at different farms. Carboxylic acids were established as a major class at the end of ripening. The relative amounts of acids and ketones increased until the 90th day of ripening, while alcohols increased for the first 30 days and tailed off during the remaining part of the ripening process. The level of esters increased gradually until the end of ripening. Butanoic, acetic and valeric acids, 2-butanol, 2-butanone, 2-heptanone, ethyl butanoate, α -pinene and toluene were the most abundant compounds, likely contributing to the characteristic aroma of this traditional cheese.

Introduction

Fermented milk products typically contain a wide range of flavors which are the result of biochemical conversions of milk components such as lactose, caseins, and milk fat. In cheese the majority of flavors are derived from proteolytic processes and the subsequent conversion of amino acids (Visser, 1993). In industrial cheese productions, starter cultures are typically added to milk fermentations, greatly accelerating the process of flavour formation, as these bacteria harbour efficient proteolytic systems and amino-acid converting enzymes, resulting in the formation of a wide range of volatile compounds during production and ripening (Yvon & Rijnen, 2001; Smit et al., 2005).

Raw milk and raw milk cheeses typically also contain lactic acid bacteria and these strains possess amino acid converting enzymes with higher or more diverse activities as compared to industrial dairy cultures, resulting in cheeses of more diverse and intense flavour (Centeno et al., 2002; Settanni & Moschetti, 2010). Moreover, other bacterial species, either naturally present in milk or contaminants added in during the handling of the milk, are present in raw milk and, albeit less controlled, play an important role in aroma development by their wide range of enzymes such as proteases and lipases (Chávez et al., 2010).

Divle Cave cheese is a semi-hard cheese made from raw semi-skimmed ewes' milk. It is produced in May and June when milk is abundant, in Karaman, a rural region in the middle of Turkey. The ripening takes place in Divle cave, which is located in the south of this region. The average temperature in the cave is 5-10°C with a humidity of 85-90%. The cheeses are produced without using any starter culture and are ripened for 4 months in goatskin bags at a depth of approximately 70 m (Ozturkoglu-Budak et al., 2016a). The surface of the goatskin bags changes from green to red during ripening in the cave and this red color is the distinguishing characteristic of Divle Cave cheese (Figure 1), when compared to other spontaneously mold growing cheeses from Turkey, e.g. Kuflu cheese. Divle Cave cheese has a crumbly texture and a pungent flavour. Moreover, a large variety of aroma compounds occur in this cheese, likely as a consequence of the contribution of the diverse bacterial community present in raw milk (Ozturkoglu-Budak et al., 2016a).

So far, studies on the flavor of Divle Cave cheese focused on the products at the end of ripening (Hayaloglu & Karabulut, 2013), but there are no studies published on the development of the volatile profile during production and ripening, which was the aim of this research. This paper presents data that contributes to a better characterization of Divle Cave cheese and this data will help to determine the authenticity of Divle Cave cheese in the future. The obtained data will also be useful for future comparative studies, both on mold ripened cheeses and on raw ewe's milk cheeses, enabling identifications of key flavor compounds that determine the unique taste of each of these cheeses.

Materials and Methods

Cheese production

Divle Cave cheeses were produced according to the traditional process and ripened in the Divle cave (Chapter 2, Ozturkoglu-Budak et al., 2016a). Standardized semi-skimmed raw ewe's milk was coagulated with calf rennet at 30-32°C for 80 min. The coagulum was cut into small sizes, heated to 55-60°C for 4-5 min and the curd was filled into cotton clothes for whey drainage for 2-3 hours. To remove the whey, pressure was applied to the cotton bag containing the curd at room temperature for one night. The resulting curd was rinsed with water for 24 hours for complete removal of the whey. Subsequently, cheese blocks were dried by overnight storage at room temperature. The curd was broken into small pieces by hand, dry-salted and tightly pressed into salted and dried goatskin bags. The goatskin bag was subsequently closed by sewing and pierced with needles in order to avoid serum loss. At the pre-ripening stage, goatskin bags were kept under cool storage conditions (15-20°C) until whey drainage stopped, in a process that took 7 to 10 days. Finally, the cheeses were left in Divle cave for 4 months for ripening.

Cheese sampling

Cheese productions were carried out in three different dairy farms (DF1, DF2, DF3) in the Karaman province of Turkey. Two independent batches were produced by each of the three farms with an interval of 15 days. We monitored and controlled all six productions and collected samples from each batch at the stages that the raw milk was obtained, whey and curd were produced, and from cheeses at day 1, 30, 60, 90 and 120 during ripening. Each cheese sample consisted of one goatskin bag

of cheese (3-5 kg). 10 goatskin bags of cheese were prepared per each batch and put in the cave. At each sampling day, duplicate goatskin bags were collected from the cave for analyses. A total of 60 different goatskin bags were analyzed in both batches produced in three DFs. All samples were transported to the laboratory at 4°C, and divided into different aliquots which were stored at -20°C prior to analysis. Analyses were performed in triplicate from different parts of cheeses in each goatskin bag. The reported values are the means of the two independent batches per DF.

Physicochemical analyses

Titrateable acidity (as % of lactic acid) was measured according to AOAC (1995). The pH was measured by direct insertion of a pH-meter electrode (Mettler Toledo MP 225) into the slurry sample obtained by maceration of 10 g sample with 10 ml distilled water. Dry matter was analyzed by the oven drying method at 102°C IDF (1982), whereas salt content was determined by titration with AgNO₃ IDF (1988). Fat content was measured by the Gerber-Van Gulik method (Ardo & Polychroniadou, 1999), and water-soluble nitrogen (WSN) as well as total nitrogen (TN) were determined by applying the micro-Kjeldahl method on fractions of the cheese prepared as described by IDF (1993). Ripening index (RI) values were calculated according to the ratio of water soluble nitrogen content to total nitrogen content ($RI = (WSN/TN) \times 100$). In the milk, whey and curd samples the pH, titration acidity, dry matter and fat content was measured, whereas in the cheese samples these measurements were complemented with water soluble and total nitrogen measurements. All analyses were performed in triplicate.

Volatile compound analyses

Volatile compounds were extracted according to the method of (Lee et al., 2003) with minor modifications. Briefly, 5 g of cheese were positioned in a 20 ml vial, followed by the addition of 5 ml NaCl (10%, w/v) and 10 µL Internal Standard (2-methyl-3-heptanone and 2-methyl-pentanoic acid). The vial was placed in a magnetic stirrer at 60°C for 30 min for equilibrium of volatile compounds in the headspace. Extraction of volatile compounds was carried out by injecting 75 µm carboxen/polydimethylsiloxane SPME (Solid Phase Micro Extraction) fiber (Supelco, Bellefonte, PA, USA) into the vial and exposure of the fiber to the headspace for 30 min at 60°C. During extraction, the sample was stirred continuously with a magnetic

stir bar. The volatile compounds were desorbed by direct insertion of the fiber into the injection port of the GC which was operated in splitless mode at 250°C for 3 min.

Volatile compound analyses were performed by employing the SPME-GC-MS method. Headspace volatile compounds were analyzed using an Agilent 7890 GC system (Agilent Technology, CA, USA) coupled to a mass spectrometer (Agilent 5975C VL MSD with Triple-Axis detector). The GC was equipped with a DB-Wax column (30m, 0.25 mm i.d and 0.25 μ m film thickness) and a split/splitless injector. Helium was used as carrier gas at a flow rate of 1 ml/min. The initial temperature program employed was isothermal at 40°C for 10 min, followed by elevation of the temperature to 110°C at a rate of 5°C/min and 240°C at a rate of 10°C/min with a final extension of 5 min at 250°C. Mass spectra were recovered in the electron impact mode at an ionization voltage of 70eV, and data were collected at a rate of 3.2 scans/s over a range of m/z 35-500. Data were analyzed under the same conditions to calculate retention indices for the volatile compounds. Identification of volatile compounds was achieved by comparison to n-alkanes (C4-C20) standards (Sigma-Aldrich, US) and by comparison of their mass spectra with those in the libraries of Wiley, NIST and Flavor (Agilent MSD Chemstation, Santa Clara, CA, US). The concentration of each compound was calculated by the ratio of the peak area of the internal standard (IS) and the unknown compound. Samples were analyzed in triplicate.

Statistical Analyses

The significance of the differences observed in the physicochemical analyses and volatile profiles during production and ripening were assessed by using the analysis of variance (ANOVA) procedure of the SPSS Statistics software version 22.0 (International Business Machines Corp., Armonk, NY). One-way analysis of variance, followed by a Duncan's multiple comparison test was used for statistical analysis to determine if there was a significant difference ($P < 0.05$) between treatments. Principal component analysis was performed using a covariance matrix and varimax rotation between production and ripening stages. The concentration of volatile compounds were used as variables.

Results and Discussion

Physicochemical parameters

The temporal development of acidity, pH, dry matter and fat during production, as well as salt and protein content during ripening are given in Table 1 and Table 2, respectively. During cheese production (from raw milk to the first day of ripening) the pH drops dramatically, similar to what is typically observed for industrial cheeses, and which is likely caused by available lactic acid bacteria (Ozturkoglu-Budak et al., 2016a) converting lactose to lactic acid. Afterwards, other organisms become more dominant and concomitantly the pH increased throughout the ripening. Specific yeast and fungi could be responsible for this pH increase, since these organisms can metabolize the lactate produced by lactic acid bacteria to CO₂ and H₂O (Schlessner et al., 1992). Moreover, deamination of amino acids in the later stages of ripening (Schlessner et al., 1992), formation of ammonia and other proteolysis products with amphoteric characteristic, as well as the fragmentation of fatty acids into methyl ketones (McSweeney, 2004) could contribute to the elevation of pH observed. Not surprisingly, a low pH typically co-occurred with a high level of acidity, and vice versa.

Table 1. Gross composition of raw milk, whey and curd during the production of Divle Cave cheese (mean $\pm$ SD; n=3)

		Production of cheese		
		Raw milk	Whey	Curd
pH	DF1	6.75 $\pm$ 0.00 ^{Ca}	6.64 $\pm$ 0.01 ^{Bc}	4.89 $\pm$ 0.07 ^{Aa}
	DF2	6.75 $\pm$ 0.01 ^{Ca}	6.61 $\pm$ 0.01 ^{Bb}	4.97 $\pm$ 0.012 ^{Aa}
	DF3	6.74 $\pm$ 0.01 ^{Ca}	6.55 $\pm$ 0.02 ^{Ba}	5.06 $\pm$ 0.017 ^{Ab}
Acidity (°SH)	DF1	12.45 $\pm$ 0.02 ^{Bb}	6.89 $\pm$ 0.18 ^{Ab}	66.65 $\pm$ 0.27 ^{Cc}
	DF2	12.87 $\pm$ 0.04 ^{Bc}	6.14 $\pm$ 0.09 ^{Aa}	62.8 $\pm$ 0.86 ^{Cb}
	DF3	11.75 $\pm$ 0.04 ^{Ba}	6.2 $\pm$ 0.12 ^{Aa}	59.45 $\pm$ 1.07 ^{Ca}
Dry matter (% w/w)	DF1	19.17 $\pm$ 0.07 ^{Bb}	8.49 $\pm$ 0.06 ^{Aa}	34.18 $\pm$ 0.48 ^{Cab}
	DF2	19.52 $\pm$ 0.10 ^{Bc}	8.78 $\pm$ 0.17 ^{Ab}	32.97 $\pm$ 0.64 ^{Ca}
	DF3	18.96 $\pm$ 0.09 ^{Ba}	8.22 $\pm$ 0.09 ^{Aa}	35.80 $\pm$ 0.85 ^{Cb}
Fat (% w/w)	DF1	4.68 $\pm$ 0.02 ^{Bb}	1.20 $\pm$ 0.07 ^{Aa}	9.38 $\pm$ 0.02 ^{Cb}
	DF2	4.60 $\pm$ 0.08 ^{Bab}	1.35 $\pm$ 0.07 ^{Aab}	9.00 $\pm$ 0.08 ^{Ca}
	DF3	4.47 $\pm$ 0.06 ^{Ba}	1.45 $\pm$ 0.08 ^{Ab}	8.85 $\pm$ 0.08 ^{Ca}

DF1: dairy farm 1, DF2: dairy farm 2, DF3: dairy farm 3

A-C Superscripted capital letters in the same row show the significant differences between ripening times, $P < 0.05$

^{a-c} Subscripted lower case letters in the same column show the significant differences between different dairy farms, $P < 0.05$

Fat, salt and dry matter content showed a slight increase during ripening. It is considered that the increase observed in salt and fat content in early days of ripening results from relative dry matter increase caused by evaporation. A further increase occurred after the 90th day of ripening which has been reported to depend on removal of fragmented products formed as a result of proteolytic and lipolytic enzyme activities, associated with the microbial community present in cheese samples (Hayaloglu et al., 2007a). A decrease in total nitrogen content was observed in the early stages of ripening while an increase was seen after day 60 until the end of the ripening period. Changes in dry matter content could impact on the nitrogen content. Water soluble nitrogen rates of cheese samples appeared to increase during the ripening period. It has previously been established that heat treatment applied to milk and the salt content can affect the water soluble nitrogen content (Guinee, 2004). Moreover, it was reported that lactic acid bacteria can perform proteolysis even after the ripening period is over, e.g. during storage, thereby affecting the nitrogen content (Carbonell et al., 2002). Taken together, despite the fact that Divle Cave cheese has a distinctly different taste as compared to industrially produced hard cheeses, the temporal development of the basic process parameters is comparable.

Table 2. Gross composition of Divle Cave cheese during ripening (mean $\pm$ SD; n=3)

		Ripening of cheese				
		Day 1	Day 30	Day 60	Day 90	Day 120
pH	DF1	4.54 $\pm$ 0.02 ^{Aa}	4.85 $\pm$ 0.04 ^{Ba}	4.89 $\pm$ 0.03 ^{Ba}	5.00 $\pm$ 0.03 ^{Ca}	5.06 $\pm$ 0.03 ^{Ca}
	DF2	4.56 $\pm$ 0.02 ^{Aab}	4.90 $\pm$ 0.02 ^{Ba}	4.87 $\pm$ 0.03 ^{Ba}	4.99 $\pm$ 0.02 ^{Ca}	5.08 $\pm$ 0.02 ^{Dab}
	DF3	4.59 $\pm$ 0.01 ^{Ab}	4.92 $\pm$ 0.02 ^{Ba}	4.97 $\pm$ 0.01 ^{Cb}	5.03 $\pm$ 0.03 ^{Ca}	5.14 $\pm$ 0.05 ^{Db}
Acidity ($^{\circ}$ SH)	DF1	99.68 $\pm$ 0.67 ^{BCa}	113.35 $\pm$ 4.84 ^{Da}	103.45 $\pm$ 0.37 ^{Cb}	95.20 $\pm$ 0.60 ^{ABc}	92.00 $\pm$ 0.79 ^{Ab}
	DF2	106.24 $\pm$ 1.60 ^{Da}	115.80 $\pm$ 0.75 ^{Ea}	103.76 $\pm$ 0.87 ^{Cb}	90.20 $\pm$ 0.32 ^{Aa}	95.01 $\pm$ 0.59 ^{Bc}
	DF3	113.72 $\pm$ 4.46 ^{Cb}	120.89 $\pm$ 1.70 ^{Da}	98.58 $\pm$ 0.82 ^{Ba}	91.80 $\pm$ 0.74 ^{Ab}	89.0 $\pm$ 0.97 ^{Aa}
Dry matter (% w/w)	DF1	54.57 $\pm$ 1.01 ^{Bb}	51.66 $\pm$ 0.42 ^{Aab}	53.20 $\pm$ 0.71 ^{Ba}	54.36 $\pm$ 0.51 ^{Bb}	53.61 $\pm$ 0.47 ^{Ba}
	DF2	54.34 $\pm$ 0.46 ^{Cb}	50.78 $\pm$ 0.50 ^{Aa}	52.67 $\pm$ 1.00 ^{Ba}	52.80 $\pm$ 0.53 ^{Ba}	52.88 $\pm$ 0.87 ^{Ba}
	DF3	52.23 $\pm$ 0.62 ^{Aa}	52.67 $\pm$ 0.52 ^{ABb}	54.09 $\pm$ 1.12 ^{BCa}	55.38 $\pm$ 0.81 ^{CDb}	56.25 $\pm$ 0.69 ^{Db}
Fat (% w/w)	DF1	16.5 $\pm$ 0.41 ^{Aa}	18.66 $\pm$ 0.23 ^{Ba}	18.23 $\pm$ 0.52 ^{Ba}	18.33 $\pm$ 0.62 ^{Ba}	18.67 $\pm$ 0.47 ^{Bb}
	DF2	18.5 $\pm$ 0.41 ^{Bb}	18.33 $\pm$ 0.23 ^{Ba}	19.16 $\pm$ 0.23 ^{BCb}	19.50 $\pm$ 0.41 ^{Ca}	16.83 $\pm$ 0.62 ^{Aa}
	DF3	18.66 $\pm$ 0.24 ^{Ab}	19.33 $\pm$ 0.23 ^{Ab}	19.33 $\pm$ 0.23 ^{Ab}	19.33 $\pm$ 0.62 ^{Aa}	19.50 $\pm$ 0.41 ^{Ab}
Fat in dry matter (%)	DF1	29.80 $\pm$ 0.57 ^{Aa}	36.17 $\pm$ 0.28 ^{Ca}	33.90 $\pm$ 0.56 ^{Ba}	33.58 $\pm$ 0.60 ^{Ba}	34.55 $\pm$ 0.93 ^{Ba}
	DF2	33.97 $\pm$ 0.77 ^{Ab}	36.13 $\pm$ 0.48 ^{Ba}	36.22 $\pm$ 0.66 ^{Bb}	36.63 $\pm$ 0.53 ^{Bb}	37.20 $\pm$ 1.31 ^{Bb}
	DF3	34.77 $\pm$ 0.31 ^{Ab}	36.31 $\pm$ 0.19 ^{Ba}	35.64 $\pm$ 0.63 ^{ABb}	35.08 $\pm$ 0.75 ^{ABa}	35.09 $\pm$ 0.83 ^{ABab}
Salt (%)	DF1	3.32 $\pm$ 0.11 ^{Bb}	3.05 $\pm$ 0.05 ^{Aa}	3.46 $\pm$ 0.05 ^{Bb}	3.40 $\pm$ 0.05 ^{Bb}	3.80 $\pm$ 0.23 ^{Ca}
	DF2	3.45 $\pm$ 0.12 ^{Bb}	3.57 $\pm$ 0.08 ^{Bb}	3.11 $\pm$ 0.07 ^{Aa}	3.46 $\pm$ 0.63 ^{Bb}	3.45 $\pm$ 0.23 ^{Ba}
	DF3	3.01 $\pm$ 0.09 ^{Aa}	2.90 $\pm$ 0.08 ^{Aa}	3.00 $\pm$ 0.07 ^{Aa}	3.10 $\pm$ 0.04 ^{Aa}	3.68 $\pm$ 0.28 ^{Ba}
Total protein (% w/w)	DF1	4.64 $\pm$ 0.08 ^{Aa}	4.42 $\pm$ 0.04 ^{Ab}	4.63 $\pm$ 0.06 ^{Ab}	4.51 $\pm$ 0.08 ^{Aa}	4.49 $\pm$ 0.19 ^{Aa}
	DF2	4.66 $\pm$ 0.09 ^{Ba}	4.17 $\pm$ 0.07 ^{Aa}	4.49 $\pm$ 0.06 ^{Bab}	4.60 $\pm$ 0.08 ^{Ba}	4.87 $\pm$ 0.09 ^{Cb}
	DF3	4.53 $\pm$ 2.09 ^{ABa}	4.63 $\pm$ 0.08 ^{BCc}	4.38 $\pm$ 0.07 ^{Aa}	4.59 $\pm$ 0.09 ^{ABa}	4.81 $\pm$ 1.15 ^{Cab}
WSN (%)	DF1	0.33 $\pm$ 0.11 ^{Aa}	0.67 $\pm$ 0.04 ^{Bab}	0.70 $\pm$ 0.04 ^{Ba}	0.72 $\pm$ 0.06 ^{Ba}	0.90 $\pm$ 0.11 ^{Ca}
	DF2	0.55 $\pm$ 0.06 ^{Ac}	0.61 $\pm$ 0.04 ^{ABa}	0.74 $\pm$ 0.05 ^{BCa}	0.76 $\pm$ 0.07 ^{Ca}	0.97 $\pm$ 0.09 ^{Da}
	DF3	0.42 $\pm$ 0.01 ^{Ab}	0.74 $\pm$ 0.05 ^{Bb}	0.80 $\pm$ 0.07 ^{Ba}	0.83 $\pm$ 0.06 ^{Ba}	0.98 $\pm$ 0.05 ^{Ca}
Ripening Index (RI)*	DF1	7.05 $\pm$ 0.04 ^{Aa}	15.07 $\pm$ 0.52 ^{Bab}	15.14 $\pm$ 0.29 ^{Ba}	15.92 $\pm$ 0.38 ^{Ca}	20.07 $\pm$ 0.45 ^{Db}
	DF2	11.69 $\pm$ 0.07 ^{Ac}	14.47 $\pm$ 0.08 ^{Ba}	16.57 $\pm$ 0.33 ^{Cb}	16.51 $\pm$ 0.51 ^{Ca}	18.95 $\pm$ 0.44 ^{Da}
	DF3	9.03 $\pm$ 0.05 ^{Ab}	15.81 $\pm$ 0.27 ^{Bb}	18.16 $\pm$ 0.37 ^{Cc}	18.11 $\pm$ 0.51 ^{Cb}	20.33 $\pm$ 0.32 ^{Db}

DF1: dairy farm 1, DF2: dairy farm 2, DF3: dairy farm 3

^{A-D} Superscripted capital letters in the same row show the significant differences between ripening times $P < 0.05$

^{a-c} Subscripted lower case letters in the same column show the significant differences between different dairy farms $P < 0.05$

WSN: Water soluble nitrogen

*Ripening index is the progress of maturation process during ripening stages.

Volatile compound profiles

We detected a total of 110 volatile compounds derived from Divle Cave cheese during production and ripening by SPME-GC-MS (Supplementary Table 1). These volatiles encompass 13 acids, 18 alcohols, 12 ketones, 15 esters, 12 terpenes, 7 aldehydes, 18 hydrocarbons and 15 miscellaneous compounds.

Figure 2 shows the variation of the volatile compounds per chemical group isolated from Divle Cave cheese produced in the 3 DFs. The chemical group encompassing the carboxylic acids displayed an increase until the 90th day of ripening in all DFs. Moreover, carboxylic acids appeared the most abundant group of volatiles in the cheese at the end of ripening. The level of alcohols tended to increase in the initial stages of ripening (day 30 in DF1 and day 60 in DF2 & 3), followed by decreasing levels in the final stages of ripening. Ketones gradually increased during 90 days, and slightly decreased in the last 30 of ripening. Esters increased gradually until the 90th day of ripening, a process that continued in the last 30 days in DF1 and DF3. The level of hydrocarbons and miscellaneous compounds was highly variable during ripening and at the different farms but tended to increase at the end of ripening. Aldehydes also showed an increasing abundance during ripening. Terpenes were found in higher amounts in raw milk and curd, whereas their levels decreased on day 1, followed by increasing levels after that time, until the end of ripening. Notably, in the raw milk used at the 3 DFs distinct differences were observed for the abundance of chemical groups such as acids and alcohols. However, these differences were virtually abolished at the curd stage and beyond, suggesting that variations in the final fermentation product are relatively minor, indicating the robustness of this wild fermentation process.

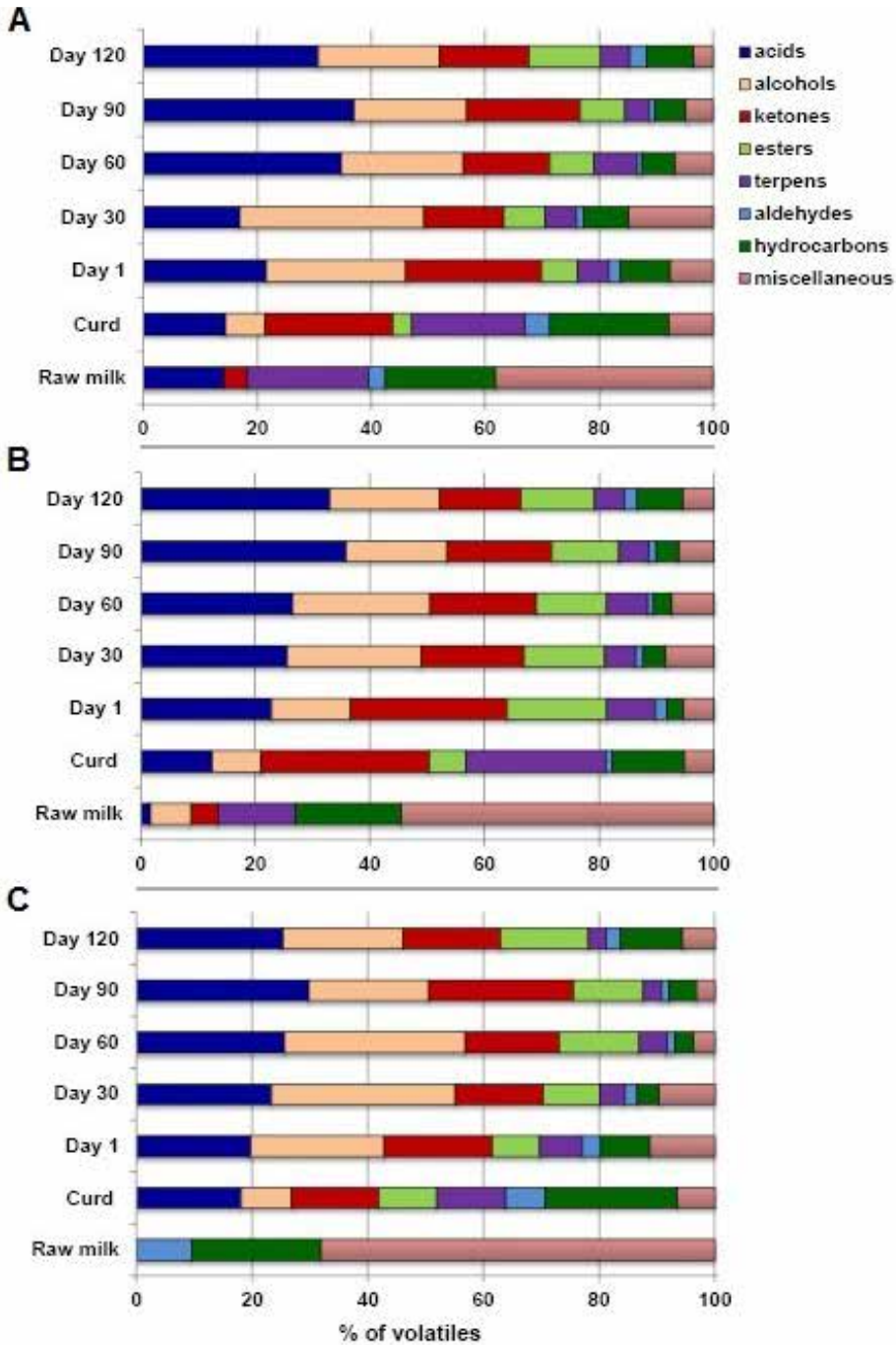


Figure 2. Variation of the main chemical groups of volatile compounds isolated from Divle Cave cheese during production and ripening. A, B and C represent the cheeses produced in DF1, DF2 and DF3, respectively.

Carboxylic acids

In Divle Cave cheese, lipolysis is thought to be the main pathway for the formation of carboxylic acids, followed by lactose metabolism. Carboxylic acids are not only aroma components as such, but can also serve as precursors for the formation of methyl ketones, alcohols, lactones, aldehydes and esters (Collins et al., 2003). Due to their low detection thresholds (<5 mg/kg), short (C4-C8) and medium-chain (C10-C14) carboxylic acids are thought to contribute significantly to the aroma profile of cheeses (Tavaria et al., 2004).

The temporal flavor profiles in the Divle Cave cheese revealed 13 carboxylic acids with comparable total volatile headspace percentages of 30.79%, 32.97% and 25.30% at the end of ripening in the 3 DFs (Supplementary Table 1, Supplementary Figure 1). Similar carboxylic acid levels were previously reported in sheep cheeses. Bergamini et al., (2010) identified 11 acids in Argentinean sheep cheeses, Hayaloglu et al. (2008) identified 11 acids in Kuflu cheese and Delgado et al., (2010) determined 13 acids in Torta Del Casar cheese made from raw ewes' milk. 50-60% percentage of acids, especially short chain fatty acids, found in Spanish sheep cheeses (Barron et al., 2005). We observed significant differences ($P < 0.05$) in total amount of carboxylic acids both throughout the ripening period in the same production as well as among different productions in different dairy farms. For specific lowly abundant carboxylic acids, different absence/presence profiles were observed in different DFs, however the highly abundant carboxylic acids were always found in all 3 DFs, suggesting that the production site only generates minor differences in flavor which is likely to be largely overruled by the much longer ripening process in Divle cave.

Among linear-chain fatty acids, that are formed by the hydrolysis of triglycerides, short chain fatty acids were found to be predominant components of aroma as in raw ewes' milk cheese such as Torta del Casar (Delgado et al., 2010) and mold-ripened cheese such as Blue cheese (Rothe et al., 1994). To this end, butanoic (butyric) and pentanoic (valeric) acid were detected at high levels at the end of ripening of cheeses in all 3 DFs. These components have been established to be present at high levels in industrial cheeses as well (Yvon & Rijnen, 2011) and butanoic acid is perceived as a rancid 'cheese-like' smell (Sable and Cottenceau, 1999). The other linear-chain acids identified in the headspace of Divle Cave cheese were hexanoic, octanoic and decanoic acids. Especially hexanoic acid was detected at high levels in Divle

Cave cheese produced at all dairy farms. Hexanoic acid has been reported to make a significant contribution to the aroma of sheep cheeses, such as Torta del Casar cheese (Delgado et al., 2010), traditional Greek Xinotyros type cheese (Bontinis et al., 2012), and Teleme cheese (Massouras et al., 2006).

Acetic acid was the second highest abundant and frequently detected acid during the ripening. Acetic acid results from the activity of lactic acid bacteria and it is perceived as a pungent aroma which gives the typical aroma to brine-cured cheese types such as Feta and Domiati (Hayaloglu & Karabulut, 2013). In our study, the increase in acetic acid content which continued until 90th day of ripening, turned into a decrease at 120th day. This result was associated with mold content which increased towards the end of ripening and lactic acid bacteria content which decreased in the last 30 days (Chapter 3).

Branched-chain fatty acids have been reported to have a characteristic effect on flavors of sheep and goat cheeses (Curioni & Bosset, 2002). In our study, it was established that 3-methyl butanoic acid (isovaleric acid) was the most abundant branched chain fatty acid in Divle Cave cheese samples and showed a continuous increase until the end of ripening. This acid is reported to give rancid, cheese-like and sweet flavoring to sheep cheeses (Yvon & Rijnen, 2001). The high amount of acids synthesized from amino acids may be associated with severe proteolysis in Divle Cave cheese due to enzymes derived from the raw milk (Carbonell et al., 2002). It was reported previously that 3-methyl butanoic acid was the most perceived acid in terms of olfactometry in a semi-hard Spanish goat cheese (Delgado et al., 2011).

Alcohols

Alcohols have been reported at high concentrations in Blue cheeses and some surface ripened cheeses such as Camembert, Brie, Gorgonzola. It has also been established that particularly enzymes secreted by *Penicillium roqueforti* and *P. camemberti* play an active role in the formation of alcohols (Molimard & Spinnler, 1996). Moreover, alcohols were reported as the main volatile component in some hard and long ripened cheeses such as Cheddar, Kaşar and Tulum (Curioni & Bosset, 2002) and in raw ewes' milk cheeses (Bintsis & Robinson, 2004; Fernández-García et al., 2004). In addition, ethanol was quantitatively determined as the main alcohol in some of these cheeses (Bontinis et al., 2012).

We detected 14-18 different alcohols, depending on the DF (Supplementary Table 1, Supplementary Figure 2), and included primary, secondary, branched-chain, unsaturated and aromatic alcohols. These findings are in agreement with the results of Wolf et al. (2010) and Bergamini et al. (2010) who found 15 and 14 alcohols in Reggiano and Argentinean sheep cheeses, respectively. Hayaloglu et al (2008) found 32 alcohols among the main volatile group in Kuflu cheese varying between 189.89 - 270.75 peak area/105 of total alcohols. Similarly, Pappa et al. (2013) reported an increase in alcohols levels during the ripening and found high amounts of alcohols at the end product of Teleme cheese. Significant ($P < 0.05$) variations were observed in total amount of alcohols both throughout the ripening period in the same production and among different productions in different dairy farms.

The use of raw milk is likely to be the reason of relatively high alcohol concentrations in Divle Cave cheese. Particularly, high ethanol concentration detected at the stages of curd, 1st and 30rd days of ripening could originated from the lactic acid bacteria, enterococci and yeast in the microbial populations coming from raw milk and the cheese processing environment (Bontinis et al., 2012). 2-butanol, ethyl alcohol and 1-pentanol were the most abundant alcohols during the production and ripening periods (Supplementary Table 1). Secondary alcohols such as 2-butanol, 2-heptanol, 2-octanol and 2-pentanol were found at the highest concentration among all alcohols at the end of ripening. 2-butanol being most abundant, followed by 2-pentanol, 2-heptanol, 2-octanol and 1-heptanol. Interestingly, 2-butanol also is the most abundant alcohol in Spanish Manchego cheese (Gómez-Ruiz et al., 2002). It is a secondary alcohol typically formed from diacetyl (2,3-butanedione) especially in raw milk cheeses. Diacetyl is degraded to acetoin by the bacterial enzymes coming from raw milk, following it is reduced to 2,3-butanediol, subsequently to 2-butanone and finally to 2-butanol (Bontinis et al., 2012). Notably, 2-pentanol has a low detection threshold and it is reported to play an important role in cheese aroma (Carbonell et al., 2002). 2-heptanol was identified as a key odorant of Gorgonzola and Grana Padano cheeses (Curioni and Bosset, 2002; Poveda and Cabezas, 2006). An increase was detected in the relative amounts of some alcohols such as ethanol, 1-pentanol, 2-butanol and 2,3-butanediol, until the 30th day of ripening, whereas the levels tended to decrease from that point in the ripening process onwards.

Ketones

Methyl ketones are formed by the enzymatic activities of filamentous fungi (Molimard & Spinnler, 1996) and have typical odours detectable at a low perception threshold. For this reason these compounds are important in the aroma of Camembert, raw ewes' milk cheese (Di Cagno et al., 2003) and Blue cheeses (Hayaloglu et al, 2007b). In total, we detected 12 ketones during the production and ripening of Divle Cave cheese and the majority of them belong to methyl ketones (Supplementary Table 1, Supplementary Figure 3). Similar results were obtained in Reggiano cheese made from ewes' milk (Wolf et. al., 2010) and in Kuflu cheese (Hayaloglu et al., 2008). 20 ketones were determined in Kuflu cheese varying between 163.50 - 285.89 peak area/105 of total ketones (Hayaloglu et al., 2008). We observed significant ($P < 0.05$) differences in total amount of ketones both throughout the ripening period in the same production and among different productions in different dairy farms.

Diacetyl (2-3-butanedione) was determined as an odour contributor compound in Cheddar, Camambert and Emmental cheeses (Yvon & Rijnen, 2001) and was also readily detected here, in a decreasing trend. Moreover, the typical ewe's milk methyl ketones 2-butanone, 2-pentanone and 2-heptanone (Gómez-Ruiz et al., 2002; Barron et al., 2007) were the most abundant ketones in all dairy farms during ripening. They were detected at a low level in milk and curd, but greatly increased after pre-ripening (on day 1) in all productions, and continued to increase until the 90th day of ripening, and tended to decrease at the end of ripening. The reason of increase observed in the concentration of methyl ketones until the 90th day of ripening could be due to the catabolism of free fatty acids, derived from the lipolysis, to methyl ketones by microorganisms (Bontinis et al., 2012), while the declines observed at the end of ripening can be explained by the enzymatic reduction of ketones to secondary alcohols depending on their intermediate compounds (Curioni and Bosset, 2002). 2-butanone and 2-heptanone have specific odours classified as herbaceous and butter scotch, respectively (Delgado et al., 2011) and have been described as primary ketones in some raw ewes' milk cheeses such as La Serena (Carbonell et al., 2002), Zamarano (Fernández-García et al., 2004), Torta del Casar (Delgado et al., 2010). 2-butanone was determined as principal ketone in Xinotyri (Bontinis et al., 2012) and Teleme (Massouras et al., 2006) cheeses. Furthermore, 2-heptanone is an important component of Emmental and Gorgonzola cheeses (Curioni & Bosset,

2002). 2-nonanone and 2-octanone, were also reported to have typical odours and have low perception threshold in dairy products (Hayaloglu et al., 2007a).

Acetoin (3-hydroxy 2-butanone) is another ketone following a decreasing trend. The reduction in the acetoin amount could be thought as a result of the decrease in diacetyl. Because, it is synthesized by the reaction of diacetyl with the enzymes from bacteria present in raw milk, or it may be produced from lactose, citrate, pyruvate by lactic acid bacteria (Di Cagno et al., 2003). For that reason, the reduction in the amount of diacetyl or lactic acid bacteria content cause a reduction in acetoin.

Esters

Esters are formed by the esterification of short-chain free fatty acids with alcohols (Delgado et al., 2010). Esters have a floral and fruity aroma and contribute to the reduction of the sharpness of fatty acids and bitterness of amines (Gallois & Langlois, 1990; Bontinis et al., 2012). Esters are highly effective in cheese aroma because of their low perception threshold values (Molimard & Spinnler, 1996).

We detected 15 esters which were not found in all dairy farms, pinpointing that subtle differences in taste observed for cheeses derived from different DFs might be caused by the variations in ester-type components (Supplementary Table 1, Supplementary Figure 4). Nevertheless, ethyl esters were the most abundant esters detected in the headspace of Divle Cave cheese. Ethyl butanoate, ethyl hexanoate, butyl acetate and isopentyl formate were detected frequently and abundantly. The increased detection of ethyl butanoate may be related to the decrease of butanoic acid at the end of the ripening. We observed an increase ($P < 0.05$) in ethyl acetate and ethyl octanoate in the first stage of ripening, which could be related to the increase in yeast and mold count and the decrease could be due to the alcohols that concomitantly decreased at the end of ripening. The increase in the concentration of ethyl hexanoate and ethyl butanoate during ripening were also observed in other ewes' cheeses (Carbonell et al., 2002; Barron et al., 2007). Similarly, ethyl acetate and ethyl hexanoate were detected in many traditional cheeses such as Cheddar, Emmental, Gorgonzola, Grana Padano, Pocorino, Ragusano (Curioni & Bosset, 2002). Moreover, other ethyl esters we identified, e.g. ethyl butyrate and phenethyl acetate were previously noticed to contribute to the odour of Cheddar and Camambert cheese, respectively (Yvon & Rijnen, 2001).

Terpenes

Terpenes are compounds related to animal feed or pasture, green fodder and meadow (Munoz et al., 2003; Revello Chion et al., 2010). Hence, it is thought that the abundance of terpenes in Divle Cave cheese results from the natural feed of the animals in the Divle district. This is corroborated by our data that show that the ripening process did not impact on the terpene concentrations. Many researchers reported that the importance of terpenes in formation of cheese aroma is not certain but these components are frequently identified in cheeses produced from goat and sheep milks (Delgado et al, 2010; Bontinis et al, 2012).

α -pinene is the primary terpene detected at highest concentration at all stages of production and ripening. Other terpenes detected at high concentrations were p-cymene, β -pinene and α -copaene. Generally, terpenes were identified starting from raw milk such as α -pinene, camphene, o-cymene and D-limonene. The highest amounts were detected in curd but the concentrations decreased during ripening, except α -pinene which concentration increased during ripening (Supplementary Table 1, Supplementary Figure 5).

The level of terpenes fluctuated with a decrease on day 1 followed by elevating levels until the end of ripening. Terpenes are lipophilic volatile compounds (Borge et al., 2016) and derive from milk fat (Viallon et al., 2000) of which the concentration also increases as a result of water loss during ripening. Several other studies showed that terpenes may also be formed by microorganisms such as yeasts and especially *Kluyveromyces lactis* (Martin et al., 2001) and fungal cultures (Agrawal & Joseph, 2000) in the milk and milk products and they might be changed as a result of microbial activity. Hence growth of diverse bacteria, yeast and filamentous fungi in Divle Cave cheese (Ozturkoglu-Budak et al., 2016a) might cause the variation in terpene amounts. Similar variations in terpene profile were also reported in milk and milk products (Pouloupoulou et al., 2012).

Aldehydes

Aldehydes have low perception thresholds and are characterized by herbaceous and green-grass like aroma (Molimard & Spinnler, 1996; Curioni & Bosset, 2002). 7 aldehyde-containing compounds including lactones, sulphurs, phenols, and amines

were identified in the volatile fraction of Divle Cave cheese (Supplementary Table 1, Supplementary Figure 6). Hexanal, 3-methyl-butanal and benzaldehyde were identified as the major compounds among the all aldehydes. 3-methyl-butanal has already been established to contribute to Cheddar, Camambert and Emmental cheese flavor (Yvon & Rijnen, 2001). Hence, our data suggest this is also the case for Divle Cave cheese.

Pentanal, n-octanal, nonanal and heptanal were irregularly detected during specific stages of production and ripening. The concentrations of aldehydes usually fluctuated during consecutive stages. These fluctuations in their amounts could be originated from their transition properties, conversion into alcohols and oxidization to their corresponding acids (Molimard & Spinnler, 1996; Munoz et al., 2003).

Hydrocarbons

Despite the frequent detection of hydrocarbons in cheeses (Delgado et al., 2010, Delgado et al., 2011; Bontinis et al., 2012), they do not contribute to cheese aroma for their low detection amounts and high perception threshold. We identified 18 hydrocarbon compounds in the volatile fraction of Divle Cave cheese (Supplementary Table 1, Supplementary Figure 7). The determined amounts were generally low and fluctuated during production and ripening. With toluene, styrene and 1,2-dichloro benzene, being the most frequently detected hydrocarbons at all stages. Taken together, it appears that hydrocarbons do not play an important role in the flavor profile of Divle Cave cheese.

Miscellaneous

We identified 15 miscellaneous compounds including lactones, sulphurs, phenols and amines in the volatile fraction of Divle Cave cheese (Supplementary Table 1, Supplementary Figure 8). Diethyl ether, p-cresol and m-cresol were frequently detected at high concentrations. Phenolic compounds such as p-cresol and m-cresol were detected starting from curd and in an increasing concentration. p-cresol (4 methyl phenol), is formed by the destruction of tyrosine (Molimard and Spinnler, 1996). Dimethyl disulphide (DMDS) was identified as the only sulphur compound. This compound was reported to contribute particularly to the aroma of mold-ripened cheeses (Bontinis et al., 2012). It originates from the degradation of methionine or methionine-containing peptides during ripening. Perception threshold value for sulphur compounds are usually very low ($\mu\text{g/kg}$) and are typically perceived as ripened cheese,

garlic, moldy cheese, broccoli, or cooked cabbage (Curioni and Bosset, 2002, Bontinis et al., 2012). It has been reported that the volatile sulphur compounds contribute to the aroma of many cheese types (McSweeney and Sousa, 2000). Microorganisms such as *Penicillium camamberti*, *Geotrichum candidum* and *Brevibacterium linens* contribute to the formation of sulphur components because of their ability to form methanethiol (Engels et al., 1997) and detected particularly in surface-and mold-ripened cheeses (McSweeney & Sousa, 2000; Molimard & Spinnler, 1996).

Effect of production/ripening stages and different dairy farms on cheese volatile profile

We performed principal component analyses (PCA) using the total concentrations of main volatile groups (acids, alcohols, ketones, esters, terpenes, aldehydes, hydrocarbones and miscellaneous) as variables to identify how the volatile profiles discriminated among the cheeses produced in each dairy farm (DF1, DF2, DF3) and during the production and ripening stages. Table 3 shows the most important loadings and the percentage accounted by the two first principal components (PC1, PC2). Scores plot obtained from PCA of raw milk, curd, 1, 30, 60, 90 and 120-days old-cheese samples defined by the two first principal components (PC1 and PC2) in three different dairy farms are shown in Figure 3.

Table 3. Principal components analysis. Loadings of the total volatile compounds for the first two principal components

Rotated Component Matrix ^a			
	Component		
	1	2	3
Total Acids	.844	.238	.186
Total Alcohols	.933	.044	-.122
Total Ketones	.909	.044	.165
Total Esters	.877	.283	.051
Total Terpenes	.285	-.039	.843
Total Aldehyde	.210	.931	.072
Total Hydrocarbones	.135	.872	.144
Total Miscellaneous	.124	-.280	-.793

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 5 iterations.

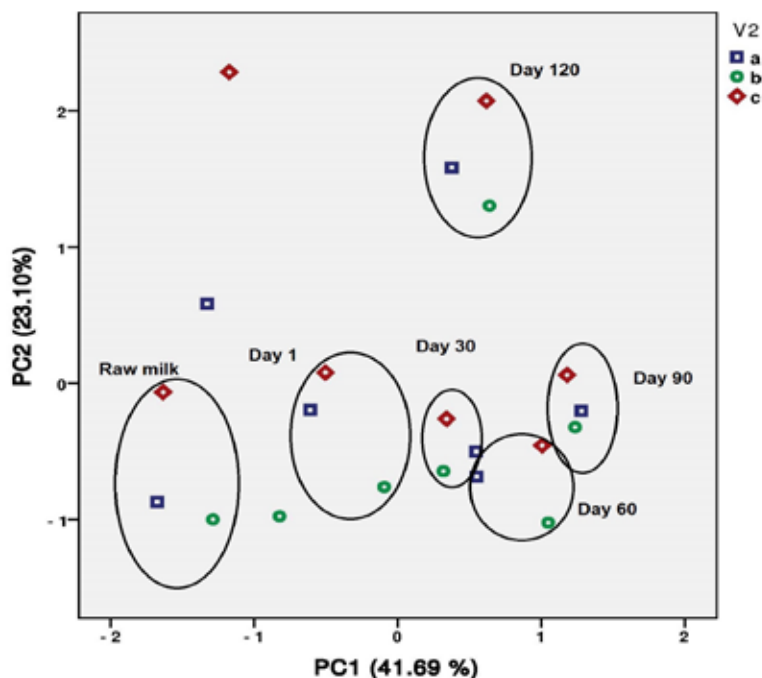


Figure 3. Scores plot obtained by principal component analysis of the individuals in the plane defined by the two first principal components (PC#1 and PC#2), a: DF1, b: DF2, c: DF3

According to the data analysis, PC1 explained 41.69% of the total variability and was mainly defined by total volatile acids, alcohols, ketones, esters, terpenes and various miscellaneous compounds. All these compounds were positioned in close proximity and positively correlated with each other. PC2 explained 23.10% of the total variability and was defined by total aldehydes and hydrocarbons. PC1 could be considered as representative of the ripening time, because all production stages (raw milk, curd) and day 1 during ripening were clustered on the negative side of PC1, all cheese samples taken on day 30, 60, 90 and 120 were located on the positive side of PC1. Production stages and cheeses at day 1 are located in the negative area of both PC1 and PC2. Cheeses at day 30 are near the axis origin. Cheeses with 60 and 90 days were located following the cheeses of day 30. PC2 could explain differences between cheeses at day 120 and the cheeses obtained in the former periods of ripening (30, 60, 90 days) because 120 days old ripened cheeses were in the upper part of the plane and the former ones were in the low part of the plane, indicating that long term ripening (over 90 days) leads to a significantly different cheese. This indicates that the volatile profile of Divle Cave cheeses were similar at the beginning of ripening

but different aromatic profiles were formed at the end of ripening in each DF. It also showed cheeses had higher contents of aldehydes and hydrocarbons at day 120. Although the milk from the three DFs cluster in our PCA analysis, the curds from the DFs appear substantially different, while at the end of the ripening the cheeses from the all DFs are highly similar. This result demonstrates that cheese production is an artisanal and variable process, but these differences are eliminated during the long ripening period in which all cheeses are placed in the same cave.

Conclusions

The study presented here established for the first time the composition of 110 volatiles of Divle Cave cheese during production and ripening, and identified the indigenous and characteristic volatile components. Carboxylic acids resulting from lipolysis of triglycerides, mainly butanoic and pentanoic acids were found to be most abundant. Alcohols, ketones and esters were determined as the other major volatile groups, likely caused by the high count of yeast and mold in Divle cheese. Short chain fatty acids, secondary alcohols, methyl ketones and ethyl esters were detected both frequently and at high concentration at different stages of the ripening process. Different terpene compounds were also frequently detected which likely reflects the natural grazing of the ewes. For some lowly abundant components, different absence/presence profiles were observed in different DFs, however highly abundant components were always found in all 3 DFs, suggesting that the production site only generates minor differences in flavor which is largely overruled by the much longer ripening process in Divle cave. This is corroborated by the fact that our PCA analyses demonstrated clustering of raw milk samples from different DFs was lost after curd production but retained in the final cheeses. The concentrations of many volatile compounds were significantly changed during the ripening period in the same production. These variations indicated that a variable cheese medium and dynamic conditions were available in the structure of cheese. These minor differences are also derived from some standardization problems during traditional production techniques. This paper presents data for determining the authentication of this traditional cheese, and can be useful for comparative purposes in future studies both on mold ripened and raw ewe's milk cheeses.

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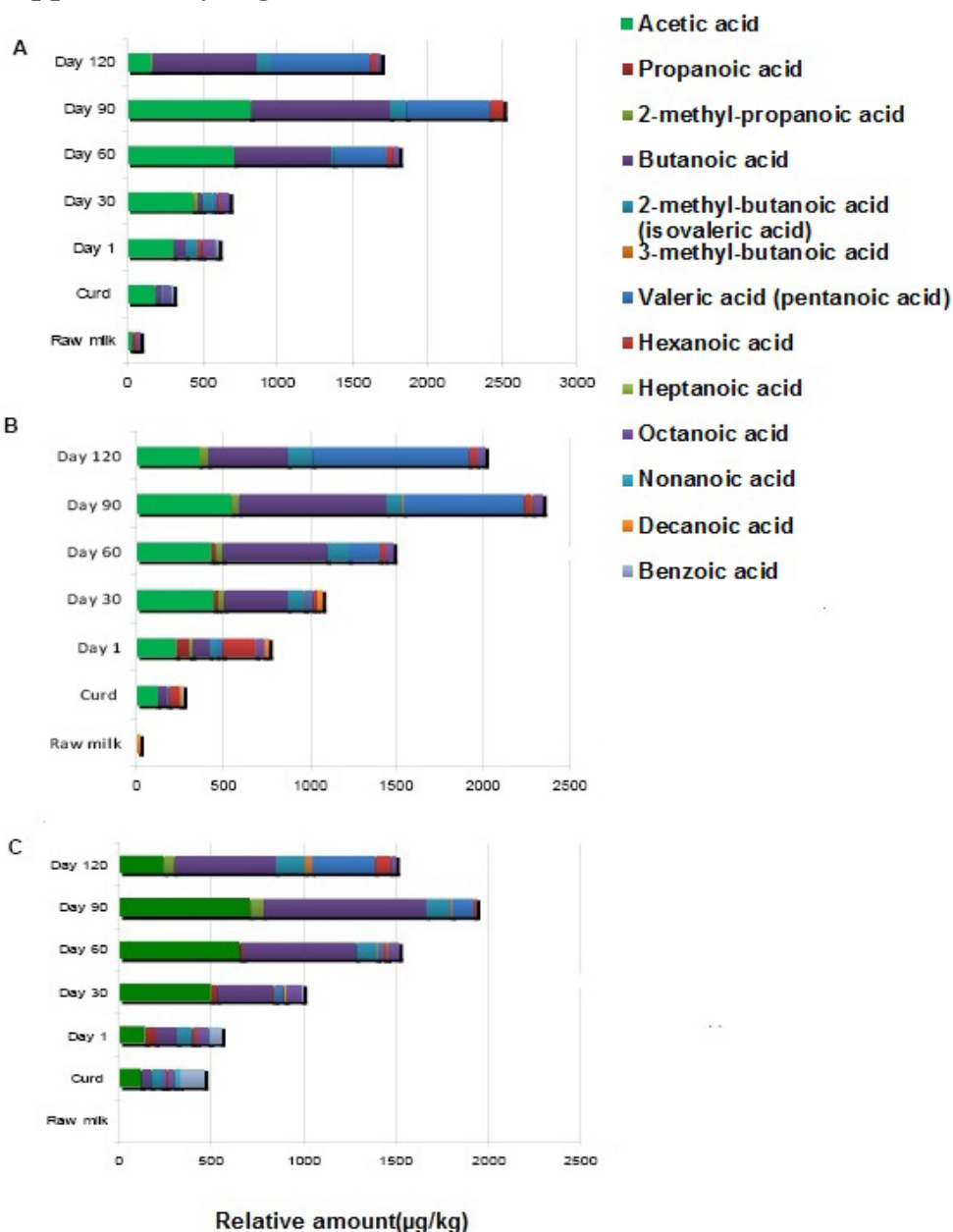
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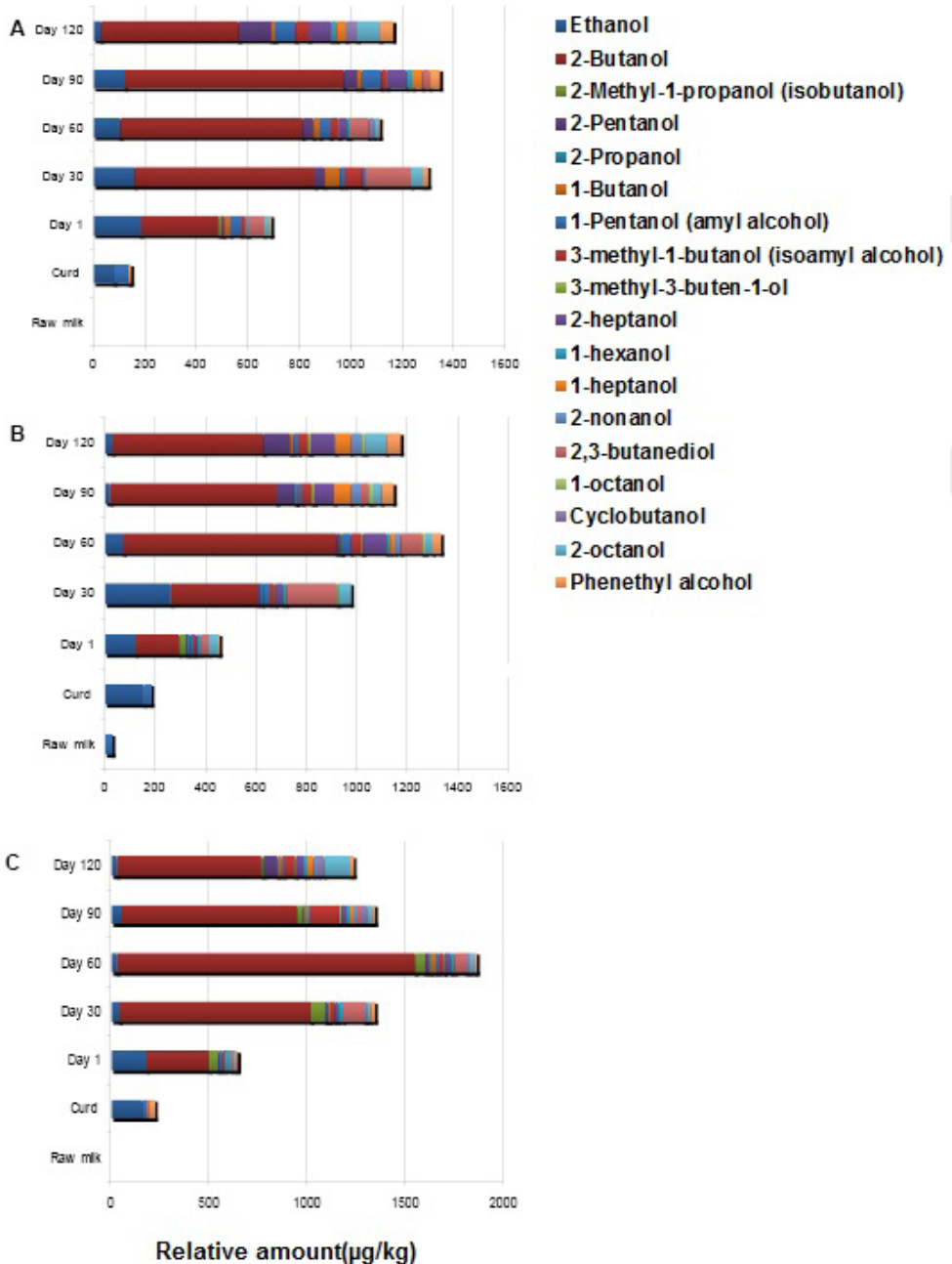
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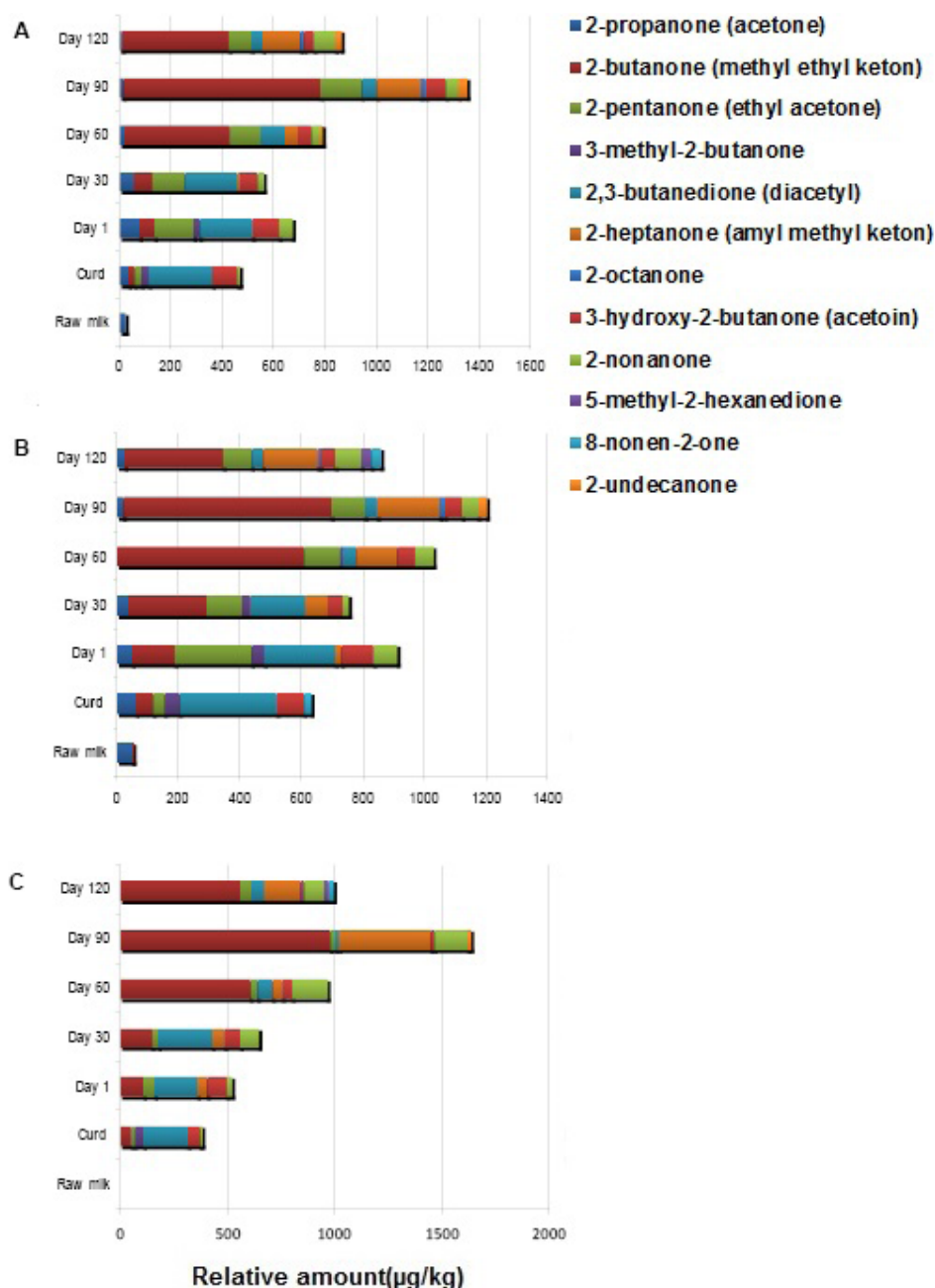
Supplementary Figures



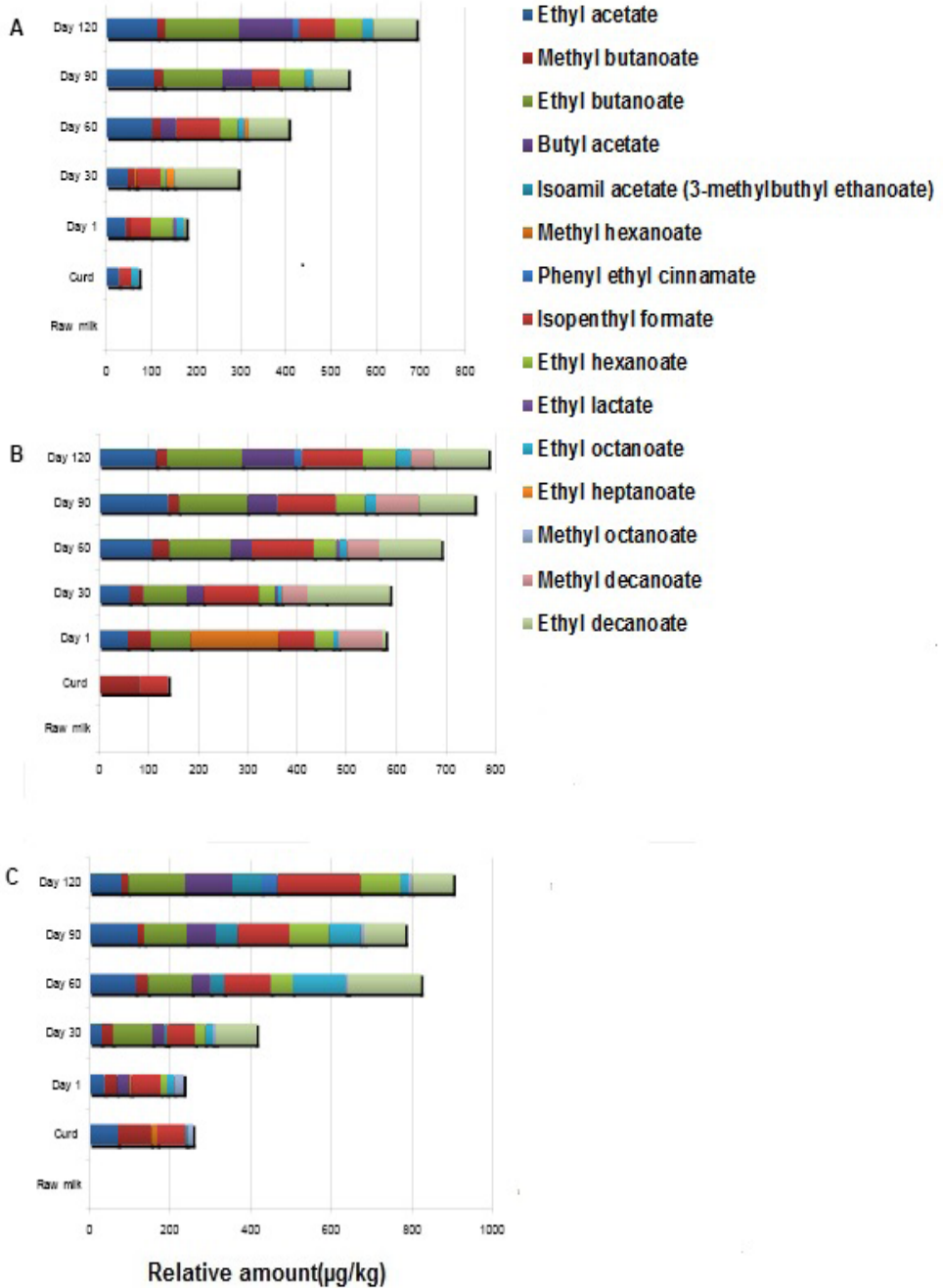
Supplementary Figure 1. Variation of the carboxylic acids detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



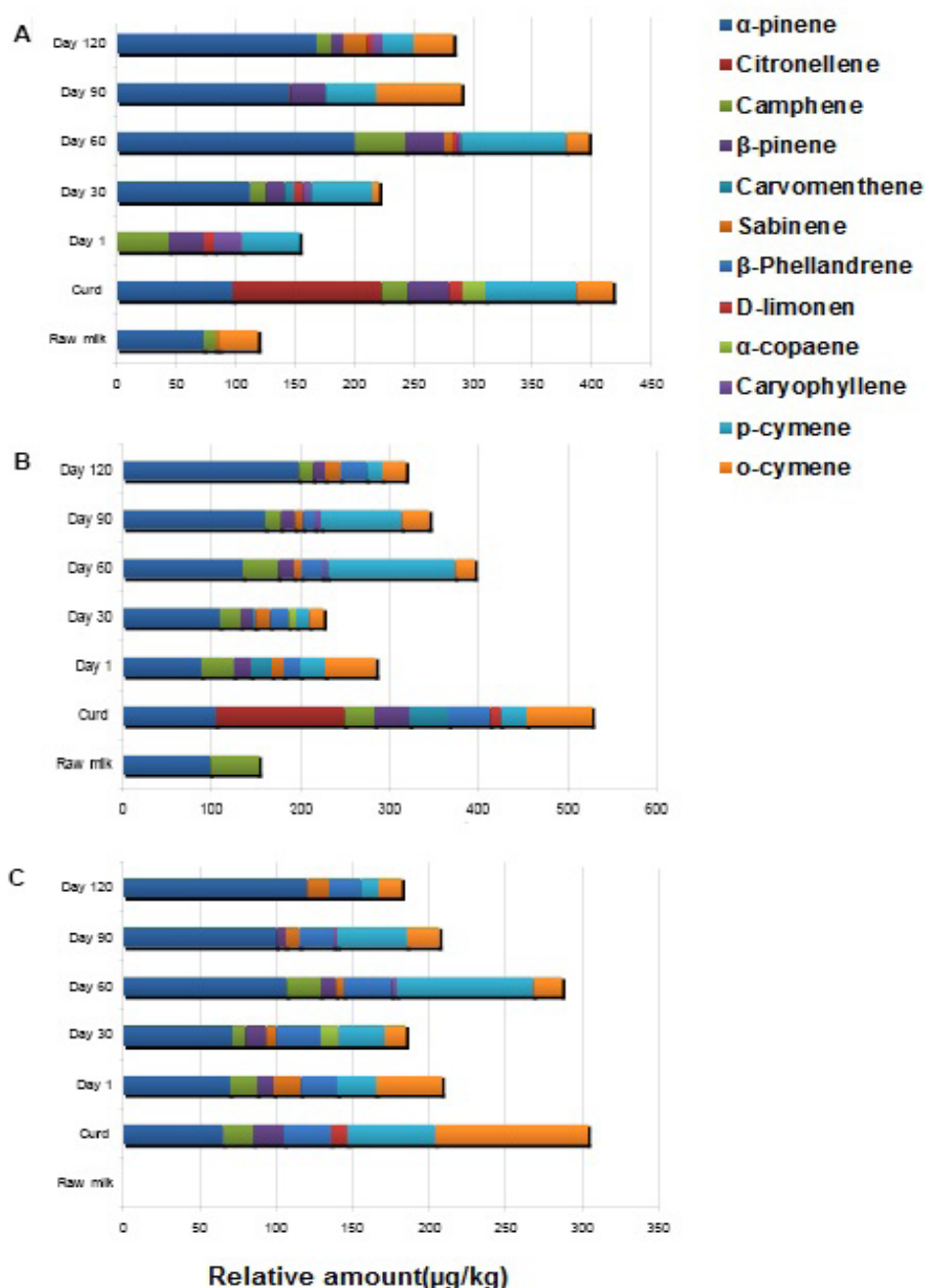
Supplementary Figure 2. Variation of the alcohols detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



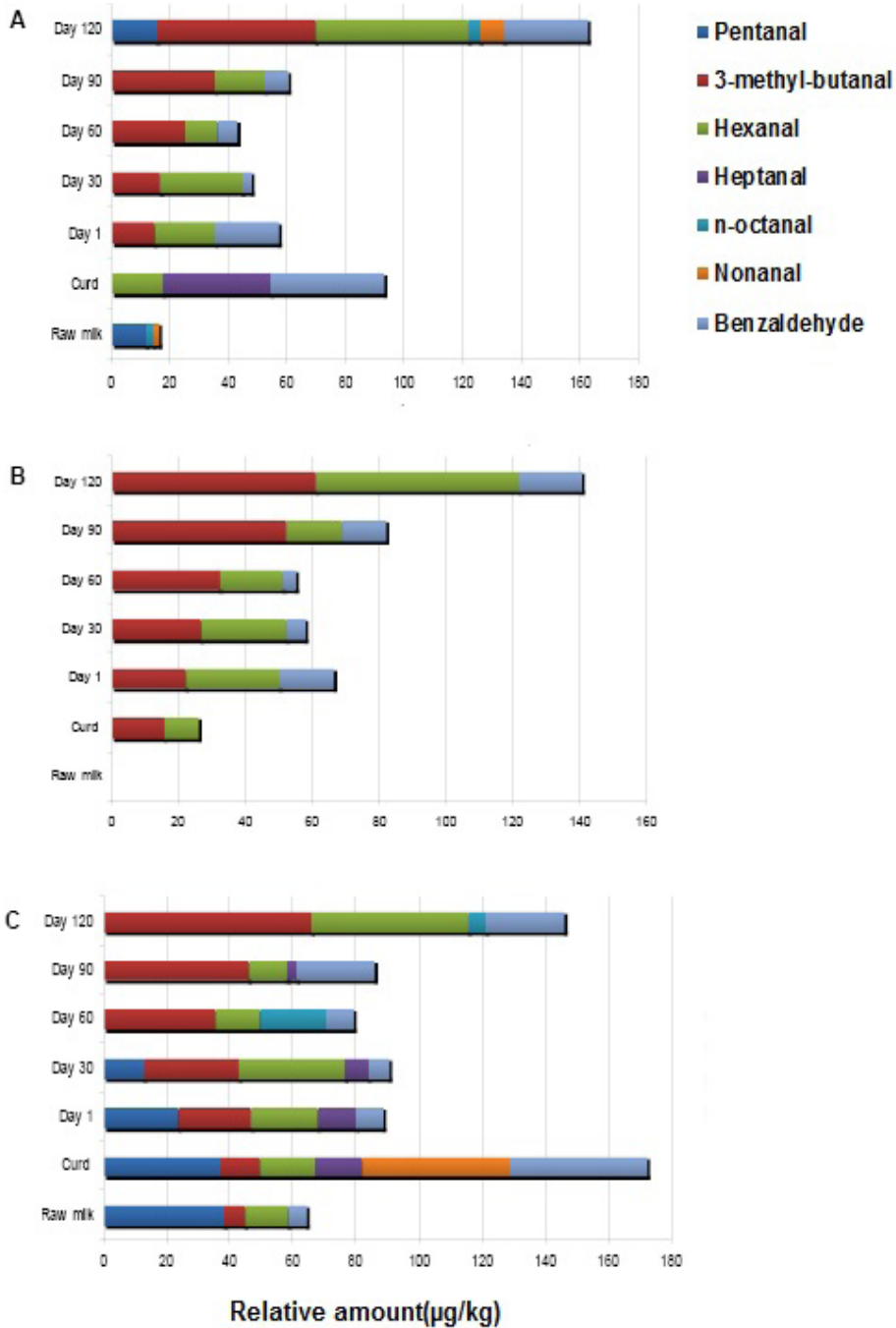
Supplementary Figure 3. Variation of the ketones detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



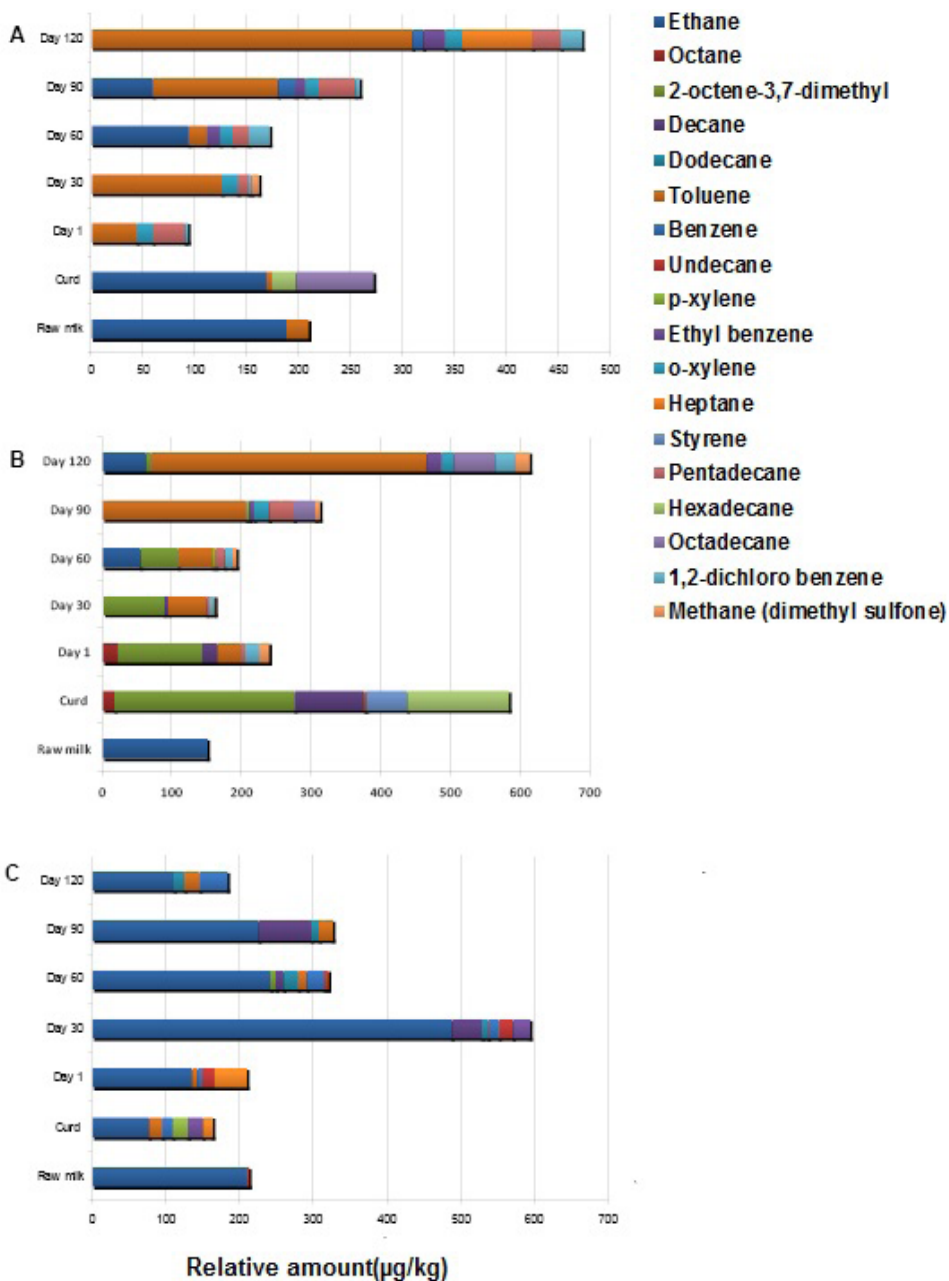
Supplementary Figure 4. Variation of the esters detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



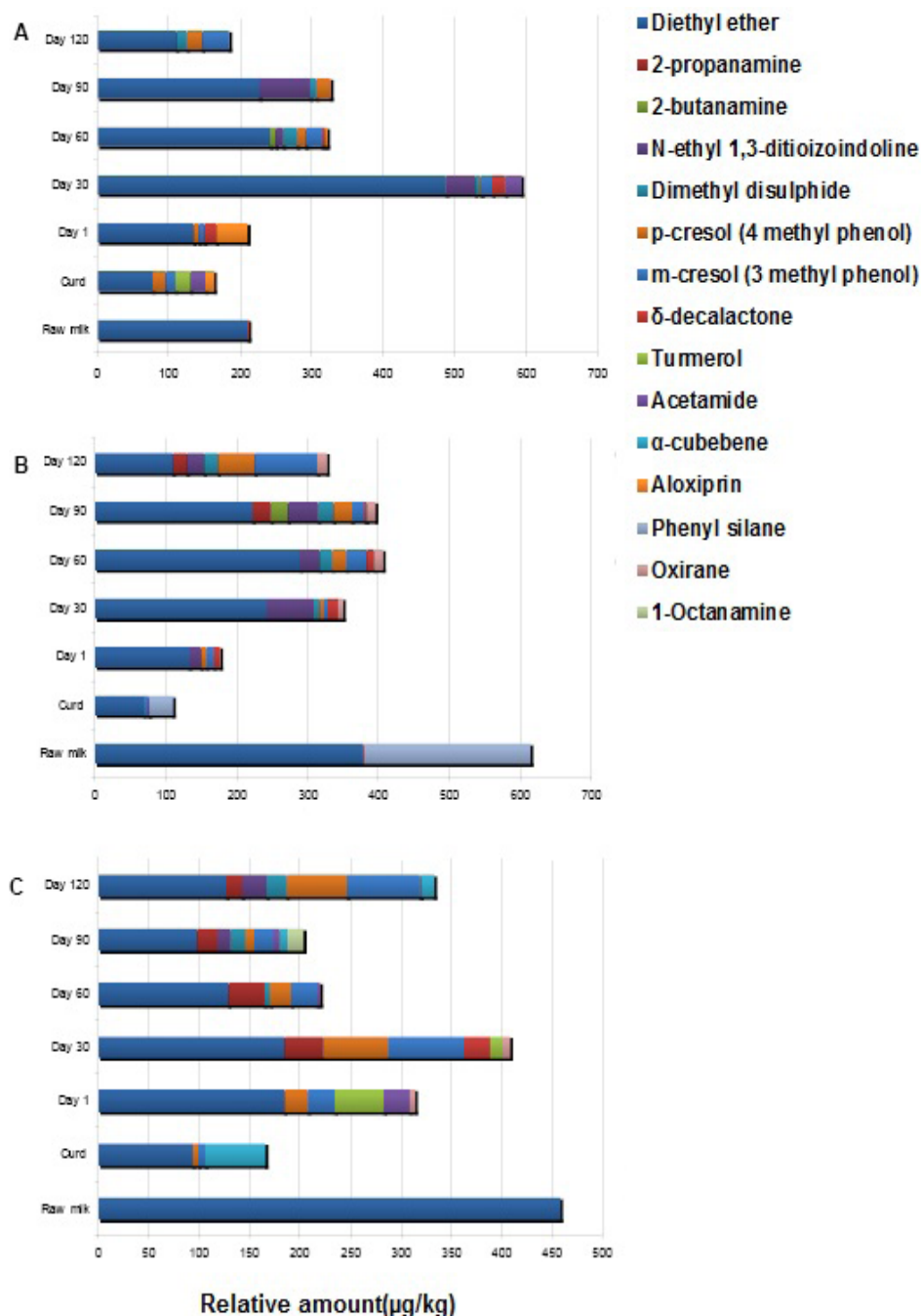
Supplementary Figure 5. Variation of the terpenes detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



Supplementary Figure 6. Variation of the aldehydes detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



Supplementary Figure 7. Variation of the hydrocarbons detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3



Supplementary Figure 8. Variation of the miscellaneous detected in DC cheese during production and ripening, A: cheese produced in DF1, B: cheese produced in DF2, C: cheese produced in DF3

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
<i>Acids</i>										
Acetic acid	1458	MS, RI	DF1	29.30±1.99 ^{ab}	181.92±11.32 ^{Ca}	298.56±3.85 ^{Db}	433.02±4.49 ^{Ea}	707.20±8.54 ^{Ec}	819.65±7.14 ^{Cc}	154.04±5.62 ^{Bb}
			DF2	ND ^{Aa}	545.85±21.09 ^{Bb}	227.97±10.95 ^{Ca}	440.15±6.79 ^{Ea}	423.87±3.89 ^{Bb}	361.90±5.75 ^{Db}	120.05±9.40 ^{Ba}
			DF3	ND ^{Aa}	651.32±12.10 ^{Ec}	495.34±5.90 ^{Ec}	703.44±12.06 ^{Gb}	116.12±0.97 ^{Ba}	237.99±1.69 ^{Ba}	140.82±2.20 ^{Cb}
Propanoic acid	1540	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	1.27±0.29 ^{Ba}	5.6±0.32 ^{Ca}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	74.13±3.66 ^{Cc}	30.59±2.80 ^{Bb}	27.78±5.56 ^{Bc}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	50.98±0.11 ^{Db}	34.98±0.09 ^{Cc}	13.29±0.31 ^{Bb}	ND ^{Aa}	ND ^{Aa}
2-Methyl- propanoic acid (isobutyric acid)	1580	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	24.71±2.45 ^{Cb}	ND ^{Aa}	ND ^{Aa}	11.53±8.78 ^{Ba}
			DF2	ND ^{Aa}	ND ^{Aa}	11.92±6.84 ^{Ab}	28.85±9.26 ^{Bb}	38.19±4.15 ^{Bb}	38.13±9.67 ^{Bb}	46.56±11.20 ^{Cb}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.81±0.56 ^{Ba}	71.23±4.75 ^{De}	55.62±1.61 ^{Cb}
Butanoic acid	1638	MS, RI	DF1	11.35±0.58 ^{ab}	39.76±2.31 ^{Aa}	89.02±1.72 ^{Ba}	36.31±0.93 ^{Aa}	648.95±9.13 ^{Cb}	931.5±10.26 ^{Ec}	690.52±34.82 ^{De}
			DF2	ND ^{Aa}	56.10±10.67 ^{Bb}	107.34±2.89 ^{Cb}	365.88±1.87 ^{De}	602.69±20.01 ^{Fa}	856.49±15.70 ^{Ga}	457.83±4.64 ^{Ea}
			DF3	ND ^{Aa}	51.03±2.66 ^{Bab}	120.77±8.02 ^{Cc}	298.87±3.05 ^{Db}	614.16±11.98 ^{Fab}	888.36±6.75 ^{Db}	551.12±4.32 ^{Db}
2-methyl-butanoic acid	1666	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	5.98±0.11 ^{Db}	3.98±0.09 ^{Cb}	ND ^{Aa}	3.29±0.31 ^{Bab}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.22±1.00 ^{ABb}	7.85±0.89 ^{Bb}	12.98 ±8.89 ^{Cb}	41.20±3.88 ^{Db}
3-methyl-butanoic acid (isovaleric acid)	1696	MS, RI	DF1	ND ^{Aa}	11.58±5.90 ^{Ab}	78.78±14.65 ^{Bb}	64.19±1.82 ^{Bb}	15.5±3.26 ^{Aa}	104.26±9.84 ^{Ca}	98.23±31.05 ^{Ca}
			DF2	ND ^{Aa}	ND ^{Aa}	35.81±2.67 ^{Ba}	97.09±8.90 ^{Cc}	129.74±10.45 ^{Bb}	93.32±3.67 ^{Ca}	142.48±6.46 ^{Ab}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Valeric acid (pentanoic acid)	1736	MS, RI	DF3	ND ^{Aa}	60.64±4.65 ^{Bc}	78.07±4.57 ^{Bb}	12.22±1.34 ^{Aa}	109.79±9.42 ^{Cb}	127.32±5.23 ^{Cb}	156.09±29.04 ^{Db}	
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	33.01±3.72 ^{Ba}	358.25±18.74 ^{Cc}	560.23±37.01 ^{Db}	661.77±10.32 ^{Eb}	
			DF2	ND ^{Aa}	11.23±1.60 ^{ABb}	30.53±2.02 ^{ABc}	43.98±6.68 ^{Bb}	177.89±65.10 ^{Cb}	697.98±11.24 ^{Dc}	905.37±7.76 ^{Ec}	
			DF3	ND ^{Aa}	13.82±2.74 ^{Cb}	6.85±1.26 ^{Bb}	36.19±2.09 ^{ABb}	28.11±2.67 ^{Ba}	112.36±2.20 ^{Aa}	339.01±5.72 ^{Ca}	
Hexanoic acid	1872	MS, RI	DF1	15.54±01.55 ^{Bb}	ND ^{Aa}	27.5±5.05 ^{Ca}	20.76±0.59 ^{BCb}	38.15±3.91 ^{Dc}	94.42±7.65 ^{Ec}	45.4±2.92 ^{Da}	
			DF2	ND ^{Aa}	53.48±1.90 ^{Dc}	191.91±7.60 ^{Eb}	26.18±3.00 ^{Bc}	29.69±1.89 ^{Bb}	40.66±2.90 ^{Cb}	41.09±3.05 ^{Ca}	
			DF3	ND ^{Aa}	15.18±1.87 ^{Bb}	18.56±4.45 ^{Ca}	13.97±0.02 ^{Ba}	13.14±0.67 ^{Ba}	16.16±1.21 ^{BCa}	84.91±3.90 ^{Db}	
Heptanoic acid	1954	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	0.33±0.15 ^{Ba}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.09±3.09 ^{Cc}	4.34±0.87 ^{Bc}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.35±8.02 ^{Db}	5.81±0.65 ^{Db}	4.46±2.21 ^{Cb}	2.91±0.56 ^{Bb}	
Octanoic acid	2074	MS, RI	DF1	22.46±1.97 ^{Bb}	51.64±2.70 ^{Cc}	87.77±2.65 ^{Ec}	60.99±20.76 ^{Db}	43.92±2.14 ^{Ca}	ND ^{Aa}	28.66±3.20 ^{Ba}	
			DF2	ND ^{Aa}	ND ^{Aa}	50.94±0.98 ^{Ca}	ND ^{Aa}	47.29±1.67 ^{Ba}	56.73±1.10 ^{Db}	49.89±2.98 ^{Cb}	
			DF3	ND ^{Aa}	34.56±4.88 ^{Bb}	67.09±14.60 ^{Cb}	84.80±5.46 ^{Dc}	69.10±15.90 ^{Cb}	ND ^{Aa}	30.16±13.88 ^{Ba}	
Nonanoic acid	2163	MS, RI	DF1	ND ^{Aa}	6.5±0.46 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	35.1±1.89 ^{Dc}	7.05±1.12 ^{Cb}	1.81±0.70 ^{Bb}	0.25±0.02 ^{Ab}	ND ^{Aa}	ND ^{Aa}	
Decanoic acid	2264	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	19.94±2.58 ^{Cb}	18.46±0.09 ^{Cb}	24.56±3.87 ^{Db}	30.18±2.59 ^{Eb}	ND ^{Aa}	3.48±0.94 ^{Bb}	ND ^{Aa}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Benzoic acid	2379	MS, RI	DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF1	ND ^{Aa}	11.05±3.46 ^{Ba}	24.48±0.99 ^{Cb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	10.64±1.60 ^{Ba}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	135.32±11.54 ^{Cb}	63.93±21.35 ^{Bc}	3.46±0.98 ^{Ab}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Total Acids (13)			DF1	78.65 ^{Ac}	302.45 ^{Ba}	607.38 ^{Ca}	678.59 ^{Ba}	1812.25 ^{Cc}	2510.06 ^{Cc}	1690.15 ^{Eb}
			DF2	19.94 ^{Ab}	695.76 ^{Bb}	761.09 ^{Cb}	1066.88 ^{Bb}	1477.14 ^{Eb}	2158.07 ^{Db}	1713.38 ^{Cc}
			DF3	ND ^{Aa}	996.97 ^{Cc}	908.64 ^{Bc}	1200.31 ^{Dc}	982.43 ^{Ca}	1470.86 ^{Fa}	1401.84 ^{Ea}
Percentage (%) ³			DF1	21.55	14.97	22.50	19.34	36.68	39.21	32.04
			DF2	2.66	27.84	24.01	27.29	28.17	35.09	30.66
			DF3	0.00	33.38	30.58	28.34	18.80	24.96	24.99
<i>Alcohols</i>										
Ethanol	936	MS, RI	DF1	ND ^{Aa}	79.88±0.45 ^{Ca}	184.19±4.58 ^{Cc}	160.89±2.80 ^{Fb}	104.81±10.6 ^{Dc}	120.65±3.4 ^{Ec}	30.35±6.5 ^{Ba}
			DF2	ND ^{Aa}	154.08±9.56 ^{Fb}	120.62±8.76 ^{Ea}	262.31±3.30 ^{Cc}	73.18±2.60 ^{Db}	18.22±1.80 ^{Ba}	31.05±1.84 ^{Ca}
			DF3	ND ^{Aa}	153.81±8.90 ^{Eb}	177.81±5.05 ^{Fb}	41.48±2.67 ^{Ca}	29.34±0.76 ^{Ba}	51.84±43.09 ^{Bb}	28.96±2.11 ^{Ba}
2-Butanol	1022	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	304.62±11.09 ^{Bb}	697.21±12.95 ^{Db}	708.87±23.01 ^{Da}	849.5±13.82 ^{Eb}	531.72±3.45 ^{Ca}
			DF2	ND ^{Aa}	ND ^{Aa}	174.47±19.70 ^{Ba}	340.78±6.97 ^{Ca}	843.47±2.89 ^{Fb}	665.16±11.90 ^{Ea}	595.68±13.24 ^{Db}
			DF3	ND ^{Aa}	ND ^{Aa}	321.5±20.60 ^{Bb}	976.5±12.08 ^{Ec}	1520.2±13.60 ^{Fc}	891.2±2.98 ^{Dc}	738.5±5.96 ^{Cc}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese			Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
2-Methyl-1-propanol (isobutanol)	1087	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	8.27±0.37 ^{Ba}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	25.81±1.15 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF3	ND ^{Aa}	ND ^{Aa}	43.83±3.7 ^{Dc}	72.71±11.09 ^{Fb}	49.64±5.43 ^{Eb}	30.16±2.67 ^{Cb}	12.65±1.90 ^{Bb}		
2-Pentanol	1123	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	9.83±0.84 ^{Bc}	37.35±3.95 ^{Cc}	40.64±8.93 ^{Cb}	50.46±3.20 ^{Db}	129.95±1.68 ^{Eb}		
			DF2	ND ^{Aa}	ND ^{Aa}	6.86±0.77 ^{ABb}	14.35±11.57 ^{Bb}	16.36±1.50 ^{Ba}	72.38±4.96 ^{Cc}	109.40±7.66 ^{Db}		
			DF3	ND ^{Aa}	ND ^{Aa}	3.95±1.00 ^{Ba}	8.93±1.67 ^{Ca}	12.66±1.25 ^{Da}	11.09±1.14 ^{Da}	66.23±5.52 ^{Ea}		
2-Propanol	1128	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF2	ND ^{Aa}	ND ^{Aa}	11.25±0.95 ^{Eb}	9.21±1.02 ^{Db}	5.78±2.73 ^{Cb}	1.18±0.86 ^{Bb}	8.91±0.95 ^{Bb}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	11.63±9.78 ^{Dc}	13.78±12.56 ^{Ec}	4.65±1.25 ^{Cc}	1.82±1.01 ^{Bb}		
1-Butanol	1137	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	23.94±5.42 ^{Cb}	62.24±2.80 ^{Dc}	23.89±12.36 ^{Cb}	20.96±1.89 ^{Cb}	9.72±1.09 ^{Ba}		
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.02±1.65 ^{Ba}	7.70±3.52 ^{Ca}	13.21±6.72 ^{Db}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	1.92±0.71 ^{Ab}	24.56±3.87 ^{Cb}	19.18±1.19 ^{Bb}	19.89±2.24 ^{Bc}		
1-Pentanol (amyl alcohol)	1238	MS, RI	DF1	ND ^a	57.62±3.62 ^{Db}	43.79±9.61 ^{Cc}	18.45±6.3 ^{Bb}	43.0±4.80 ^{Cc}	74.6±1.95 ^{Ec}	80.99±2.06 ^{Ec}		
			DF2	79.09±2.98 ^{Eb}	28.61±1.06 ^{CDa}	8.72±0.79 ^{Aa}	27.94±1.15 ^{Cc}	31.40±3.09 ^{Db}	20.09±2.12 ^{Bb}	18.02±0.72 ^{Bb}		
			DF3	ND ^a	28.95±2.20 ^{Fa}	17.5±1.05 ^{Eb}	4.13±2.01 ^{Ba}	15.77±0.09 ^{Ea}	12.33±1.08 ^{Da}	9.45±0.65 ^{Ca}		
3-methyl-1-butanol (isoamyl alcohol)	1200	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	15.97±6.9 ^{Bb}	63.85±9.81 ^{Fb}	31.05±16.23 ^{Da}	22.19±9.56 ^{Ca}	54.09±5.07 ^{Eb}		
			DF2	ND ^{Aa}	ND ^{Aa}	11.86±0.89 ^{Ba}	15.88±5.17 ^{Ca}	45.23±2.67 ^{Eb}	38.46±10.56 ^{Db}	40.94±3.40 ^{Da}		
			DF3	ND ^{Aa}	ND ^{Aa}	11.91±2.2 ^{Ba}	20.05±10.08 ^{Ca}	28.03±1.89 ^{Da}	140.21±3.4 ^{Ec}	56.17±4.60 ^{Eb}		

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
3-methyl-3-buten-1-ol	1241	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.42±1.90 ^{Bb}	4.98±1.46 ^{Cb}	8.18±5.14 ^{Dc}	7.98±2.06 ^{Dc}
			DF3		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.14±3.01 ^{Bb}	6.78±2.01 ^{Db}	5.95±1.48 ^{Cb}
2-heptanol	1320	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	9.87±0.74 ^{Ba}	31.26±0.99 ^{Ca}	77.52±1.65 ^{Db}	88.90±1.23 ^{Eb}
			DF2		ND ^{Aa}	9.28±1.09 ^{Bc}	28.52±1.45 ^{Cc}	90.08±1.79 ^{Eb}	75.11±4.90 ^{Db}	95.46±0.95 ^{Ec}	
			DF3		ND ^{Aa}	2.45±0.98 ^{Ab}	21.87±2.66 ^{Bb}	28.97±3.09 ^{Ca}	31.51±2.89 ^{Ca}	42.66±4.01 ^{Da}	
1-hexanol	1348	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	12.01±6.17 ^{Ca}	7.29±2.26 ^{Ba}	12.88±0.85 ^{Ca}	24.72±0.06 ^{Ec}	15.4±0.66 ^{Db}
			DF2		ND ^{Aa}	11.49±1.67 ^{Ba}	14.54±1.10 ^{Cb}	15.06±2.89 ^{Cb}	ND ^{Aa}	ND ^{Aa}	
			DF3		ND ^{Aa}	30.24±1.75 ^{Db}	22.34±4.34 ^{Cc}	17.5±3.64 ^{Bc}	16.79±4.87 ^{Bb}	15.61±1.50 ^{Bb}	
1-heptanol	1450	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	37.7±1.45 ^{Bb}	40.15±12.27 ^{Cb}
			DF2		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	19.65±2.25 ^{Bb}	69.11±4.76 ^{Dc}	65.78±23.46 ^{Cc}
			DF3		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	18.49±1.43 ^{Ba}	34.16±6.69 ^{Ca}
2-nonanol	1518	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	3.8±0.09 ^{Ab}	23.57±2.38 ^{Bb}	39.02±2.80 ^{Cc}	40.05±4.00 ^{Cc}
			DF3		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	1.45±0.82 ^{Aa}	18.74±2.66 ^{Bb}	22.73±1.90 ^{Cb}
2,3-butanediol	1535	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	58.17±7.63 ^{Cc}	174.75±19.85 ^{Eb}	73.51±5.5 ^{Db}	25.94±3.30 ^{Bb}	ND ^{Aa}
			DF2		ND ^{Aa}	30.96±1.97 ^{Bb}	198.02±3.07 ^{Dc}	90.00±2.01 ^{Cc}	34.02±10.56 ^{Bc}	ND ^{Aa}	
			DF3		ND ^{Aa}	10.94±2.11 ^{Bb}	18.42±1.24 ^{Ca}	106.22±7.56 ^{Ea}	59.74±3.06 ^{Da}	18.81±1.14 ^{Ca}	ND ^{Aa}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese			Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
1-octanol	1550	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	9.86±0.90 ^{Cb}	6.79±2.06 ^{Bb}	15.77±1.60 ^{Db}	15.79±6.12 ^{Db}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
Cyclobutanol	1574	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	16.45±3.65 ^{Bb}	ND ^{Aa}	43.11±3.99 ^{Cc}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	14.15±0.78 ^{Bb}	21.48±5.87 ^{Cc}	29.36±8.87 ^{Db}	31.08±10.66 ^{Eb}		
2-octanol	1658	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	23.71±2.44 ^{Cb}	49.54±3.21 ^{De}	19.74±0.72 ^{Ba}	ND ^{Aa}	88.77±2.82 ^{Eb}		
			DF2	ND ^{Aa}	ND ^{Aa}	37.40±2.00 ^{Cc}	44.44±2.76 ^{Db}	32.17±1.46 ^{Bc}	31.88±2.45 ^{Bc}	81.07±3.65 ^{Ea}		
			DF3	ND ^{Aa}	ND ^{Aa}	12.79±4.5 ^{Ba}	19.67±5.08 ^{Ca}	23.44±1.05 ^{Db}	28.05±5.51 ^{Eb}	128.69±3.66 ^{Fc}		
Phenethyl alcohol	1927	MS, RI	DF1	ND ^{Aa}	8.64±5.32 ^{Cb}	5.28±1.74 ^{Ba}	18.36±5.74 ^{Db}	7.44±2.66 ^{Ca}	43.5±15.85 ^{Eb}	50.6±0.29 ^{Fb}		
			DF2	ND ^{Aa}	ND ^{Aa}	7.54±0.80 ^{Cc}	4.57±6.02 ^{Ba}	32.57±3.03 ^{De}	49.81±12.89 ^{Ec}	60.63±1.60 ^{Fc}		
			DF3	ND ^{Aa}	28.66±1.77 ^{Fc}	5.85±0.90 ^{Bb}	21.63±1.89 ^{Ec}	9.98±3.54 ^{Cb}	15.51±6.96 ^{Da}	20.94±1.67 ^{Ea}		
Total Alcohols (18)			DF1	ND ^{Aa}	146.14 ^{Ba}	689.78 ^{Cc}	1299.8 ^{Eb}	1113.5 ^{Da}	1347.74 ^{Eb}	1163.75 ^{Da}		
			DF2	79.09 ^{Ab}	182.69 ^{Ba}	456.26 ^{Ca}	978.64 ^{Da}	1335.233 ^{Fb}	1146.09 ^{Ea}	1175.06 ^{Ea}		
			DF3	ND ^{Aa}	222.36 ^{Ba}	646.25 ^{Cb}	1343.23 ^{Ec}	1861.68 ^{Fc}	1344.7 ^{Eb}	1235.49 ^{Db}		
Percentage (%)			DF1	0,00	7,23	25,55	37,05	22,54	21,05	22,06		
			DF2	10,56	7,31	14,40	25,03	25,46	18,63	21,02		
			DF3	0,00	7,45	21,75	31,71	35,63	22,82	22,02		

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
<i>Ketones</i>											
2-propanone (acetone)	848	MS, RI	DF1	22.65±3.1 ^{Cb}	36.13±3.88 ^{Db}		79.94±27.9 ^{fc}	55.16±11.18 ^{Ec}	21.82±8.84 ^{Cb}	16.75±5.38 ^{Bb}	9.42±11.5 ^{Ab}
			DF2	49.51±3.01 ^{De}	59.12±2.76 ^{Ec}	48.33±18.45 ^{Db}	36.78±9.12 ^{Cb}	ND ^{Aa}	21.96±6.15 ^{Bc}	22.79±8.95 ^{Bc}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
2-butanone (methyl ethyl keton)	903	MS, RI	DF1	ND ^{Aa}	20.71±5.88 ^{Ba}		59.01±6.87 ^{Ca}	697.76±22.6 ^{Ec}	406.34±20.8 ^{Da}	761.48±35.75 ^{Fb}	413.08±54.12 ^{Db}
			DF2	3.25±1.40 ^{Ab}	59.49±13.81 ^{Bc}	139.17±42.66 ^{Cc}	255.49±23.10 ^{Db}	608.56±29.71 ^{Fb}	675.42±20.70 ^{Ga}	319.33±63.61 ^{Ea}	
			DF3	ND ^{Aa}	51.23±11.88 ^{Bb}	107.43±43.65 ^{Cb}	149.05±10.08 ^{Da}	608.21±34.56 ^{Fb}	976.32±24.98 ^{Gc}	557.23±15.21 ^{Ec}	
2-pentanone (ethyl acetone)	972	MS, RI	DF1	ND ^{Aa}	24.18±5.51 ^{Bb}		147.33±23.18 ^{Fb}	128.15±39.56 ^{Ec}	116.98±21.9 ^{Db}	162.05±10.35 ^{Gc}	88.59±28.25 ^{Cb}
			DF2	ND ^{Aa}	34.08±2.07 ^{Bc}	250.58±17.65 ^{Ec}	112.09±20.04 ^{DEb}	117.71±17.65 ^{Eb}	108.27±15.28 ^{Db}	94.69±3.09 ^{Cc}	
			DF3	ND ^{Aa}	19.08±2.00 ^{Ba}	51.3±5.46 ^{Ea}	25.71±2.55 ^{Ca}	31.84±14.01 ^{Da}	24.24±10.98 ^{Ca}	55.14±19.07 ^{Fa}	
3-methyl-2-butanone	1008	MS, RI	DF1	ND ^{Aa}	32.52±21.9 ^{Ca}		28.34±1.79 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	50.65±8.12 ^{Ec}	37.45±4.35 ^{De}	26.75±3.40 ^{Cb}	8.02±1.14 ^{Bb}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	40.05±1.32 ^{Cb}	3.12±1.12 ^{Ba}	0.28±0.02 ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
2,3-butanedione (diacetyl)	970	MS, RI	DF1	ND ^{Aa}	247.03±76.71 ^{Fb}		204.45±39.43 ^{Ea}	205.37±4.49 ^{Eb}	97.71±10.52 ^{De}	61.80±5.97 ^{Cc}	43.08±4.98 ^{Bb}
			DF2	ND ^{Aa}	312.88±50.54 ^{Ec}	230.66±44.48 ^{Db}	178.09±36.13 ^{Ca}	43.16±7.13 ^{Ba}	40.16±5.43 ^{Bb}	38.33±6.77 ^{Ba}	
			DF3	ND ^{Aa}	202.33±42.98 ^{Ea}	199.81±64.79 ^{Ea}	254.75±27.09 ^{Ec}	68.15±4.45 ^{Db}	14.98±3.79 ^{Ba}	56.23±5.30 ^{Cc}	
2-heptanone (amyl methyl keton)	1178	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}		ND ^{Aa}	9.95±1.4 ^{Ba}	51.58±7.1 ^{Cb}	167.13±4.56 ^{Ea}	149.22±6.29 ^{Da}
			DF2	ND ^{Aa}	ND ^{Aa}	20.54±0.78 ^{Bb}	75.23±3.46 ^{Cc}	132.57±9.70 ^{De}	203.83±5.60 ^{Fb}	175.59±10.24 ^{Ec}	

Supplementary Table 1. Relative abundance of volatile compounds (µg/kg) isolated from Divle Cave cheese during production and ripening (mean ± SD)

Compounds	RI	ID method ¹	Production of cheese			Ripening of cheese					
			Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120		
2-octanone	1276	MS, RI	DF3	ND ^{Aa}	41.88±3.09 ^{Bc}	55.1±7.61 ^{Db}	48.81±3.09 ^{Ca}	428.12±23.09 ^{Fe}	163.32±11.67 ^{Eb}		
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	20.01±2.9 ^{Cc}	15.54±1.78 ^{Bc}		
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	14.44±3.12 ^{Ch}	10.66±2.55 ^{Bb}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.15±0.95 ^{Ba}		
3-hydroxy-2-butanone (acetoin)	1280	MS, RI	DF1	ND ^{Aa}	97.22±16.8 ^{Fe}	100.94±22.9 ^{Ch}	69.93±7.19 ^{Bb}	52.72±5.4 ^{Ch}	78.43±10.6 ^{Ec}	39.59±18.83 ^{Bb}	
			DF2	ND ^{Aa}	89.13±96.04 ^{Eb}	106.58±48.88 ^{Fe}	47.75±12.78 ^{Ca}	57.39±0.90 ^{De}	55.41±2.63 ^{Db}	44.98±9.66 ^{Bc}	
			DF3	ND ^{Aa}	56.01±85.01 ^{Ea}	95.12±31.19 ^{Ga}	77.88±9.01 ^{Fe}	43.43±1.09 ^{Da}	21.04±7.11 ^{Ca}	15.35±3.74 ^{Ba}	
			DF1	ND ^{Aa}	11.54±3.9 ^{Bb}	51.02±7.73 ^{Eb}	23.94±20.6 ^{Ch}	35.54±9.05 ^{Da}	50.73±6.66 ^{Ea}	79.80±10.82 ^{Fa}	
2-nonanone	1390	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	76.99±5.00 ^{Ec}	18.78±10.03 ^{Ba}	58.66±9.90 ^{Db}	55.10±9.89 ^{Ch}	88.45±9.78 ^{Fb}	
			DF3	ND ^{Aa}	15.27±1.77 ^{Bc}	22.56±4.13 ^{Ca}	85.05±7.58 ^{De}	164.97±38.97 ^{Gc}	153.40±24.77 ^{Fe}	95.51±24.02 ^{Ec}	
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	32.36±2.65 ^{Bc}	
5 - m e t h y l - 2 - hexanedione	1414	MS, RI	DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	23.7±6.01 ^{Bb}	
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	27.19±8.65 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	29.53±12.20 ^{Cc}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	19.66±10.07 ^{Bb}	
8-nonen-2-one	1442	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	25.06±1.23 ^{Cc}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	2.08±0.97 ^{Bb}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
2-undecanone	1608	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.74±2.10 ^{Bc}	31.90±6.90 ^{De}		
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	2.15±1.05 ^{Bb}	25.50±5.12 ^{Ch}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Total ketones (12)			DF3		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	1.80±0.89 ^{Ba}	13.65±3.04 ^{Ca}	ND ^{Aa}
			DF1		22.65 ^{Ab}	469.33 ^{Bb}	671.03 ^{Cb}	1190.34 ^{Fc}	790.43 ^{Da}	1350.28 ^{Db}	863.38 ^{Ea}
			DF2		52.76 ^{Ac}	632.54 ^{Bc}	910.3 ^{Ec}	750.96 ^{Cb}	1028.22 ^{Fc}	1200.09 ^{Ea}	858.79 ^{Da}
			DF3		ND ^{Aa}	383.97 ^{Ba}	521.22 ^{Ca}	647.82 ^{Da}	967.21 ^{Eb}	1631.75 ^{Gc}	993.29 ^{Fb}
Percentage (%)			DF1		6.21	23.23	24.86	16.03	16.00	20.69	16.21
			DF2		7.04	25.31	28.72	19.21	19.61	19.77	15.26
			DF3		0.00	12.86	17.54	15.29	18.51	27.69	17.64
<i>Esters</i>											
Ethyl acetate	894	MS, RI	DF1		ND ^{Aa}	27.61±8.18 ^{Bb}	41.37±4.61 ^{Cb}	46.66±1.54 ^{Db}	99.65±17.72 ^{Ea}	105.23±12.17 ^{Fa}	113.12±1.95 ^{Gb}
			DF2		ND ^{Aa}	ND ^{Aa}	55.17±6.09 ^{Bc}	60.25±7.10 ^{Cc}	105.56±14.45 ^{Db}	138.26±9.21 ^{Fc}	114.60±1.23 ^{Fb}
			DF3		ND ^{Aa}	70.36±9.44 ^{Dc}	37.31±3.28 ^{Ca}	27.67±5.57 ^{Ba}	113.74±24.97 ^{Fc}	117.90±14.88 ^{Gb}	77.77±13.06 ^{Ea}
Methyl butanoate	988	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	12.92±0.82 ^{Ba}	14.87±0.88 ^{Ca}	21.61±0.5 ^{Ea}	20.35±2.92 ^{Fb}	17.27±1.05 ^{Da}
			DF2		ND ^{Aa}	82.78±2.26 ^{Fb}	47.82±2.96 ^{Ec}	28.16±3.45 ^{Cb}	35.05±0.41 ^{Dc}	21.30±1.90 ^{Bb}	22.65±7.24 ^{Bb}
			DF3		ND ^{Aa}	82.88±6.66 ^{Eb}	30.98±5.13 ^{Db}	29.50±6.78 ^{Dc}	30.81±7.03 ^{Db}	15.59±2.45 ^{Ba}	18.67±0.84 ^{Ca}
Ethyl butanoate	1034	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	132.07±11.76 ^{Bb}	162.87±19.90 ^{Cc}
			DF2		ND ^{Aa}	ND ^{Aa}	80.71±9.17 ^{Bb}	87.69±7.91 ^{Cb}	123.41±15.89 ^{Dc}	139.12±19.23 ^{Ec}	150.45±42.30 ^{Fb}
			DF3		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	98.24±8.65 ^{Bc}	109.11±13.42 ^{Db}	105.66±10.90 ^{Ca}	138.46±22.65 ^{Ea}
Butyl acetate	1072	MS, RI	DF1		ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	34.09±3.90 ^{Ba}	65.97±9.61 ^{Cb}	121.03±16.06 ^{Db}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Isoamil acetate (3-methylbutyl ethanoate)	1124	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	33.09±2.06 ^{Bb}	42.55±4.40 ^{Cb}	59.70±7.45 ^{Da}	106.24±12.90 ^{Ea}	
			DF3	ND ^{Aa}	ND ^{Aa}	29.60±5.14 ^{Bb}	31.09±6.53 ^{Bb}	45.59±8.45 ^{Cb}	73.09±10.61 ^{Dc}	120.21±10.05 ^{Eb}	
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.23±1.79 ^{Bb}	35.31±4.13 ^{Cb}	54.62±9.78 ^{Db}	71.45±10.13 ^{Eb}	
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.32±1.16 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
Methyl hexanoate	1182	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	176.80±18.40 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
Phenyl cinnamate	19,12	MS, RI	DF3	ND ^{Aa}	14.85±1.35 ^{Db}	5.21±2.65 ^{Cb}	3.51±1.27 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	16.31±3.09 ^{Ba}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	14.99±1.09 ^{Ba}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	38.54±2.64 ^{Bb}	
			DF1	ND ^{Aa}	26.98±1.75 ^{Ba}	44.56±5.68 ^{Ca}	54.39±3.50 ^{Da}	97.69±10.09 ^{Ea}	60.79±5.64 ^{Ea}	77.89±6.55 ^{Fa}	
			DF2	ND ^{Aa}	55.14±5.40 ^{Bb}	74.33±9.69 ^{Cc}	113.24±3.89 ^{Dc}	125.66±15.42 ^{Fc}	120.42±11.40 ^{Eb}	122.56±13.23 ^{EFb}	
Ethyl hexanoate	1226	MS, RI	DF3	ND ^{Aa}	69.05±13.4 ^{Cc}	71.33±1.44 ^{Cb}	65.75±6.53 ^{Bb}	113.78±2.05 ^{Db}	129.34±11.40 ^{Ec}	205.81±0.67 ^{Fc}	
			DF1	ND ^{Aa}	ND ^{Aa}	49.1±2.84 ^{Dc}	8.89±1.61 ^{Ba}	39.78±3.54 ^{Ca}	57.23±1.28 ^{Ea}	59.89±4.9 ^{Fa}	
			DF2	ND ^{Aa}	ND ^{Aa}	36.94±3.30 ^{Cb}	31.24±2.90 ^{Bc}	45.8±2.29 ^{Db}	57.68±9.67 ^{Ea}	66.54±1.12 ^{Fb}	
Ethyl lactate	1454	MS, RI	DF3	ND ^{Aa}	ND ^{Aa}	17.91±1.00 ^{Ba}	26.22±0.38 ^{Cb}	56.43±2.16 ^{Dc}	97.13±8.33 ^{Eb}	100.01±2.26 ^{Fc}	
			DF1	ND ^{Aa}	ND ^{Aa}	7.47±1.36 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Ethyl octanoate	1475	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.79±2.06 ^{Bb}	6.99±2.78 ^{Cb}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	ND ^{Aa}	16.16±8.10 ^{De}	16.38±2.20 ^{Bb}	3.46±2.50 ^{Ba}	13.42±6.12 ^{Ca}	17.06±9.78 ^{Da}	25.17±1.49 ^{Eb}
			DF2	ND ^{Aa}	ND ^{Aa}	10.64±5.09 ^{Ba}	9.90±1.02 ^{Bb}	13.47±2.78 ^{Ca}	21.38±2.60 ^{Bb}	31.2±1.45 ^{Ec}
			DF3	ND ^{Aa}	6.67±0.08 ^{Bb}	16.15±0.64 ^{Cb}	19.61±2.00 ^{Cc}	130.07±14.77 ^{Fb}	79.15±8.91 ^{Ec}	19.33±1.88 ^{Da}
Ethyl heptanoate	1529	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	17.76±7.37 ^{Bb}	8.45±2.31 ^{Ch}	ND ^{Aa}	1.24±0.42 ^{Bb}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Methyl octanoate	1380	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	11.51±0.65 ^{Eb}	23.17±3.09 ^{Fb}	8.05±1.75 ^{Cb}	5.12±4.56 ^{Bb}	10.15±4.23 ^{Bb}	9.89±2.33 ^{Bb}
Methyl decanoate	1588	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	87.66 ±7.28 ^{Eb}	50.87±7.57 ^{Cb}	65.90±12.61 ^{Db}	87.52±11.32 ^{Eb}	45.16±3.05 ^{Bb}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Ethyl decanoate	1636	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	5.36±0.65 ^{Bb}	141.16±17.20 ^{Fb}	89.12±10.34 ^{Ba}	78.08±8.56 ^{Ca}	94.12±7.67 ^{Ea}
			DF2	ND ^{Aa}	ND ^{Aa}	7.19±0.04 ^{Bc}	167.73±24.09 ^{Ec}	124.77±11.45 ^{Bb}	111.90±13.09 ^{Cc}	110.05±12.23 ^{Cc}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	100.20±9.11 ^{Ba}	180.56±12.78 ^{Cc}	100.67±11.20 ^{Bb}	101.88±10.43 ^{Bb}
Total esters (15)			DF1	ND ^{Aa}	70.75 ^{Ba}	177.16 ^{Ca}	291.51 ^{Da}	403.81 ^{Ea}	536.78 ^{Fa}	688.91 ^{Ga}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese			Ripening of cheese					
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120		
Percentage (%)			DF2	ND ^{Aa}	137.92 ^{Bb}	577.26 ^{Ca}	586.96 ^{Dc}	689.16 ^{Eb}	757.3 ^{Fb}	784.44 ^{Gb}		
			DF3	ND ^{Aa}	255.32 ^{Cc}	231.66 ^{Bb}	414.07 ^{Db}	820.52 ^{Fc}	783.34 ^{Ec}	902.02 ^{Gc}		
			DF1	0,00	3,50	6,56	8,31	8,17	8,22	13,06		
			DF2	0,00	5,52	18,21	15,01	13,14	12,48	14,04		
			DF3	0,00	8,55	7,80	9,78	15,70	13,29	16,08		
<i>Terpens</i>												
α -pinene	1024	MS, RI	DF1	72.14±5.88 ^{Bb}	97.14 ±6.85 ^{Cb}	ND ^{Aa}	112.09±15.71 ^{De}	199.78±21.6 ^{Gc}	144.79±15.6 ^{Eb}	167.07±14.70 ^{Fb}		
			DF2	98.54±10.35 ^{Fc}	103.66±14.32 ^{Cc}	87.12±9.88 ^{Ac}	107.65±11.24 ^{Db}	134.75±20.09 ^{Eb}	159.36±8.88 ^{Fc}	197.46±10.89 ^{Gc}		
			DF3	ND ^{Aa}	65.19±0.56 ^{Ba}	69.49±4.81 ^{Cb}	70.65±0.78 ^{Ca}	106.28±2.84 ^{Ea}	99.93±11.19 ^{Ba}	119.78±12.68 ^{Fa}		
Citronellene	1066	MS, RI	DF1	ND ^{Aa}	125.31±1.91 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	1.58±0.19 ^{Ab}	ND ^{Aa}		
			DF2	ND ^{Aa}	145.01±8.72 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
Camphene	1080	MS, RI	DF1	10.56±1.15 ^{Bb}	22.53±0.6 ^{Db}	43.72±3.89 ^{Ec}	13.53±0.9 ^{Cb}	43.05±2.16 ^{Fc}	ND ^{Aa}	12.16±1.08 ^{Bc}		
			DF2	53.72±4.80 ^{Fc}	34.08±2.26 ^{Cc}	36.55±2.66 ^{Db}	23.41±2.03 ^{Bc}	38.73±1.18 ^{Eb}	18.01±3.00 ^{Ab}	16.18±1.12 ^{Ac}		
			DF3	ND ^a	18.95±9.06 ^{Ba}	17.42±2.00 ^{Ca}	8.47±1.17 ^{Ba}	22.12±3.16 ^{Ea}	ND ^{Aa}	ND ^{Aa}		
β -pinene	1110	MS, RI	DF1	ND ^{Aa}	34.51±2.9 ^{Bb}	28.54±3.31 ^{De}	14.61±1.47 ^{Ca}	32.39±1.9 ^{Ec}	28.76±1.29 ^{De}	10.56±1.09 ^{Bb}		
			DF2	ND ^{Aa}	38.48±2.89 ^{Fc}	19.68±3.14 ^{Eb}	15.44±1.60 ^{Ca}	17.68±2.81 ^{Db}	15.63±0.95 ^{Cb}	12.48±2.05 ^{Bc}		
			DF3	ND ^{Aa}	20.73±3.31 ^{Ea}	11.09±2.32 ^{Ca}	13.92±1.97 ^{Ba}	10.4±1.485 ^{Ca}	5.6±1.20 ^{Ba}	ND ^{Aa}		

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Carvomenthene	1021	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	19.71±1.66 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	8.81±1.26 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	11.35±2.54 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Sabinene	1134	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	23.84±1.10 ^{Eb}	6.13±0.54 ^{Cb}	3.57±0.18 ^{Ba}	ND ^{Aa}	8.17±0.40 ^{Db}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.35±2.09 ^{Bb}	7.00±1.11 ^{Cc}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	3.21±1.19 ^{Ba}	2.81±0.75 ^{Bb}	ND ^{Aa}
β-Phellandrene	1185	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.90±1.75 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	44.11±3.05 ^{Db}	23.65±3.29 ^{Cb}	3.05±0.04 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
D-limonen	1200	MS, RI	DF1	3.72±1.56 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.19±0.1 ^{Cb}	ND ^{Aa}	19.69±1.72 ^{Dc}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	13.43±1.03 ^{Cb}	16.05±0.08 ^{Dc}	9.36±0.05 ^{Bc}	9.37±2.14 ^{Bb}	17.92±1.78 ^{Eb}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	17.52±1.65 ^{Ec}	7.04±1.04 ^{Cb}	5.27±0.06 ^{Ba}	9.67±1.23 ^{Db}	14.66 ± 1.86 ^{Ea}
α-copaene	1374	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	46.87±6.01 ^{Fc}	18.78±2.71 ^{Cb}	19.76±3.18 ^{Cb}	23.42±2.06 ^{Db}	13.05±1.08 ^{Bb}	29.19±0.67 ^{Ec}
			DF3	ND ^{Aa}	ND ^{Aa}	30.52±2.44 ^{DEb}	23.67±3.89 ^{Cc}	28.59±4.05 ^{Dc}	30.79±0.21 ^{Ec}	21.66±2.11 ^{Bc}	20.04±3.88 ^{Bb}
Caryophyllene	1417	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	10.37±0.58 ^{Ea}	9.18±1.56 ^{Db}	8.70±0.88 ^{Db}	4.33±0.56 ^{Db}	ND ^{Aa}	5.28±1.02 ^{Cb}
			DF2	ND ^{Aa}	ND ^{Aa}	11.88±1.10 ^{Ba}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	10.55±21.04 ^{Ba}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese			Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
p-cymene	1274	MS, RI	DF1	ND ^{Aa}	77.21±11.07 ^{Ec}	48.12±3.17 ^{De}	51.77±2.27 ^{Ec}	88.65±8.17 ^{Ga}	42.35±10.24 ^{Ga}	26.3±1.65 ^{Be}		
			DF2	ND ^{Aa}	28.94±2.02 ^{Da}	27.96±1.19 ^{Db}	15.37±1.83 ^{Ba}	143.21±10.56 ^{Fb}	90.97±7.09 ^{Ec}	17.86±3.18 ^{Ch}		
			DF3	ND ^{Aa}	57.63±6.59 ^{Fb}	25.29±3.91 ^{Ca}	30.56±2.16 ^{Fb}	90.19±15.15 ^{Ga}	45.60±7.76 ^{Fb}	12.17±1.45 ^{Ba}		
o-cymene	1278	MS, RI	DF1	32.11±2.88 ^{Fb}	29.38±1.96 ^{Da}	ND ^{Aa}	4.89±0.19 ^{Ba}	18.09±1.00 ^{Ca}	71.99±5.40 ^{Ec}	33.35±2.28 ^{Ec}		
			DF2	ND ^{Aa}	73.68±3.06 ^{Ch}	56.76±3.63 ^{Ec}	16.25±1.20 ^{Be}	21.73±2.46 ^{Ch}	29.80±4.60 ^{Fb}	26.43±1.76 ^{Fb}		
			DF3	ND ^{Aa}	99.71±11.05 ^{Gc}	43.22±5.77 ^{Fb}	13.60±6.51 ^{Fb}	18.72±1.39 ^{Da}	20.65±3.09 ^{Ea}	15.52±2.08 ^{Ca}		
Total terpenes (12)			DF1	118.53 ^{Ab}	416.16 ^{Fb}	153.4 ^{Ba}	219.62 ^{Ch}	396.05 ^{Fb}	289.47 ^{Fb}	282.58 ^{Fb}		
			DF2	152.26 ^{Ac}	526.71 ^{Gc}	283.93 ^{Cc}	225.79 ^{Be}	395.23 ^{Fb}	343.19 ^{Ec}	317.52 ^{De}		
			DF3	ND ^{Aa}	303.28 ^{Ea}	207.7 ^{Ch}	184.18 ^{Ba}	286.98 ^{Da}	205.92 ^{Ca}	182.17 ^{Ba}		
Percentage (%)			DF1	32.48	20.60	5.68	6.26	8.02	4.52	5.36		
			DF2	20.33	21.08	8.96	5.78	7.54	5.58	5.68		
			DF3	0.00	10.15	6.99	4.35	5.49	3.49	3.25		
<i>Aldehydes</i>												
Pentanal	840	MS, RI	DF1	11.8±6.79 ^{Fb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	15.45±1.30 ^{Ch}		
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF3	38.06±8.78 ^{De}	36.88±4.50 ^{Fb}	23.43±3.09 ^{Ch}	12.66±1.50 ^{Fb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
3-methyl-butanal	912	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	14.7±3.61 ^{Ba}	16.44±2.30 ^{Ca}	25.19±3.61 ^{Da}	35.19±5.61 ^{Ea}	54.14±8.74 ^{Fa}		
			DF2	ND ^{Aa}	15.60±3.01 ^{Be}	22.03±2.94 ^{Ch}	26.69±4.06 ^{Fb}	32.14±3.92 ^{Fb}	52.14±10.92 ^{Ec}	61.09±10.40 ^{Fb}		

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Production of cheese			Ripening of cheese				
				Cheese ²	Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
2-methyl propanal	1076	MS, RI	DF3	6.66±1.34 ^{Ab}	12.45±2.83 ^{Bb}	23.27±3.81 ^{Cb}	30.08±3.82 ^{Cc}	35.22±4.63 ^{Ec}	45.82±9.63 ^{Fb}	65.81±7.75 ^{Gc}	
			DF1	ND ^{Aa}	37.11±2.24 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	98.66±084 ^{Cb}
			DF3	ND ^{Aa}	14.56±1.90 ^{Eb}	12.26 ±1.15 ^{Db}	7.46±1.34 ^{Cb}	ND ^{Aa}	3.13±0.68 ^{Bb}	8.43±0.98 ^{Cb}	
Hexanal	1171	MS, RI	DF1	ND ^{Aa}	17.28±3.28 ^{Cb}	20.31±0.95 ^{Da}	27.89±1.92 ^{Eb}	10.84±1.55 ^{Ba}	17.04±9.25 ^{Cb}	52.03±7.70 ^{Fb}	
			DF2	ND ^{Aa}	10.27±2.08 ^{Ba}	28.1±2.00 ^{Fb}	25.38±1.90 ^{Ea}	18.80±2.03 ^{De}	16.83±1.05 ^{Cb}	60.50±11.90 ^{Gc}	
			DF3	13.46±7.81 ^{ABb}	17.70±1.68 ^{Cb}	20.86±3.97 ^{Da}	33.47 ±4.56 ^{Ec}	14.07±1.88 ^{Bb}	12.28±1.65 ^{Aa}	49.58±9.78 ^{Fa}	
			DF1	2.51±0.35 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	8.21±3.51 ^{Cb}	
n-octanal	1288	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	1.68±0.97 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.25±0.62 ^{Cb}	
Nonanal	1381	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	47.27±5.64 ^{Db}	ND ^{Aa}	ND ^{Aa}	21.16±3.80 ^{Cb}	ND ^{Aa}	5.34±0.90 ^{Bc}	
			DF1	ND ^{Aa}	38.54±0.79 ^{Cb}	21.91±2.68 ^{Ec}	3.42±0.24 ^{Ba}	6.71±1.10 ^{Cb}	8.22±1.76 ^{Da}	28.61±2.49 ^{Ec}	
Benzaldehyde	1504	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	16.05±1.60 ^{Eb}	5.71±1.27 ^{Cb}	4.18±0.56 ^{Ba}	13.02±0.99 ^{Db}	18.95±1.66 ^{Fa}	
			DF3	6.18±2.09 ^{Ab}	43.33±3.90 ^{De}	8.51±0.97 ^{Ba}	6.65±1.08 ^{Ae}	8.78±0.34 ^{Bc}	24.61±2.89 ^{Gc}	25.38±1.83 ^{Cb}	
			DF1	15.99 ^{Ab}	92.93 ^{Fb}	56.92 ^{Da}	47.75 ^{Ca}	42.74 ^{Ba}	60.45 ^{Ea}	162.69 ^{Gc}	
Total aldehydes (7)			DF2	ND ^{Aa}	25.87 ^{Ba}	66.18 ^{Eb}	57.78 ^{Db}	55.12 ^{Cb}	81.99 ^{Fb}	140.54 ^{Ca}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Production of cheese			Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
Percentage (%)			DF3	64.369 ^{Ae}	172.19 ^{Fe}	88.33 ^{CDe}	90.32 ^{De}	79.23 ^{Bc}	85.84 ^{Cc}	146.11 ^{Eb}	
			DF1	4.64	4.60	2.13	1.35	0.86	0.92	3.02	
			DF2	0.00	1.03	2.07	1.47	1.04	1.34	2.47	
			DF3	29.96	5.76	2.97	2.12	1.50	1.45	2.56	
<i>Hydrocarbones</i>											
Ethane		MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	188.21±10.28 ^{Ec}	168.82±13.54 ^{Db}	ND ^{Aa}	ND ^{Aa}	93.98±2.89 ^{Cc}	58.82±10.54 ^{Bb}	ND ^{Aa}	
			DF3	150.45±8.26 ^{Db}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	54.67±3.33 ^{Bb}	ND ^{Aa}	62.82±12.89 ^{Cb}	
Octane	803	MS, RI	DF1	8.54±2.89 ^{Cb}	7.32±1.10 ^{Bb}	ND ^{Aa}	ND ^{Aa}	11.77±1.60 ^{Db}	ND ^{Aa}	14.68±0.91 ^{Eb}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	16.74±2.55 ^{Cc}	21.86±3.05 ^{Db}	2.06±0.38 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
2-octene-3,7-dimethyl	965	MS, RI	DF1	ND ^{Aa}	133.11±11.36 ^{Eb}	ND ^{Aa}	68.51±4.02 ^{Db}	ND ^{Aa}	33.97±2.86 ^{Cb}	18.44±0.70 ^{Bc}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	259.05±19.56 ^{Fe}	120.56±13.68 ^{Eb}	87.18±5.51 ^{De}	54.41±2.79 ^{Cb}	ND ^{Aa}	6.77±4.78 ^{Bb}	
Decane	1008	MS, RI	DF1	40.28±7.74 ^{Cb}	36.18±2.44 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	100.02±8.23 ^{De}	21.45±6.63 ^{Cb}	5.36±0.14 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
Dodecane	1089	MS, RI	DF1	45.79±3.55 ^{Bb}	50.38±4.49 ^{Cb}	47.3±4.91 ^{Bb}	50.05±2.12 ^{Cb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Toluene	1042	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF1	ND ^{Aa}	12.2±1.13 ^{Bc}	48.90±6.32 ^{Cc}	141.12±9.89 ^{Dc}	233.18±10.65 ^{Ec}	264.40±3.98 ^{Ec}	333.69±8.71 ^{Gb}
			DF2	20.54±6.12 ^{Ch}	4.38±0.95 ^{Ab}	44.3±3.76 ^{Db}	125.47±7.20 ^{Fb}	17.33±1.09 ^{Ba}	120.58±2.27 ^{Ea}	309.14±4.67 ^{Ca}
			DF3	ND ^{Aa}	2.85±1.16 ^{Aa}	34.51±5.78 ^{Ba}	53.66±4.99 ^{Ca}	49.54±1.54 ^{Ch}	206.43±12.57 ^{Dh}	395.7±4.77 ^{Ec}
Benzene	1065	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	13.45±1.37 ^{Bc}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	16.63±1.60 ^{Cb}	10.11±0.91 ^{Bb}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Undecane	1100	MS, RI	DF1	ND ^{Aa}	84.49±3.94 ^{Eb}	22.12±1.98 ^{Db}	ND ^{Aa}	2.88±1.0 ^{Bb}	ND ^{Aa}	6.43±1.0 ^{Cb}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
p-xylene	1149	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	20.11±0.92 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	33.38±0.90 ^{Cb}
			DF2	ND ^{Aa}	ND ^{Aa}	14.73±1.10 ^{Bb}	15.68±2.28 ^{Bb}	20.46±0.98 ^{Dh}	16.85±1.88 ^{Cb}	25.63±1.75 ^{Ea}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	22.17±1.46 ^{Bc}	38.73±0.54 ^{Cc}
Ethyl benzene	1154	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	11.93±1.50 ^{Cb}	9.72±3.37 ^{Bc}	21.91±2.20 ^{Bb}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.43±0.49 ^{Bb}	21.53±3.08 ^{Cb}
o-xylene	1182	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	20.06±4.11 ^{Ch}	18.66±2.07 ^{Cc}	19.50±2.35 ^{Cc}	12.14±1.23 ^{Bb}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese				
					Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Heptane	1201	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	4.74±1.02 ^{Cb}	4.21±0.44 ^{Bb}	ND ^{Aa}	
			DF1	ND ^{Aa}	ND ^{Aa}	68.42±8.27 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	67.22±10.40 ^{Bb}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	ND ^{Aa}	ND ^{Aa}	16.95±2.86 ^{Bb}	19.90±4.70 ^{Cc}	16.36±1.15 ^{Bb}	50.14±3.60 ^{Bb}	14.51±1.11 ^{Bb}	
Styrene	1263	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	30.96±2.03 ^{Ec}	9.57±3.66 ^{Bb}	15.84±2.06 ^{Cb}	35.62±2.14 ^{Fa}	28.52±2.53 ^{Dc}	
			DF3	ND ^{Aa}	ND ^{Aa}	7.21±1.08 ^{Ca}	6.11±1.87 ^{Ba}	12.25±4.97 ^{Da}	35.25±2.66 ^{Ea}	ND ^{Aa}	
			DF1	ND ^{Aa}	47.88±4.00 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
Pentadecane	1504	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	59.73±7.81 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	ND ^{Aa}	13.11±1.21 ^{Ba}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
Hexadecane	1600	MS, RI	DF2	ND ^{Aa}	24.19±0.85 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	144.84±10.14 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	ND ^{Aa}	16.73±0.48 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
Octadecane	1805	MS, RI	DF2	ND ^{Aa}	73.86±5.48 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF1	ND ^{Aa}	27.38±1.43 ^{Bb}	12.03±2.02 ^{Bb}	35.07±1.64 ^{Fc}	9.98±0.87 ^{Ca}	30.54±0.65 ^{Bb}	58.88±4.16 ^{Cb}	
1,2-dichloro benzene	1519	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.17±0.50 ^{Ba}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese		Ripening of cheese					
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
Methane (dimethyl sulfone)	1877	MS, RI	DF2	ND ^{Aa}	ND ^{Aa}	2.72±0.04 ^{Ba}	3.39±0.03 ^{Ba}	19.58±1.75 ^{Ch}	3.75±0.03 ^{Bb}	20.11±1.98 ^{Ch}	
			DF3	ND ^{Aa}	ND ^{Aa}	19.11±14.58 ^{Dc}	5.89±1.24 ^{Bb}	10.16±1.08 ^{Ca}	ND ^{Aa}	29.70±0.87 ^{Ec}	
			DF1	12.88±0.95 ^{Eb}	7.73±0.15 ^{Ch}	10.69±2.27 ^{Db}	4.09±0.35 ^{Bb}	7.80±0.96 ^{Cc}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.14±0.71 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF3	ND ^{Aa}	ND ^{Aa}	14.43±1.59 ^{Dc}	1.42±0.04 ^{Ba}	6.58±0.99 ^{Ch}	6.83±0.83 ^{Ch}	20.71±1.11 ^{Eb}	
Total hydrocarbones (18)			DF1	107.49 ^{Aa}	436.51 ^{Fb}	246.52 ^{Bc}	318.74 ^{Db}	300.63 ^{Cc}	368.01 ^{Ec}	454.08 ^{Ga}	
			DF2	208.75 ^{Dc}	271.25 ^{Fa}	92.71 ^{Aa}	161.25 ^{Ba}	179.12 ^{Ca}	261.88 ^{Ea}	483.32 ^{Gb}	
			DF3	150.45 ^{Ab}	583.23 ^{Ec}	239.13 ^{Db}	161.68 ^{Ba}	192.35 ^{Ch}	311.86 ^{Eb}	635.47 ^{Gc}	
Percentage (%)			DF1	29.45	21.61	9.13	9.09	6.08	5.75	8.61	
			DF2	27.87	10.85	2.93	4.12	3.42	4.26	8.65	
			DF3	54.88	19.53	8.05	3.82	3.68	5.29	11.33	
<i>Miscellaneous</i>											
Diethyl ether		MS, RI	DF1	210.25±15.98 ^{Da}	76.74±8.89 ^{Ab}	133.61±12.43 ^{Ca}	487.19±35.60 ^{Gc}	241.44±9.91 ^{Fb}	226.05±18.51 ^{Ec}	110.25±9.90 ^{Ba}	
			DF2	377.29±10.34 ^{Gb}	68.29±11.20 ^{Aa}	132.67±9.65 ^{Ca}	241.56±19.58 ^{Eb}	289.06±14.69 ^{Fc}	221.47±16.01 ^{Dh}	109.56±10.11 ^{Ba}	
			DF3	458.07±21.56 ^{De}	93.13±7.65 ^{Ac}	184.43±15.51 ^{Ch}	183.75±13.90 ^{Ca}	128.65±10.20 ^{Ba}	97.94±8.39 ^{Aa}	126.74±6.45 ^{Bb}	
2-propanamine		MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	25.78±1.95 ^{Cc}	19.58±2.46 ^{Bc}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	38.9±2.12 ^{Fb}	35.23±5.89 ^{Dh}	19.93±1.19 ^{Ch}	15.48±3.01 ^{Cb}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Cheese ²	Production of cheese			Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120	
2-butanamine		MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.71±1.90 ^{Bb}	ND ^{Aa}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	24.78±2.10 ^{Bb}	ND ^{Aa}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	
N-ethyl 1,3-dithiozindoline		MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	41.5±1.63 ^{Cb}	10.08±0.97 ^{Bb}	71.05±6.79 ^{Dc}	ND ^{Aa}	
			DF2	ND ^{Aa}	ND ^{Aa}	15.87±0.98 ^{Bb}	66.12±16.09 ^{Fe}	26.88±3.10 ^{Dc}	41.92±2.18 ^{Eb}	24.27±1.57 ^{Cb}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	12.57±0.94 ^{Ba}	23.56±4.11 ^{Cb}	
Dimethyl disulphide	1074	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	1.35±0.08 ^{Bb}	6.78±0.15 ^{Cb}	20.15±1.42 ^{Cc}	9.55±3.49 ^{Ba}	14.71±1.31 ^{Ea}	
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.46±1.65 ^{Bc}	17.99±2.10 ^{Cb}	21.16±2.11 ^{Ec}	19.64±3.04 ^{Db}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.38±0.49 ^{Ba}	14.68±1.80 ^{Cb}	20.65±2.56 ^{Db}	
p-cresol (4 methyl phenol)	2089	MS, RI	DF1	ND ^{Aa}	17.99±1.67 ^{Ec}	7.37±0.85 ^{Ca}	1.89±0.77 ^{Ba}	11.1±1.80 ^{Da}	19.42±1.79 ^{Bb}	20.66±1.4 ^{Ea}	
			DF2	ND ^{Aa}	ND ^{Aa}	7.13±0.055 ^{Ba}	7.36±0.62 ^{Bb}	19.65±1.67 ^{Cb}	25.90±3.42 ^{Dc}	51.65±2.87 ^{Eb}	
			DF3	ND ^{Aa}	5.31±0.07 ^{Bb}	23.22±1.15 ^{Eb}	64.05±2.47 ^{Gc}	21.2±5.64 ^{Db}	8.96±3.21 ^{Ca}	59.35±0.94 ^{Ec}	
m-cresol (3 methyl phenol)	2115	MS, RI	DF1	ND ^{Aa}	14.26±1.65 ^{Cb}	6.30±1.16 ^{Ba}	14.4±1.56 ^{Cb}	23.66±0.8 ^{Da}	ND ^{Aa}	38.47±1.6 ^{Ea}	
			DF2	ND ^{Aa}	6.50±2.28 ^{Ba}	10.69±1.03 ^{Cb}	6.23±0.52 ^{Ba}	28.64±1.88 ^{Cc}	17.88±2.92 ^{Db}	87.58±1.68 ^{Ec}	
			DF3	ND ^{Aa}	7.41±1.24 ^{Ba}	25.92±2.24 ^{Dc}	75.49±0.95 ^{Ec}	25.94±1.18 ^{Db}	19.33±3.95 ^{Cb}	72.58±3.13 ^{Eb}	
δ-decalactone		MS, RI	DF1	1.66±0.54 ^{Bc}	ND ^{Aa}	16.74±2.77 ^{Dc}	18.63±3.25 ^{Eb}	4.63±2.14 ^{Cb}	ND ^{Aa}	ND ^{Aa}	
			DF2	0.97±0.09 ^{Bb}	ND ^{Aa}	10.15±2.31 ^{Db}	13.08±1.54 ^{Ea}	11.05±1.46 ^{Ec}	3.86±0.88 ^{Cb}	ND ^{Aa}	
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	26.14±4.50 ^{Bc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds		RI	ID method ¹	Production of cheese		Ripening of cheese				
				Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120
Turmerol	MS, RI	DF1	ND ^{Aa}	20.97±3.06 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF3	ND ^{Aa}	ND ^{Aa}	49.33±4.51 ^{Cb}	12.35±6.26 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Acetamide	MS, RI	DF1	ND ^{Aa}	20.26±0.83 ^{Bc}	ND ^{Aa}	22.86±3.20 ^{Cb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF2	ND ^{Aa}	1.55±0.32 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF3	ND ^{Aa}	ND ^{Aa}	25.47±5.46 ^{Cb}	ND ^{Aa}	2.85±1.13 ^{Cb}	6.1±0.37 ^{Bb}	1.12±0.46 ^{Bb}	
α-cubebene	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF3	ND ^{Aa}	59.11±4.29 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	8.37±1.22 ^{Bb}	12.73±3.18 ^{Cb}	
Aloxiprin	MS, RI	DF1	ND ^{Aa}	12.37±1.13 ^{Cb}	44.85±2.92 ^{Bb}	ND ^{Aa}	2.09±0.69 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Phenyl silane	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF2	235.32±6.38 ^{Cb}	33.67±2.05 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	1.26±1.05 ^{Ab}	
		DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
Oxirane	MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}
		DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	7.82±0.85 ^{Bc}	12.83±1.05 ^{Cb}	12.57±2.04 ^{Cb}	13.01±1.66 ^{Cb}	
		DF3	ND ^{Aa}	ND ^{Aa}	5.76±1.10 ^{Bb}	7.21±0.92 ^{Cb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}

Supplementary Table 1. Relative abundance of volatile compounds ($\mu\text{g/kg}$) isolated from Divle Cave cheese during production and ripening (mean $\pm$ SD)

Compounds	RI	ID method ¹	Production of cheese			Ripening of cheese					
			Raw milk	Curd	Day 1	Day 30	Day 60	Day 90	Day 120		
1-Octanamine		MS, RI	DF1	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	27.42 $\pm$ 2.62 ^{Bb}	ND ^{Aa}	ND ^{Aa}		
			DF2	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}		
			DF3	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	14.97 $\pm$ 2.65 ^{Bb}	ND ^{Aa}		
Total Miscellaneous (15)		DF1	211.91 ^{Ca}	162.59 ^{Ab}	210.22 ^{Cb}	593.27 ^{Cc}	347.3 ^{Bb}	326.07 ^{Db}	184.09 ^{Ba}		
		DF2	613.58 ^{Cc}	109.00 ^{Aa}	176.51 ^{Ba}	349.64 ^{Ba}	406.10 ^{Cc}	395.33 ^{Cc}	326.55 ^{Cb}		
		DF3	458.07 ^{Cb}	164.96 ^{Ab}	314.13 ^{De}	407.89 ^{Fb}	219.25 ^{Ca}	202.84 ^{Ba}	332.20 ^{Fb}		
Percentage (%)		DF1	0.45	4.25	2.81	2.68	2.21	1.63	1.49		
		DF2	31.54	1.26	1.29	2.58	2.23	2.85	4.01		
		DF3	0.00	2.08	4.27	5.05	1.73	1.78	3.49		

Mean $\pm$ SD values of the two replicate productions for each dairy farm analysed in triplicate, expressed as relative abundance to internal standard.

¹ Method of identification: MS, Mass spectrometry using the libraries of Wiley, NIST and Flavor; RI, retention index of n-alkanes (C8-C20) under the same chromatographic conditions

² Cheese produced in DF1: Dairy Farm 1, DF2: Dairy Farm 2, DF3: Dairy Farm 3

³ Percentage (%): percentage of total volatile compounds of each group in each ripening stage of cheese

ND: not detected

^{A-G}Superscripted upper case letters in the same row show the significant differences between ripening times (P<0.05)

^{a-c} Superscripted lower case letters in the same column show the significant differences among different dairy farms (P<0.05)

CHAPTER 5

Protease and lipase activities of fungal and bacterial strains derived from an artisanal raw ewe's milk cheese

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Abstract

We previously identified the microbiota present during cheese ripening (Chapter 2, Ozturkoglu Budak et al., 2016a) and observed high proteolytic and lipolytic activity in Divle Cave cheese (Chapter 3). Here we analysed a panel of species representing this microbiota for their proteolytic and lipolytic ability. In total, 17 fungal, 5 yeast and 18 bacterial strains, previously isolated from Divle Cave cheese, were included in this analysis. Qualitative protease and lipase activities, were performed on skim-milk agar and spirit-blue lipase agar, respectively and resulted in a selection of strains for quantitative assays. For the quantitative assays, the strains were grown on minimal medium containing irradiated Divle Cave cheese, obtained from the first day of ripening. Out of 16 selected filamentous fungi, *Penicillium brevicompactum*, *Penicillium cavernicola* and *Penicillium olsonii* showed the highest protease activity, while *Mucor racemosus* was the best lipase producer. *Yarrowia lipolytica* was found to be the best yeast with respect to protease and lipase activity. From the 18 bacterial strains, 14 and 11 strains showed positive proteolytic and lipolytic reactions in agar plates, respectively. *Micrococcus luteus*, *Bacillus stratosphericus*, *Brevibacterium antiquum*, *Psychrobacter glacincola* and *Pseudomonas proteolytica* displayed the highest protease and lipase activity. The proteases of yeast and filamentous fungi were identified as mainly aspartic protease by specific inhibition with Pepstatin A, whereas inhibition by PMSF indicated that most bacterial enzymes belongs to serine type protease. Our results demonstrated that milk-clotting enzymes providing proteolysis (mainly aspartic proteases) during ripening are predominantly derived from fungal strains, and therefore fungal enzymes appear to be more suitable enzyme for employment in cheese industry.

Introduction

Proteases catalyze the hydrolysis of peptide bonds in proteins molecules resulting in peptides and aminoacids. Proteases are among the most important industrial enzymes and account for over 65% of the total global sales of industrial enzymes (Hsiao et al., 2014; Rao et al., 1998). They have applications in detergent, pharmaceutical and biotechnological industries, as well as food and dairy industry (Sabotic & Kos, 2012; Yegin et al., 2011). During cheese manufacture, proteases are commonly used as milk-clotting agents and affect cheese quality by improving the taste, flavor and functional properties (Yegin et al., 2011).

Proteases can be categorized depending upon the aminoacids present in the active sites such as aspartic, serine and cysteine or as metalloprotease if a metal ion is required for catalytic activity (Sumantha et al., 2006). They are present in all living beings and are widely distributed in nature (Sumantha et al., 2006), while mainly secreted by fungi and bacteria (Quintero & Bermudes, 2014), in a variable ratio from one strain to another (Boutrou, Kerriou, et al., 2006).

Lipases are another important group of enzymes with a considerable physiological significance and industrial potential. Lipases mainly catalyze the hydrolysis of fats and oils to glycerol and fatty acids (Mohamed et al., 2011). They are produced by various plants, animals and microorganisms. Lipases from microbial origin, bacterial and fungal, are most commonly used in biotechnological applications (Ülker & Karaoglu, 2012). The most commonly used bacterial genera for lipase applications are *Bacillus*, *Arthrobacter* and *Pseudomonas*. Filamentous fungi are also used as a source of lipase as they are able to produce higher amounts of extracellular lipases (Saxena et al., 1999) and the most commonly used fungal genera are *Mucor*, *Penicillium*, *Aspergillus*, *Geotrichum* and *Rhizopus* (Ghosh et al., 1996). The most commonly used species from the genus *Penicillium* are *P. cyclopium*, *P. brevicompactum*, *P. chrysogenum*, *P. crustosum*, *P. roqueforti* and *P. verrucosum* (Li & Zong, 2010). Similar to proteases, lipases have also been widely applied in detergent, pharmaceutical, food and dairy industries, especially during cheese ripening (Gupta et al., 2004; Hasan et al., 2006). Lipases are the third largest group of enzymes based on total sales market, following proteases and carbohydrases (Liu et al., 2008).

The unique character of artisanal dairy products is partly due to the large number of microorganisms and enzymes associated with raw milk. Specific microorganisms and natural starters derived from raw milk contribute to the sensory properties and quality of cheeses (Chaves-Lopez et al., 2006). Raw milk and associated microorganisms were analysed as a source of proteases and lipases. Specifically, psychrotrophic bacteria isolated from raw milk were examined to determine their spoilage potential during storage and refrigeration (Baur et al., 2015; Champagne et al., 1994; Hantsis-Zacharov & Halpern, 2007; Munsch-Alatossava & Alatossava, 2006).

Many studies addressed the production, purification and characterisation of an individual protease or lipase produced by a bacterial or fungal strain. The objective of these studies was generally to determine the potential of the enzyme for specific applications. In relation to dairy applications, the proteolytic activity of *P. roqueforti* strains were confirmed to contribute to the texture and organoleptic properties of different varieties of blue cheeses (Fernandez-Bodega et al., 2009). Also, synergistic protease activities of *Geotrichum candidum* and *P. camamberti* were demonstrated in mixed cultures of Camambert cheese whey, which showed the contribution of proteolytic activities of these strains in soft cheese ripening (Boutrou, Aziza, et al., 2006). In addition, the presence of proteolytic and lipolytic enzymes produced by different yeast strains such as *Candida zeylanoides*, *Y. lipolytica* were reported in dairy products and *C. zeylanoides* was found to show the highest lipase activity among the yeasts isolated from milk and dairy products (Corbo et al., 2001b). However, proteases and lipases also have applications in other industrial processes. Proteases from *Debaryomyces hansenii* have a particular role in food fermentation processes and accelerated the proteolysis in fermented meat products (Bolumar et al., 2008), while purified proteases from *Bacillus cereus* were applied in laundry detergents (Banik & Prakash, 2004).

Despite the many studies on protease and lipase production from fungi and bacteria, no studies have been reported that specifically attempt to produce enzymes from the associated microorganisms of the original cheese substrate to improve its utilization mainly during ripening. Divle Cave cheese is an artisanal raw milk cheese ripened in a cave in goat skin bags and during ripening a variety of yeasts, filamentous fungi and bacteria was observed (Chapter 2, Ozturkoglu Budak et al., 2016a). These

cheese microorganisms derived from raw milk, environment and cave develop and contribute to the sensory properties such as aroma and flavour of the cheese (Chapter 4, Ozturkoglu-Budak et al., 2016b), in part by releasing extracellular enzymes such as proteases and lipases. Fungi growing on the surface of goatskin bags usually secrete their enzymes into the inner part of cheese.

The aim of this study was to determine protease and lipase production by yeasts, filamentous fungi and bacteria previously isolated from Divle Cave cheese. To simulate the real ripening conditions, the enzyme activities were quantified in submerged cultivations of the isolates in medium made from irradiated cheese at conditions adapted from cheese ripening. The effects of protease inhibitors were also monitored in terms of the inhibition of the proteolytic activities of strains to determine the class of proteases.

Materials and methods

Strains and growth conditions

17 filamentous fungi, 5 yeast and 18 bacteria which are most frequently isolated from Divle Cave cheese in our previous study (Ozturkoglu Budak et al., 2015) were used to examine their proteolytic and lipolytic activities. Yeast and fungal strains were grown on Malt Extract Agar (Merck, Darmstadt, Germany) and incubated at 28°C for 5-7 days until good sporulation had occurred. Bacterial strains were grown on Tryptic Soya Agar (Merck, Darmstadt, Germany) at 35°C for 48 h.

Fresh yeast cells and fungal spores were harvested by gentle agitation in 10 ml ACES (acid buffer). Cell and spore counts of suspensions were determined using a haemocytometer (Burker-Turk, Brand, Germany) under a microscope (Axioplan, Zeiss, Jena, Germany). Microbial density of bacterial cells were also measured at 660 nm using a UV-visible spectrophotometer (Perkin-Elmer, Massachusetts, US). Cell and spore suspensions of yeast and fungal strains were adjusted to 5.10^5 cells/ml and spores/ml with ACES. Bacterial cells were adjusted to 1.10^6 cfu/ml with physiological saline.

Utilization of carbohydrates by fungal strains were performed by growth tests using minimal medium (MM) prepared according to (de Vries et al., 2004). Carbon sources

were added to a final concentration of 25 mM for D-glucose and lactose and 1% for casein (Sigma-Aldrich, Germany). Plates of all media contained 1.5 % agar. Strains were inoculated with 2 μ L of spore suspensions (500 spores/ μ L) prepared before and incubated at 15°C for 5 days.

Qualitative enzymatic activities in plates

Protease plate assay

The qualitative evaluation of proteolytic activities of all strains were determined on skim-milk agar plates, prepared with 10% skim-milk powder (Oxoid, Hampshire, UK), 1% yeast-extract (Merck, Darmstadt, Germany) and 2% agar (Merck, Darmstadt, Germany). Plates inoculated with fungal and bacterial strains were incubated at 15°C for 10 and 7 days, respectively. A lower temperature (15°C) than for normal cultivation (30°C) was used to mimic the low temperature of cave in which the cheese is produced. The activity was evaluated after incubation by the formation of a transparent halo around the colonies, as a result of the hydrolysis of milk proteins (Mayerhof et al., 1973). The strains showing such a halo were selected for the quantitative protease assays.

Lipase plate assay

Both fungal and bacterial strains were evaluated in terms of their lipolytic activities on Spirit Blue agar (Sigma-Aldrich, St. Louis, US) which includes the pancreatic digest of casein (1%), yeast extract (0.5%), agar (2%) and spirit blue (0.02%). Lipase reagent (Sigma-Aldrich, St. Louis, US) containing tributyrin and triglycerides, was added into the sterilized medium aseptically. Plates inoculated with fungal and bacterial strains were incubated at 15 °C for 10 and 7 days, respectively. The lipolysis was assessed with halos indicating that microorganisms metabolized the lipids.

Quantitative enzymatic activities

Culture medium preparation

Five kilograms of homogenized cheese from three independent batches were mixed, sterilized and used throughout the study. To sterilize the cheese samples gamma irradiation (9.36 kG) was applied for 8h by the Turkish Atomic Energy Authority, Saraykoy Nuclear Research and Training Center of Turkey.

Culture medium for strain inoculation was prepared by mixing 5 g irradiated cheese and 10 ml MM. The prepared slurries were poured into plates and inoculated with spore suspensions of 5.10^5 spore/ml of each fungal strain and cell suspension of 1.10^6 cells/ml for bacterial strains. To determine the extracellular enzyme production, plates inoculated with yeast and fungi were incubated at 15°C for 10 days, while the plates were incubated for 7 days at 15°C for bacteria. As shown in Figure 1, the liquid phase under the solid fungal layer was taken from the plates and centrifuged at $14000 \times g$ for 10 min (Eppendorf, UK). The supernatant was separated after centrifugation and stored at -20°C until the measurements of protease activity. The same stages and incubation period were applied to a negative control plate without any inoculation and no colonies observed during the same incubation period. Duplicate inoculations were carried out and experiments were repeated in triplicate, throughout the study.

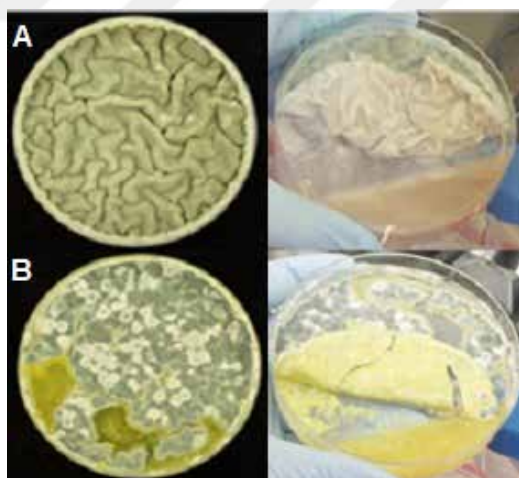


Figure 1. Growth of strains on cheese culture medium for quantitative assay and separation of the liquid phase under the solid fungal layer. **A.** *P. brevicompactum*, **B.** *P. chrysogenum*.

Quantitative protease activity

The quantitative protease activity assay was performed according to the procedures mentioned in the protocol of the Pierce Fluorescent Protease Assay Kit (Pierce Biotechnology, Thermo Fischer Scientific, USA). The level of protease activity in the supernatants of all strains over 72 h were quantified using a fluorescein isothiocyanate (FITC)-labeled casein assay according to the manufacturer's instructions. Relative fluorescence of the samples were measured by optical density (OD) using a plate reader (Fluostar Optima, BMG LABTECH) with excitation at 485 nm and emission

at 530. A standard curve of trypsin was prepared at the following concentrations: 0.25, 0.50, 1.0, 2.0, 3.0, 4.0 $\mu\text{g/ml}$ and used for conversion of relative fluorescence units (RFU) to protease activity ($\mu\text{g protease/ml}$). Control sample value (3.32 $\mu\text{g/ml}$) which represented the proteases arising from raw milk and cheese were subtracted from measurements of culture filtrates. pH 5 was selected for protease activity measurements due to the pH value of Divle Cave cheese which was in the range of 4.85 and 5.19 during ripening.

Quantitative lipase activity

The quantitative lipase activity was evaluated by the Lipase Activity Assay Kit (Sigma-Aldrich, St. Louis, US) according to the manufacturer's instructions. The absorbance of the culture supernatants were measured by optical density (OD) using a plate reader (Fluostar Optima, BMG LABTECH) at 570 nm to determine lipase activity. The enzyme activity was expressed as $\text{nmole/min/ml} = \text{milliunits/mL}$ in which one unit of lipase is the amount of enzyme that generates 1 μmol of glycerol released from triglycerides per minute at 37°C .

SDS-polyacrylamide gel electrophoresis (PAGE)

Supernatants obtained from the culture mediums were separated with SDS-PAGE. This detection provided information on the production of proteases by each strain with showing the cleavage of the proteins present in cheese. Culture medium supernatant without inoculation of any microorganism was used as control for detection of the native proteins present in milk and cheese.

12% running gel and 5% stacking gels were used during analyses. 12% separating gel was prepared according to Laemmli (1970) with some modifications. Gel was including 30% acrylamide-bismonomer, 1.5 M Tris-HCl (pH 8.8), 10% SDS, 10% ammonium persulphate and TEMED. The stacking gel (4.8%) was made using 0.5 M Tris-HCl (pH 6.8), 20% SDS, 10% ammonium persulphate and TEMED. The samples were diluted to 1:4 in distilled water and 30 μL were mixed with 10 μL loading buffer (12% glycerol, 1.2% SDS, 5.4% b-mercaptoethanol and saturated bromophenol blue) and boiled at 100°C for 5 min in a dry block heater. The boiled samples were kept on ice before loading on the gel. 7 μL of sample was loaded onto the gel with loading buffer.

Electrophoresis was performed in Mini Protean-Tetra Cell (Bio-Rad, Hercules, California, USA) with a voltage of 50 V for stacking and 100 V for separation. After fixing the gel with 45% (v/v) methanol and 10% (v/v) acetic acid, bands were visualized by silver staining (Thermo Fischer Scientific, USA). The gel was washed with distilled water until the background was clear prior to view. Proteins were identified according to the molecular weight marker (BioRad, Precision Plus Protein Unstained Standard) with recombinant prestained protein standards (BioRad, California, USA).

Inhibition of proteases

The effect of protease inhibitors bestatin, pepstatin A, PMSF and EDTA on proteolytic activity gave an estimation to determine the class of proteases produced from each microorganism. Protease inhibitors were prepared to give final concentrations of 50 mM for PMSF and EDTA, 1 mM for Pepstatin A and Bestatin as instructed by the manufacturers (Sigma-Aldrich, St. Louis, US). 2 μ L protease inhibitors were added to the assay mixture and incubated for 60 min at room temperature prior to performing the assay. Non-treated culture supernatants were used as control. Each measurement was performed in triplicate at pH 5.

Results and Discussion

Qualitative protease and lipase activities of fungal and bacterial strains

Five yeast, 17 fungal and 18 bacterial strains isolated from Divle Cave cheese were selected and tested to determine their proteolytic and lipolytic activities by employing a screening technique on agar plates containing skim-milk and tributyrin/triglycerides as substrate, respectively. The fungal and bacterial strains with the largest halos in skim-milk and spirit blue lipase agar plates are presented in Figure 2 and Figure 3, respectively. In Figure 2, the transparent halo indicated the hydrolysis of milk protein, while in Figure 3 halos occurred as a result of lipase activity.

Protease and lipase production varied greatly among the strains (Table 1). As an example, from the five yeast strains, only *Y. lipolytica* and *D. hansenii* produced proteases. Out of 17 fungal strains, 16 produced halos of variable sizes in plates, while the highest protease production was observed in three strains; *P. brevicompactum*, *P. olsonii* and *P. cavernicola*. From the 18 bacterial strains 14 showed protease halos. The main bacterial protease producers were *Micrococcus luteus*, *Bacillus stratosphericus*, *Brevibacterium antequum* and *Staphylococcus equorum subsp. equorum*.

All tested yeast and fungal strains produced lipase. The highest extracellular lipase activity for the yeasts was detected for *Y. lipolytica*, while *P. cavernicola*, *P. biforme*, *P. olsonii*, *P. cyclopium*, *P. brevicompactum*, *P. roqueforti* and *P. commune* were the best fungal lipase producers. The best bacterial strains were *B. stratosphericus*, *B. antiquum*, *P. glacincola* and *P. proteolytica* (Table 1).

Table 1. The main ptease and lipase producer strains according to qualitative plate assays

Class	Species	GenBank Accession Number	Halo on Skim-milk agar	Halo on Lipase Agar
Yeast				
	<i>Yarrowia lipolytica</i>	AF_335977	medium (++)	very strong (+++++)
	<i>Debaryomyces hansenii</i>	AF_210327	weak (slight)	medium (+++)
	<i>Kluyveromyces lactis</i>	NR_131273	no zone	medium (++)
	<i>Candida zeylenoides</i>	AF_335977	no zone	medium (++)
	<i>Geothricum candidum</i>	KF_112070	weak (slight)	weak (slight)
Filamentous Fungi				
	<i>Penicillium olsonii</i>	AY_674444	strong (+++++)	strong (++++)
	<i>Penicillium brevicompactum</i>	AY_674437	strong (+++++)	strong (++++)
	<i>Penicillium cavernicola</i>	KJ_834439	strong (++++)	very strong (+++++)
	<i>Penicillium cyclopium</i>	AY_674309	medium (+++)	strong (++++)
	<i>Penicillium roqueforti</i>	AY_674381	medium (+++)	strong (++++)
	<i>Penicillium rubens</i>	JX_091546	medium (+++)	strong (++++)
	<i>Penicillium commune</i>	AY_674361	medium (++)	strong (++++)
	<i>Penicillium biforme</i>	FJ_930951	weak (+)	strong (+++++)
	<i>Penicillium chrysogenum</i>	AY_495981	medium (++)	medium (+++)
	<i>Penicillium corylophilum</i>	JX_141042	medium (++)	medium (+++)
	<i>Penicillium spinulosum</i>	HM_103380	medium (+++)	medium (+++)
	<i>Penicillium polonicum</i>	AF_001206	weak (+)	medium (++)
	<i>Penicillium crustosum</i>	FJ_004401	weak (+)	medium (+++)
	<i>Penicillium fuscoglaucum</i>	FJ_930977	weak (+)	medium (+++)
	<i>Mucor racemosus</i>	FJ_345353	weak (slight)	medium (++)
	<i>Mucor flavus</i>	JN_206467	weak (slight)	weak (+)
	<i>Trichoderma</i> spp.	GQ_221185	no zone	weak (+)

Class	Species	GenBank Accession Number	Halo on Skim-milk agar	Halo on Lipase Agar
Bacteria				
	<i>Bacillus stratosphericus</i>	NR_042339	very strong (+++++)	strong (++++)
	<i>Micrococcus luteus</i>	NR_075062	very strong (+++++)	medium (+++)
	<i>Brevibacterium antiquum</i>	NR_029079	strong (++++)	strong (++++)
	<i>Staphylococcus equorum</i> subsp. <i>equorum</i>	NR_027520	strong (++++)	medium (++)
	<i>Lactobacillus paraplantarum</i>	NR_025447	medium (+++)	medium (+++)
	<i>Psychrobacter glacincola</i>	NR_042076	medium (+++)	medium (++)
	<i>Enterococcus faecium</i>	NR_114742	medium (++)	no zone
	<i>Lactobacillus brevis</i>	NR_116238	medium (++)	no zone
	<i>Brachyбактерium tyrofermentans</i>	NR_026272	medium (++)	medium (++)
	<i>Brevibacterium</i> spp.	AM_398220	medium (++)	medium (++)
	<i>Pseudomonas proteolytica</i>	NR_025588	medium (+++)	no zone
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	NR_113960	weak (+)	weak (+)
	<i>Arthrobacter arilaitensis</i>	NR_074608	weak (+)	weak (slight)
	<i>Staphylococcus warneri</i>	NR_025922	weak (slight)	no zone
	<i>Solibacillus silvestris</i>	NR_028865	no zone	no zone
	<i>Microbacterium halotolerans</i>	NR_036935	no zone	medium (+++)
	<i>Kocuria salsicia</i>	NR_117299	no zone	no zone
	<i>Lactobacillus coryniformis</i>	NR_029018	no zone	no zone

The fungal and bacterial strains with the largest halos in skim-milk and spirit blue lipase agar plates are presented in Figure 2 and Figure 3, respectively. In Figure 2, the transparent halo indicated the hydrolysis of milk protein, whereas halos in Figure 3 represent lipid metabolism by lipase producer/lipolytic microorganisms.

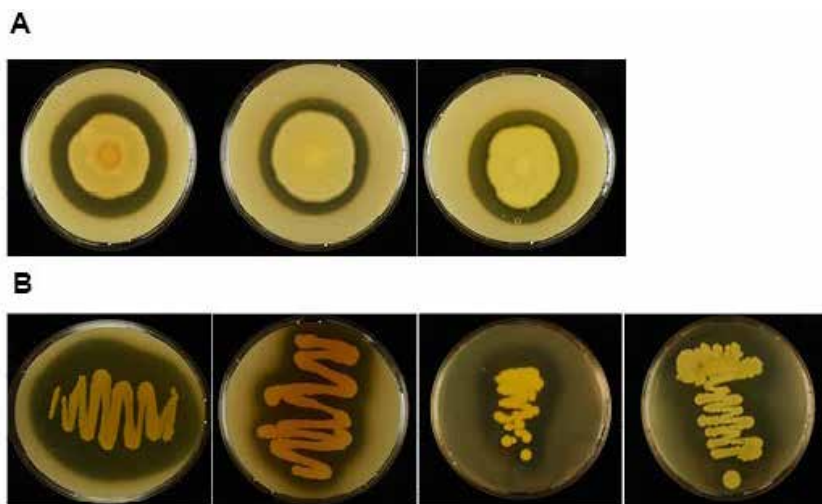


Figure 2. Fungal and bacterial strains showing the largest halos in skim-milk agar. **A.** Fungal strains from left to right: *Penicillium brevicompactum*, *Penicillium olsonii*, *Penicillium cavernicola*. **B.** Bacterial strains from left to right: *Micrococcus luteus*, *Brevibacterium anticum*, *Staphylococcus equorum*, *Bacillus stratosphericus*.

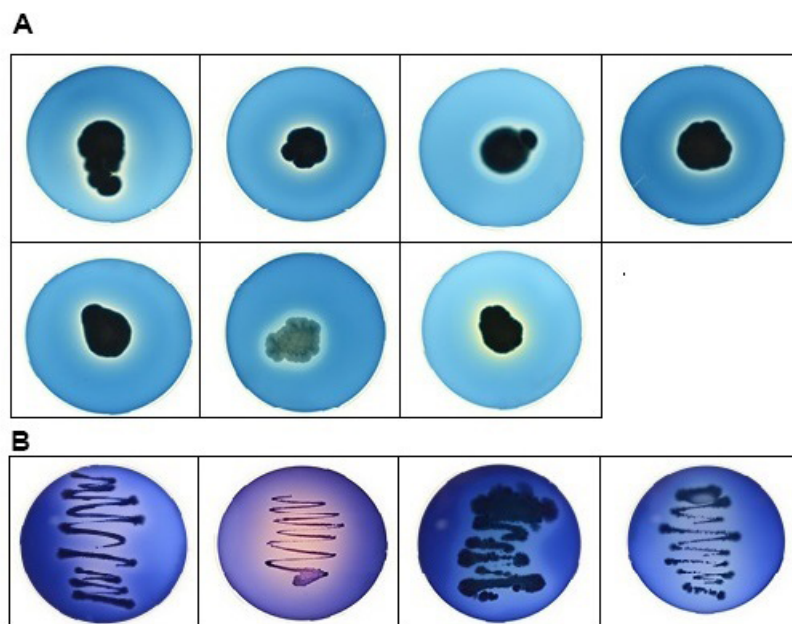


Figure 3. Fungal and bacterial strains showing the largest halos on spirit blue lipase agar. **A.** Fungal strains from left to right: *Penicillium rubens*, *Penicillium bifforme*, *Penicillium cavernicola*, *Penicillium cyclopium*, *Penicillium roqueforti*, *Yarrowia lipolytica* and *Penicillium corylophilum*. **B.** Bacterial strains from left to right: *Bacillus stratosphericus*, *Brevibacterium anticum*, *Psychrobacter glacincola*, *Pseudomonas proteolytica*.

The qualitative enzyme activities of different microorganisms in dairy products were investigated previously (Hantsis-Zacharov & Halpern, 2007; Marchand et al., 2009; Suzzi et al., 2001). Visualising enzyme activity in agar plate technique provides information in a relatively fast manner, however the quantification of enzyme activity is not possible. Moreover, microorganisms may show different growth characteristics on solid media than by growth as a submerged culture (Baur et al., 2015). According to Hantsis-Zacharov & Halpern (2007), among the strains isolated from raw milk *Microbacterium* spp. was highly lipolytic and proteolytic, *Pseudomonas* spp. showed mainly lipolytic activity and the lactic acid bacteria displayed very minor enzymatic ability. Suzzi et al. (2001) found that various strains of *Y. lipolytica* isolated from different cheese types showed variations in terms of lipase activity. However, the authors concluded that biochemical activities of *Y. lipolytica* strains can play important roles in the organoleptic features of cheeses, due to the production of aromatic compounds and/or their precursors such as methyl ketones, alcohols, lactones and esters.

Quantitative activities of fungal strains

To confirm the results of the qualitative assay and to quantify the enzyme amounts, strains showing positive reactions on plates were selected for quantitative assays to identify the strains with the highest proteolytic and lipolytic activity.

Protease activities

The fungal and yeast strains that were selected for quantitative protease assays are indicated in Figure 4A. Highly diverse levels of protease activity were observed for the yeasts and filamentous fungi. The highest protease activity was observed for *P. brevicompactum* (14.19 µg/ml), which was also one of the strains showing the largest halo in skim-milk agar plates. *P. brevicompactum* protease activity was followed by *P. corylophilum* (13.44 µg/ml) and *P. chrysogenum* (11.85 µg/ml). Similar to most microbial strains, *G. candidum* produced proteolytic enzymes, with variable activity from one strain to another. However, the extracellular proteolytic activity of *G. candidum* has been reported to be low compared to its intracellular activity (Desmaures, 2014). The yeasts *Y. lipolytica* and *D. hansenii*, showed a low enzymatic activity compared to most of the fungal strains.

Similar to our results, *P. brevicompactum* was isolated from coffee fruit in a previous study and a high proteolytic activity of that strain was determined quantitatively (Rodarte et al., 2011). Moreover, *P. roqueforti* was reported to have the highest proteolytic activity in blue cheese and it was suggested that this strain might be an industrial starter (Fernandez-Bodega et al., 2009).

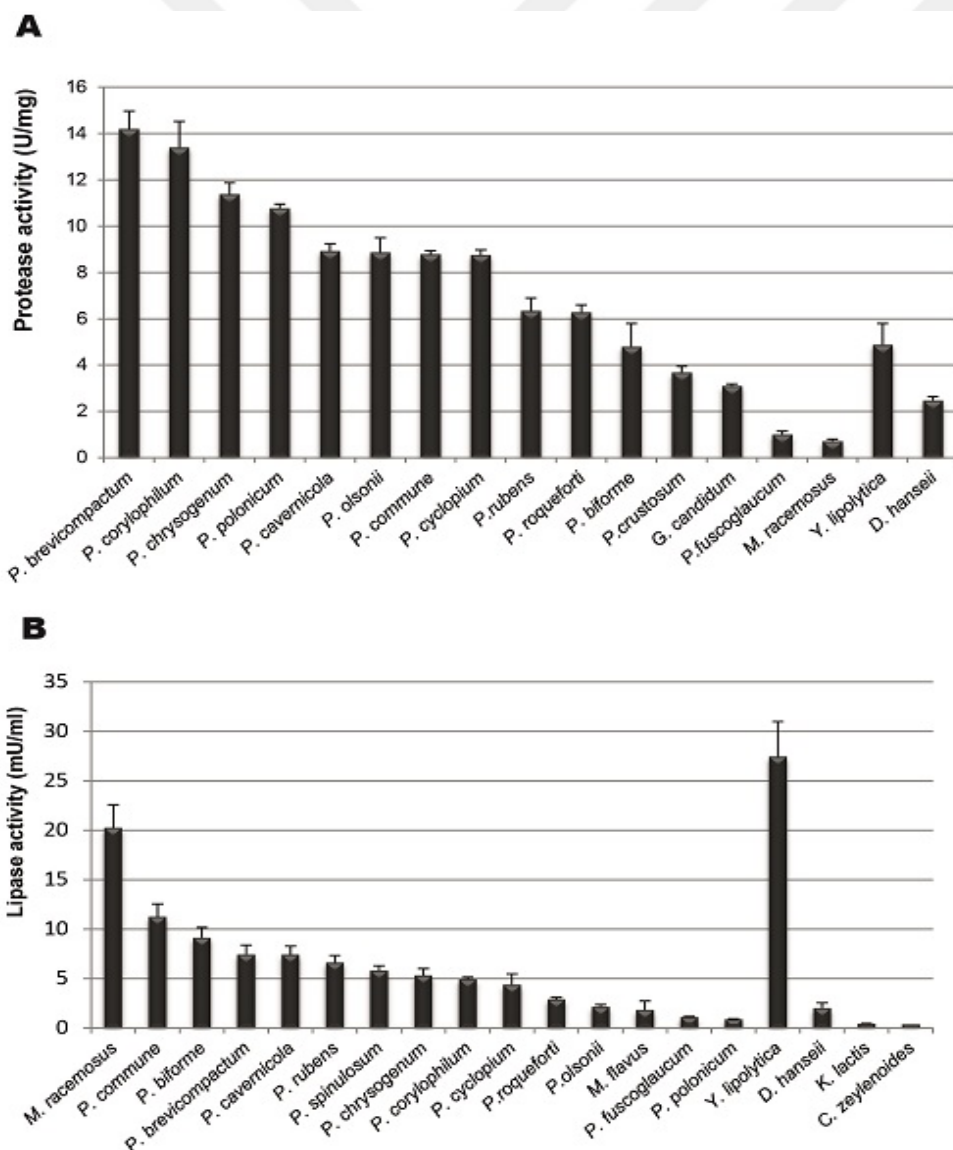


Figure 4. Quantitative determination of protease (A) and lipase (B) activities of yeasts and filamentous fungi.

Some differences were identified between the results of the qualitative and quantitative protease assays. The reason of those differences are thought to be that medium prepared from irradiated cheeses includes some ingredients peculiar to Divle Cave cheese which are absent in ready-to-use mediums used for qualitative assay. The presence of original cheese could stimulate growth and enzyme production of certain microbial strains that adapted to that medium. The differences between two media used in qualitative and quantitative assay could also induce the enzymes at different levels. According to the growth profile of fungal strains, casein was found to promote the growth of strains much more than lactose and glucose (Figure 5). Furthermore on casein-added media production of extrudates with different colours were observed for several strains, e.g. *P. brevicompactum*, *P. chrysogenum*, *P. commune*, *P. corylophilum* and *P. olsonii* (Figure 1). Moreover, during the quantitative assays performed in submerged cultivations, incubations were simulated to the available conditions of cave such as low temperature (10-15°C), due to the strains were cold-adapted during ripening.

Lipase activities

All the fungal and yeast strains displayed positive reactions on lipase plate and were selected for the quantitative lipase assay (Figure 4B). *M. racemosus* was the fungal strain showing the highest lipase production (20.26 miliunits/ml) followed by *Penicillium* spp., while among the yeast strains *Y. lipolytica* was the best lipase producer (27.44 miliunits/ml), and was even better than the fungal strains. Other studies also showed that specific *Mucor* strains such as *M. racemosus* (Mohamed et al., 2011) and *M. hiemalis* isolated from soil (Ülker & Karaoğlu, 2012), produced high lipase activity. In another study performed on soil isolates, *P. chrysogenum* was reported to be the best lipase producer (Bancerz et al., 2005). Yeasts such as *Candida*, *Kluyveromyces* and *Pichia* were isolated from raw milk as a good lipase producers (Cocolin et al., 2002). *Y. lipolytica* has also been reported as a producer of both extracellular protease and lipase (Suzzi et al., 2001). However, while in our study *C. zeylenoides* was determined as the lowest lipase producer, another study reported that *C. zeylenoides* showed the highest lipase activity among all the strains isolated from raw milk (Baur et al., 2015; Corbo et al., 2001a). This difference could be due to strain differences and cultivation differences, demonstrating the importance of analyzing the actual strains that were isolated from the cheese that is being studied.

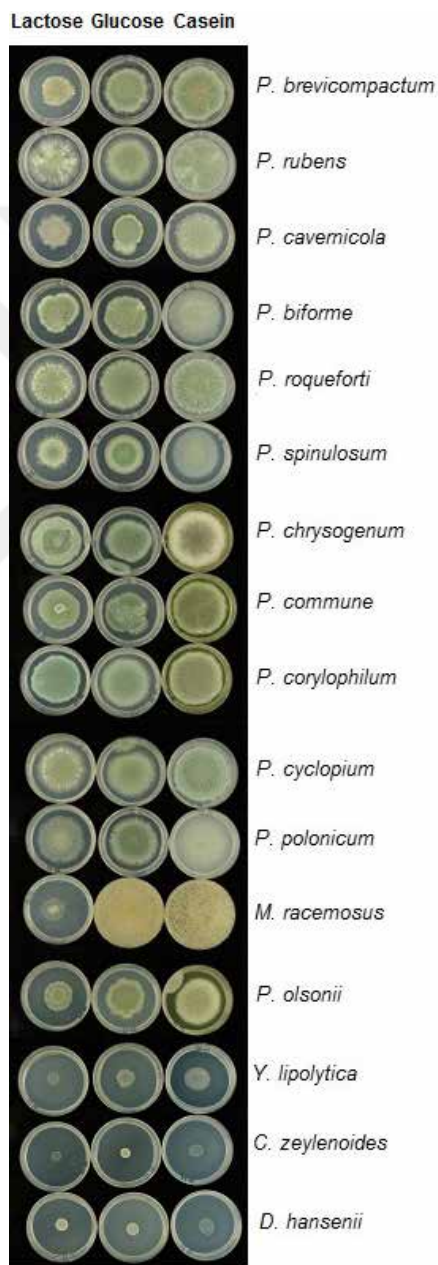


Figure 5. Growth of fungal strains on various carbon sources.

Protein profiles of fungal strains

SDS-PAGE profiles allow the visualization the protein sets produced by the fungi as well as the degradation of large and medium size peptides (Boutrou, Aziza, et

al., 2006). In our study, the intensity of the protein patterns of *P. corylophilum*, *P. chrysogenum*, *P. brevicompactum* and *P. polonicum* strains were significantly lower than in the control. Specifically, the bands corresponding to 20-40 kDa peptides had almost disappeared at the end of the culture (Figure 6). The intensity of bands higher than 50 kDa remained largely unaltered in most culture supernatants except for *P. corylophilum* and *P. polonicum*. Reduction of specific bands indicated a higher protease activity in the respective culture supernatant. The SDS-PAGE results are in agreement with quantitative assay results, as both methods indicate *P. corylophilum*, *P. brevicompactum*, *P. polonicum* and *P. chrysogenum* as the strongest protease producers.

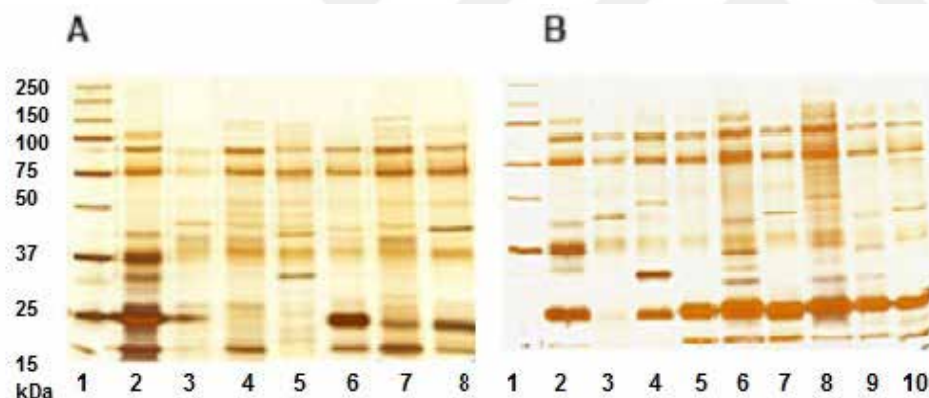


Figure 6. SDS-PAGE profiles of fungal strains. **A.** 1: Ladder, 2: Control, 3: *P. corylophilum*, 4: *P. chrysogenum*, 5: *P. brevicompactum*, 6: *P. cavernicola*, 7: *P. commune*, 8: *P. cyclopius*; **B.** 1: Ladder, 2: Control, 3: *P. polonicum*, 4: *P. olsonii*, 5: *P. rubens*, 6: *P. biforme*, 7: *P. roqueforti*, 8: *M. racemosus*, 9: *P. fuscoglaucum*, 10: *P. crustosum*.

Effect of protease inhibitors on proteolytic activity of fungal strains

To characterize the proteases produced by microbial strains, the effects of protease inhibitors such as Bestatin, Pepstatin A, PMSF and EDTA were determined on enzyme activity (Figure 7). Protease activity of most yeasts and filamentous fungi was strongly inhibited by Pepstatin A, which suggests that the majority of their proteases were aspartic (acid) proteases. Reduced activity in the presence of PMSF revealed that some strains also produced serine proteases. Metalloprotease activity was only detected for *P. polonicum* as for this strain both EDTA and PMSF reduced the protease activity. *P. rubens* and *D. hansenii* were the only strains where the overall protease activity was reduced in the presence of Bestatin, which indicated that these strains produced a significant amount of aminopeptidases.

It has also been previously determined that fungal strains are good microbial sources of aspartic and serine proteases. Production of aspartic proteases from *Penicillium* spp. (Hashimoto et al., 1973), *Mucor* spp. (Yegin et al., 2011) and *Rhizopus oryzae* (Hsiao et al., 2014) were reported, while serine proteases have been extracted from various filamentous fungi such as *Penicillium*, *Aspergillus* and *Mucor* spp. (Ramírez-Zavala et al., 2004).

Milk clotting requires enzymes that cleave the bovine- κ -casein Phe105-Met106 bond and belong to the class of aspartic proteases (Llorente et al., 2014; Visser, 1993). The utilization of aspartic proteases from microbial sources as milk clotting enzymes during cheese ripening was reported before (Claverie-Martin & Vega-Hernández, 2007). Particularly, fungal strains showing the highest aspartic protease content could be used as a commercial source following testing during cheese production. *Rhizomucor miehei* has gained broad industrial application as a microbial milk-clotting enzyme producer that substitutes bovine-derived chymosin (Claverie-Martin & Vega-Hernández, 2007). Aspartic protease from *Mucor circinelloides* was also found to be so similar to commercial rennet and it has been widely applied in the cheese industry (Sathya et al., 2010).

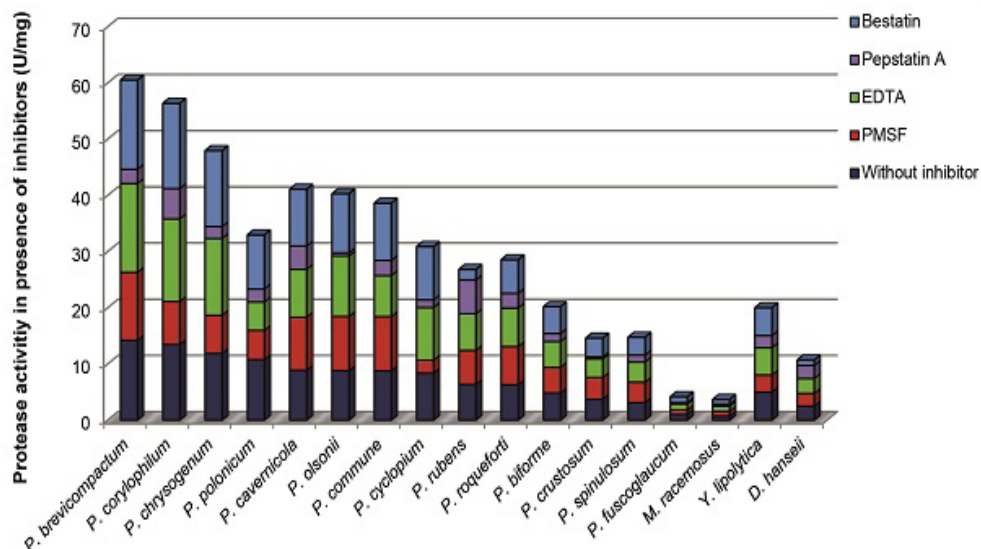


Figure 7. Effect of inhibitors on protease activities of yeast and filamentous fungi.

Quantitative activities of bacterial strains

Protease activities

The protease activity produced by bacteria is given in Figure 8A. Out of 18 bacterial strains, 14 were evaluated for their protease levels. The highest proteolytic activity was observed for *M. luteus* (6.46 $\mu\text{g/ml}$), which was one of the strains showing the largest halo in skim-milk agar plates. *B. antiquum*, *B. stratosphericus* and *P. glacincola* also secreted high protease levels, but less than *M. luteus*.

The genus *Microbacterium* has been previously isolated from raw milk and reported to be proteolytically active (Baur et al., 2015; Hantsis-Zacharov & Halpern, 2007). Growth of psychrotolerant bacteria is often correlated with the production of extracellular enzymes (Champagne et al., 1994). *Pseudomonas* is the predominant genus of psychrotrophs in raw milk. In previous studies, *Pseudomonas* species were frequently isolated from dairy products and associated with proteolysis and lipolysis by their ability to produce extracellular proteases and lipases (De Jonghe et al., 2011; Hantsis-Zacharov & Halpern, 2007; Marchand et al., 2009).

Lipase activities

The amount of lipase activity produced by bacteria is given in Figure 8B. Bacterial strains were overall poor lipase producers. Of 18 bacterial isolates, only 11 showed halos indicating lipolysis on lipase agar plates, and these were evaluated with the quantitative lipase assay. 10 of these strains secreted lipase. *B. antiquum* was the best lipase producing species (1.83 miliunits/ml), followed by *B. stratosphericus* (1.57 miliunits/ml), *P. proteolytica* (0.98 miliunits/ml) and *P. glacincola* (0.77 miliunits/ml).

Similar to their protease activity, lipase activity of *Pseudomonas* spp. has also been described in previous studies (Baur et al., 2015; Hantsis-Zacharov & Halpern, 2007). When bacterial strains isolated from Antarctic environments were screened for lipase activity the highest activity was observed for *Pseudomonas* spp., *Bacillus* spp. and *Psychrobacter* spp. (Loperena et al., 2012). In our study, *P. proteolytica* only produced low levels of proteolytic activity, but had a more pronounced lipolytic activity. The genus *Bacillus* is also known for its production of extracellular proteases and lipases in dairy products (Montanhini et al., 2013).

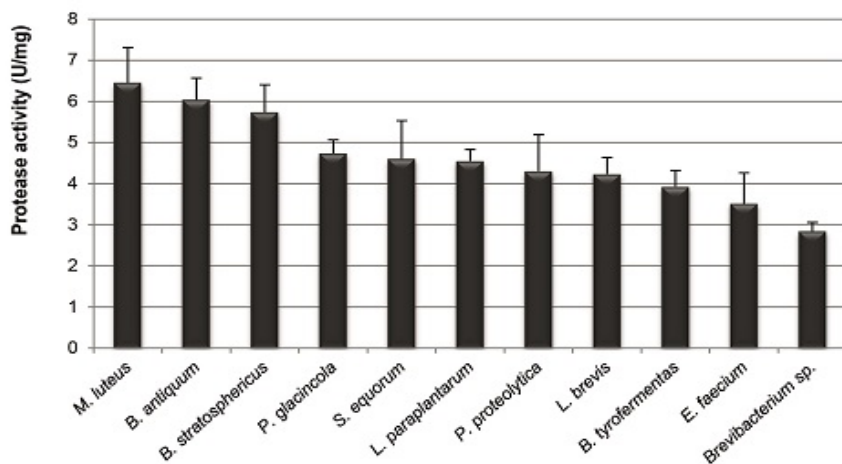
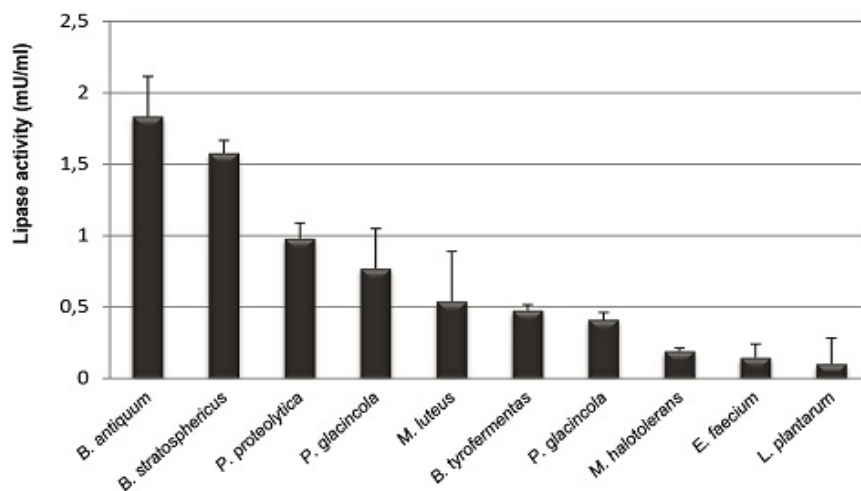
A**B**

Figure 8. Quantitative determination of protease (A) and lipase (B) activities of bacteria.

Effect of inhibitors on bacterial strains

The proteases of all bacterial isolates were inhibited by PMSF (Figure 9). The strongest inhibition was observed for *M. luteus*, *B. antiquum*, *B. stratosphericus* and *Brevibacterium* spp., for which protease activity was almost completely inhibited by PMSF. Thus, our inhibition studies revealed that the proteases produced by the bacterial strains mainly belongs to the serine protease class.

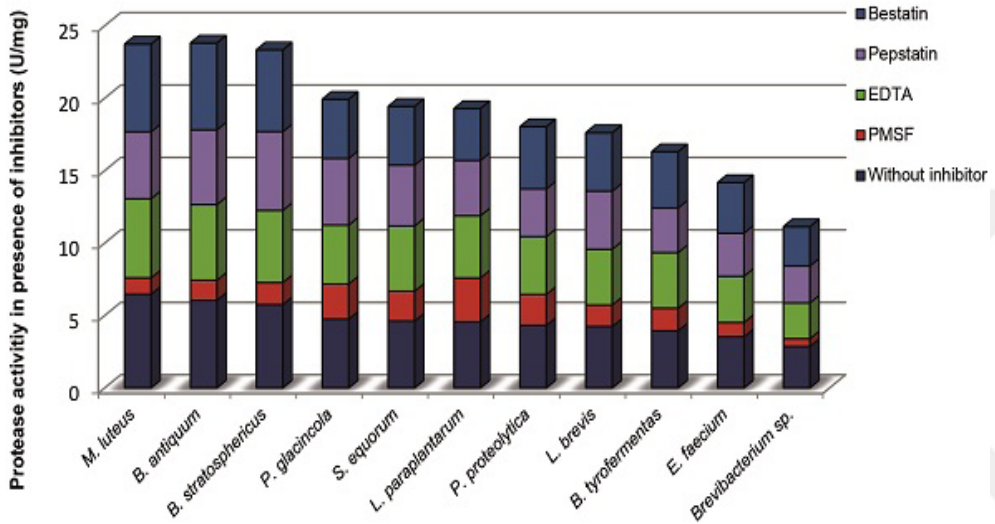


Figure 9. Effect of inhibitors on protease activities of bacteria.

Conclusion

The aim of this study was the quantification of protease and lipase activity secreted by cheese microbiota isolated previously from the same cheese. The detection of these activities of strains isolated from Divle Cave cheese is crucial to obtain better inside into their contribution to cheese ripening. The current study confirmed the high frequency of enzymatically active strains in Divle Cave cheese by identifying the best protease and lipase producer strains. Fungal strains were determined to have higher protease and lipase activities than bacteria. Especially LAB strains showed low values. The proteases and lipases of LAB are intracellular enzymes. They have been reported to release into the cheese matrix due to cell lysis (autolysis), caused by various stressors including mechanical, physical and chemical stress (El-Soda et al., 2000). This could be the reason why the activity of the bacteria is lower than those of the fungi in relation to a possible lower incidence of cell lysis in culture medium. In addition, proteases of LAB can also be plasmid encoded and unstable when cultured outside milk (Kok, 1991). Therefore, the genes encoding these enzymes could have been lost by the strains and this could be another reason their activities appear low. In the future, secreted enzymes from the individual strains could be purified and tested for their suitability for well-defined applications in the dairy industry. Moreover, the native strains studied in this research can be selected as potential starter or adjunct

cultures for the production of industrial cheeses to improve the sensory, textural, physiological and biochemical properties while protecting the traditionality of cheese.

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CHAPTER 6

A genomic survey of proteases in *Aspergilli*

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Abstract

Proteases can hydrolyze peptides in aqueous environments. This property has made proteases the most important industrial enzymes by taking up about 60% of the total enzyme market. Microorganisms are the main sources for industrial protease production due to their high yield and a wide range of biochemical properties. Several *Aspergilli* have the ability to produce a variety of proteases, but no comprehensive comparative study has been carried out on protease productivity in this genus so far. We have performed a combined analysis of comparative genomics, proteomics and enzymology tests on seven *Aspergillus* species grown on wheat bran and sugar beet pulp. Putative proteases were identified by homology search and Pfam domains. These genes were then clusters based on orthology and extracellular proteases were identified by protein subcellular localization prediction. Proteomics was used to identify the secreted enzymes in the cultures, while protease essays with and without inhibitors were performed to determine the overall protease activity per protease class. All this data was then integrated to compare the protease productivities in *Aspergilli*. Genomes of *Aspergillus* species contain a similar proportion of protease encoding genes. According to comparative genomics, proteomics and enzymatic experiments serine proteases make up the largest group in the protease spectrum across the species. In general wheat bran gives higher induction of proteases than sugar beet pulp. Interesting differences of protease activity, extracellular enzyme spectrum composition, protein occurrence and abundance were identified for species. By combining *in silico* and wet-lab experiments, we present the intriguing variety of protease productivity in *Aspergilli*.

Introduction

Proteases form a complex family of enzymes that possess different catalytic mechanisms with various active sites and divergent substrate specificities (Fedatto et al., 2006; Vishwanatha et al., 2009). Proteases hydrolyze peptides in aqueous environments (Horikoshi, 1996; Tunga et al., 2003) and for years this ability has been utilized in industrial processes like food processing, waste treatment, textiles/detergent applications, and photography/chemical processing (Gessesse & Gashe, 1997; Hernandez-Martinez et al., 2011; Kaur et al., 2001; Kembhavi et al., 1993; Kim & Lei, 2005). Proteases can be classified into four major groups: aspartic, cysteine, metallo and serine proteases (Fedatto et al., 2006). Protease inhibitors for each of these classes have been described (Xue et al., 2006). These inhibitors regulate the activity of proteases by binding to the enzyme and eliminating unwanted proteolysis (Y. Li et al., 2012; Macedo et al., 2011). In recent years, proteases and protease inhibitors have gained additional interests in many health related areas as e.g. pathogenic agents by allergy, asthma and obese related illness (Sabotic & Kos, 2012). Proteases have been recognized as the most important industrial enzymes accounting for about 60% of the total enzyme market (Charles et al., 2008).

Proteases can be obtained from animal, plant and microbial sources (Hernandez-Martinez et al., 2011). However, microorganisms are the most important sources for industrial applications (Horikoshi, 1996; Tunga et al., 2003) due to their high yield and productivity and a wide range of biochemical and catalytic properties (Horikoshi, 1996). The genus *Aspergillus* represents a diverse group of filamentous ascomycetous fungi (Fedorova et al., 2008), including human, animal and plant pathogens, but also species with a major role in industrial biotechnology (Braaksma et al., 2010). Several *Aspergillus* species have the ability to produce a variety of proteases (Conesa et al., 2001; Kunert & Kopecek, 2000; Machida et al., 2008; Markaryan et al., 1994; Su & Lee, 2001; Wang et al., 2008).

In this study we have performed a genome survey of several *Aspergilli* based on the protein sequences of verified proteases and Pfam domains. Curated putative proteases were fed to a combination of protein subcellular localization (SCL) predictors to identify the potentially secreted proteins. The results of this *in silico* comparative secretomics were then tested by enzyme activity assays and proteomic

experiments on samples from cultures grown on wheat bran and sugar beet pulp. Protease inhibitors were used to determine the contribution of the various protease classes to the total protease activity. Finally, by combining comparative genomes, proteomics and enzymology tests, we demonstrate the intriguing variety of protease productivity in the *Aspergilli*.

Materials and Methods

Genome mining, clustering and extracellular protein prediction

The genome sequences were extracted from AspGD (Arnaud et al., 2012) (version May 2014). Used genome information is listed in Table 1. The pre-calculated protease clusters in AspDG were retrieved from the *Aspergillus*10-way-comparative database. Additional homologs were added to the clusters by homolog searches using majority vote of BLASTP (Mount, 2007), Jaccard (Gajera & Vakharia, 2010) (cutoff E-value e^{-20} and alignment coverage 85%) and OrthoMCL (L. Li et al., 2003) (E-value $1E^{-10}$, inflation level 1 and sequence coverage 40%) results. Gene models were double checked with manual curation combining literature searches.

Six protein subcellular localization (SCL) predictors, Phobius (Kall et al., 2007), SignalP (Petersen et al., 2011), PrediSi (Hiller et al., 2004), CELLO (Yu et al., 2004), MultiLOC (Hoglund et al., 2006) and WoLF-PSORT (Horton et al., 2007; Lum & Min, 2011) were used to predict the extracellular proteases. Default settings of each SCL predictor were used, with the species parameter as “Eukaryotic” or “Fungi”. Majority votes were applied to combine the results of each SCL prediction.

Protease inhibitor information extraction and other bioinformatics analysis

The specific enzyme inhibitor information was retrieved by AspGD gene annotation repository and literature researches. Protein functional domain prediction was performed by HMMER v.3.0 (Johnson et al., 2010) using the complete Pfam-A and Pfam-B models (Bateman et al., 2000) (data retrieved from Pfam database, version November 2012) with the trust cutoff and the gathering cutoff. The resulting Pfam predictions were pooled.

Table 1. Summary of putative proteases in *Aspergilli*. The numbers of extracellular proteins are provided in brackets following each category.

Species	Genome reference	Total Genes	Putative Proteases	Serine proteases	Aspartic proteases	Metallo proteases	Amino proteases	Miscellaneous proteases
<i>A. fumigatus</i> AT293	(Nierman et al., 2005)	9781	301 (45)	75 (16)	17 (9)	48 (8)	28 (5)	133 (7)
<i>A. flavus</i> NRLL 3357	(Yu et al., 2008)	12604	336 (63)	88 (23)	21 (15)	61 (9)	24 (4)	142 (12)
<i>A. oryzae</i> RIB40	(Machida et al., 2005)	12030	336 (57)	85 (21)	21 (14)	66 (11)	25 (5)	139 (6)
<i>A. terreus</i> NIH 2624	unpublished	10406	306 (44)	73 (16)	18 (9)	58 (6)	26 (4)	131 (9)
<i>N. fischeri</i> CBS 544.65	(Nierman et al., 2005)	10406	307 (45)	76 (15)	15 (11)	50 (7)	26 (4)	140 (8)
<i>A. nidulans</i> FGSC A4	(Galagan et al., 2005)	10680	302 (40)	72 (13)	16 (8)	50 (6)	24 (5)	140 (8)
<i>A. niger</i> ATCC 1015	(Andersen et al., 2011)	11162	314 (53)	84 (22)	19 (15)	57 (7)	25 (3)	129 (6)

Strains and media

The fungal strains used in this study are listed in Table 1. All strains were grown on Malt Extract Agar and incubated at 30 °C for 3–4 days until good sporulation had occurred. Spores were harvested by gentle agitation in 10 ml ACES (acid buffer) and solutions were taken into sterile tubes. Twenty times dilution of each solution were counted using a haemocytometer (Burker-Turk) under microscope (Axioplan, Zeiss). Liquid media was prepared in 250 ml conical flasks containing 50 ml Minimal medium (MM) (de Vries et al., 2004). Five different culture conditions were prepared for the determination of protease activity in different mediums. Below substrates were added into 250 ml conical flasks containing 50 ml MM and a) 1% wheat bran, b) 1 % wheat bran +1% glucose, c) 1% sugar beet pulp, d) 1% glucose + 1% sugar beet pulp and e) 1% glucose + 1% casein. All prepared media were autoclaved at 121 °C for 20 min. For each strain, sterile liquid culture media were inoculated with 5×10^8 spore/ml in 250 ml erlenmeyer flask and incubated for 72 h at 30 °C on a shaker at 250 rpm for the production of proteases. During the growth of fungi, 2 ml of aliquots were taken from cultures at 48h, 72h and 96h. Those were centrifuged and used for all the experiments. Cultures were established in duplication for biological repetition and triplicated for technical repetition. The pH of most samples on was 7 except for *A. nidulans* on SBP (pH=8) and *A. niger* on WB (pH = 5-6) and SBP (pH = 4-5).

Protease activity assay

A pilot experiment was performed on *A. nidulans* growing on WB, WB+Glc, SBP and SBP+Glc and protease activities were measured on 48, 72 and 96 h post-inoculation. From the analysis the best day with highest protease activity was found to be day 3 (72 h post-inoculation, Supplementary Table 7).

For all experiments, protease activities of the cultures were measured after 72 h post-inoculation in liquid-state fermentation. 2 ml samples were taken from flasks and centrifuged at $14000 \times g$ for 10 min (Eppendorf Centrifuge, 5417R). Supernatant was separated after centrifugation and stored at -20 °C until the measurements of protease activity.

The protease activity assay was performed according to the procedures mentioned in protocol of Pierce Fluorescent Protease Assay Kit (Kit number: 23266, Pierce

Biotechnology, Thermo Scientific, USA). The levels of protease activity in the supernatants of 7 strains over 72 h were compared using a fluorescein isothiocyanate (FITC)-labeled casein assay according to the manufacturer's instructions. Fluorescence of the samples were measured by optical density (OD) using the plate reader (Fluostar Optima, BMG LABTECH) with excitation at 485 nm and emission at 530 nm to determine protease activity. The enzyme activity was expressed as micromoles of trypsin released per minute per milligram of total protein in culture filtrate (unit: U/mg, 1 μ mol trypsin min⁻¹).

pH 7.2 was required for the Pierce in light of the TBS solution stability. A pilot experiment was performed testing this kit on pH 4, 6, and 8 (Supplementary Table 6). According to the result of this test, pH 6 was selected for protease activity measurements.

Inhibition of proteases

Protease inhibitors were prepared to give final concentrations of 50 mM for PMSF and EDTA (Sigma), 1 mM for Pepstatin A and Bestatin (Sigma) as instructed by the manufacturer. 2 μ L protease inhibitors were added into the assay mixture and incubated for 60 min at room temperature prior to performing the assay. Culture supernatants treated alone was used as negative control. Each assay was performed in triplicate. All measurements were performed under pH 6.

Neutral carbohydrate composition

Neutral carbohydrate composition of wheat straw and sugar beet pulp was analysed according to Englyst (Englyst & Cummings, 1984) using inositol as an internal standard. Samples were treated with 72% (w/w) H₂SO₄ (1h, 30°C) followed by hydrolysis with 1M H₂SO₄ for 3h at 100°C and the constituent sugars released were derivatised and analysed as their alditol acetates using gas chromatography (GC).

Proteomics experiments

Protein Digestion

Protein from 3 ml of incubation medium was precipitated with cold TCA/Acetone. Protein sample determination was carried out with the RCDC kit assay (BioRad, Mississauga, Ont). Five μ g of protein was incubated in 100 mM ammonium bicarbonate, 0.1% AALS II (Morgantown, WV) and 5 mM dithiothreitol for 30 min. followed by

the addition of Iodoacetamide to a final 25 mM concentration and incubated for an additional 30 min at 37°C. 200 ng of trypsin was added to each sample and the solution totaling 70 μ l was incubated for 18 h at 37°C. The digestion solutions were acidified with trifluoroacetic acid (1% final) then put through two rounds of desalting using C18 ziptips™ (Millipore, Billerica, MA). Eluted peptides were dried in a SpeedVac and resuspended in a 60 μ l solution of 5% ACN, 0.1% FA and 4fmol/ μ l of predigested Bovine Serum Albumin (Michrom, Auburn, CA) used as an internal standard.

LC-MS/MS Analysis

5 μ l of peptide digest was loaded onto 15 cm x 75 μ m i.d PicoFrit column (New Objective, Woburn, MA) packed with Jupiter 5 μ m, 300 Å, C18 resin (Phenomenex, Torrance, CA) connected in-line with a Velos LTQ-Orbitrap mass spectrometer (Thermo-Fisher, San Jose, CA). Peptide separation was done using a linear gradient generated by an Easy-LC II Nano-HPLC system (Thermo-Fisher) using a mixture of solvent A (3% ACN:0.1% FA) and solvent B (99.9% ACN:0.1%FA). The gradient started at 1% B, was set to reach 27% B in 85 min, ramped to 52 % B in 15 min and 90% B in 5 min then held at 90% for 5 min.

The mass spectrometer used was a Velos LTQ-Orbitrap (Thermo-Fisher, San Jose, CA). The capillary voltage on the nanospray source was adjusted to get the best spraying plume at 10% B and typically ranged from 1.9 to 2.1 kV. MS survey scan spanning the 350 to 2000 m/z range was done at 60000 resolution. The top 10 doubly, triply or quadruply charged ions with intensity higher than 5000 counts were considered candidates to undergo CID MS/MS fragmentation in the LTQ-Velos ion trap. Quantification was based on MS precursor ion signal using the precursor ion detection workflow from Proteome Discoverer Quant 1.3 (Thermo-Fisher). Briefly, extracted ion chromatograms were generated to compute the peptide area value associated to each identified precursor ion. A Protein Area value is subsequently calculated as the average of the three most intense, distinct, peptides assigned to a protein. Protein area values were expressed as a fold value of the protein area value calculated for Bovine Serum Albumin (BSA) which was spiked as an internal standard in each individual sample. For spectral count-based comparisons, the number of assigned spectra for each protein was reported as a fold value of the total number of spectra assigned to BSA in each sample.

Bioinformatics Data Processing

LC-MS/MS data was processed using Proteome Discoverer Quant 1.3 (Thermo-Fisher) and spectral data was searched against *Aspergillus* protein databases downloaded from the *Aspergillus* Genome Database (AspGD). Search parameters used were 0.80 Da for fragment ion tolerance of and 10.0 ppm for parent ion tolerance, fixed iodoacetamide cysteine modification and variable methionine oxidation. Quantitation analysis based on MS precursor ion signal was done using the precursor ion detection workflow from Proteome Discoverer Quant 1.3 (Thermo-Fisher). Briefly, extracted ion chromatograms are generated to compute the peptides area values for precursor ions identified from the Mascot search using. A Protein Area value is subsequently calculated as the average of the 3 most intense distinct peptides identified for the protein. Protein area values were normalized using the reported values in each individual sample. Confidence filters were applied to satisfy a 1% FDR at the Peptide and Protein level. Protein grouping was applied so as to satisfy the principles of parsimony. The normalized protein areas of a protein were used as the measurement of abundance level of the protein. The abundance of a protein represents the productivity of the protein in an organism under that specific circumstance while measured. The areas are also used as the measurement of protease productivity.

The unique counts of peptides to each identified protein were used as evidences of the occurrence of the protein. For any protein that has more than one uniquely mapped peptide it is considered occurred in the supernatant. The total amount/number of proteases in a sample was calculated by the sum of proteins which have more than one uniquely mapped peptide.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaino et al., 2013) with the dataset identifier PXD000982.

Results and Discussion

Genome mining and extracellular protein clustering

The genomes of seven *Aspergillus* species, *Aspergillus niger* ATCC 1015 (Andersen et al., 2011), *Aspergillus nidulans* FGSC A4 (Galagan et al., 2005), *Aspergillus*

oryzae RIB40 (Machida et al., 2005), *Aspergillus flavus* NRLL 3357 (Yu et al., 2008), *Aspergillus terreus* NIH 2624, *Neosartorya fischeri* CBS 544.65 (Nierman et al., 2005) and *Aspergillus fumigatus* AF293 (Nierman et al., 2005) (Table 1, data retrieved from AspGD (Arnaud et al., 2012)), were included in the genomic comparison of protease-encoding genes. On the basis of putative protease clusters (588 proteins, 478 clusters) already existing in AspGD, additional putative proteases were found by homology. Gene models were manually corrected by multiple sequence alignments. A thorough Pfam domain detection was carried out on the *Aspergillus* genomes. Proteins containing no known protease-related Pfam domain(s) were removed when no additional literature support could be found. At the end, 1558 extra putative proteases were added to the original set of AspGD protein clusters by Jaccard (Gajera & Vakharia, 2010) and OrthoMCL (Fischer et al., 2011) (in total 2146 proteins, 478 clusters) (Supplementary Table 1). While investigating the gene presence/absence patterns, genome scale ortholog clusters were utilized to identify species-specific genes. 236 out of the 478 clusters appeared to be ubiquitous, by containing at least 1 protein from each species. 56 clusters contained only a single member with no homologs in other species, and were therefore considered “orphan genes” (Khalturin et al., 2009; Tautz & Domazet-Loso, 2011). The other clusters cover the species partially (Supplementary Table 2).

Six different protein SCL predictors were applied to all 2146 putative proteases. By using majority vote 335 proteins were considered extracellular, among which 277 were in the original AspGD protease clusters (Supplementary Table 3).

Further classification of proteases was determined by combined manual literature search and Pfam annotations. At the end, most putative proteases were classified into four major groups, namely amino, aspartic, metallo and serine, while the remaining genes formed the miscellaneous group (Supplementary Table 1).

Effect of wheat bran and sugar beet pulp on extracellular protease induction in *Aspergilli*

Two cultivation media, minimal medium with 1% wheat bran (WB) and minimal medium with 1% sugar beet pulp (SBP), were used to induce extracellular protease production in *Aspergilli*, resulting in an interesting variability of protease activity

(Figure 1A). Among the tested species, *N. fisheri* produced the highest protease activity on SBP, *A. fumigatus* produced the highest activity on WB whereas *A. flavus* had the most moderate activities in both substrates. In all cases WB induced more protease activity than SBP. This was particularly true for *A. flavus* and *A. fumigatus*, where the extracellular protease activities on WB were around twice as high as those on SBP. In contrast, for *N. fisheri* only a small difference (<10%) was detected.

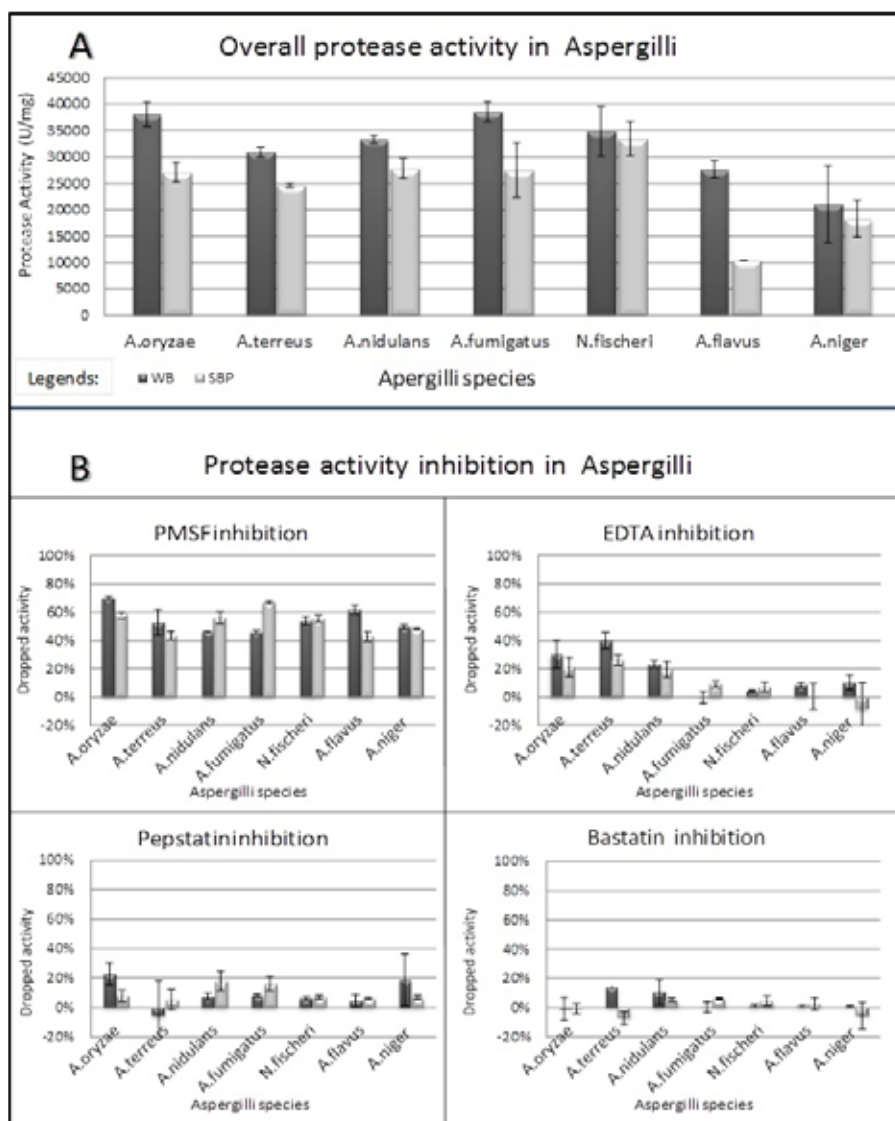


Figure 1. Protease activity with and without inhibitor in *Aspergilli* using WB and SBP as carbon sources. **1A: Overall protease activity in *Aspergilli* growing on WB and SBP.** Seven *Aspergillus* species were grown on WB or SBP on 30°C and sampled at 72h, protease activities were measured for each sample in 2 biological replications with technical triplicates. **1B: Protease activity inhibition in *Aspergilli*.** With the same settings described for Figure 1A, the protease activity was measured after adding corresponding inhibitors. The ratio of dropped activity was calculated by $\text{PercentageDroppedActivity} = [1 - (\text{activity after adding inhibitor} / \text{original activity without inhibitor})] \%$. This dropped activity indirectly represents the proportion of corresponding protease activity in the supernatants, higher this number, bigger proportion of such type of protease takes the overall activity. Legends: WB and SBP: protease activity in wheat bran and sugar beet pulp, respectively.

Extracellular proteases in 7 *Aspergilli* confirmed by proteomics

In order to confirm protease production variability by *Aspergillus* species on different carbon sources, we performed proteomics experiments. In total, 133 putative proteases were identified (at least 2 unique peptides found per protein, Supplementary Table 4). The identified proteases were then mapped to the extended protease clusters created by comparative genomics, resulting in the presence of 45 orthologous groups (OG) in the cultures (Table 2).

From all identified proteins, 93 were found on both WB and SBP, while 38 were found uniquely on WB and only two (dipeptidyl-peptidase Afu4g09320 (Sharon et al., 2009; Sriranganadane et al., 2010) and neutral protease I NFIA_099860) were found uniquely on SBP. Twenty-five out of these 133 identified proteases were not predicted to be extracellular according to our combined SCL predictions. Some of them may be secreted through alternative (non-classical) secretion systems, as suggested for the spermidine synthase (AO090012000528) from *A. oryzae*.

Table 2. Putative proteases identified by proteomics in 7 *Aspergilli* on wheat bran and sugar beet pulp

Ortholog Groups	<i>A. nidulans</i>	<i>A. niger</i>	<i>A. oryzae</i>	<i>A. terreus</i>	<i>A. favus</i>	<i>N. fisheri</i>	<i>A. fumigatus</i>	Protease group	Inh.	Ref.
OG-1	AN2572	185799	-	ATEG_09875	AFL2G_05512	NFIA_084570	Afu2g09030	amino	B	(Schinko et al., 2010)
OG-2	AN3918	214665	AO090012000110	ATEG_09990	AFL2G_03045	NFIA_032410	Afu2g00220	amino	B	(Guillemette et al., 2007; Hartmann et al., 2011)
OG-3	AN6438	-	AO090023000602	ATEG_05269	AFL2G_04433	NFIA_106690	Afu4g09320	amino	B	(Szilagyi et al., 2013)
OG-4	AN8445	-	AO090003000354	ATEG_09137	AFL2G_02631	NFIA_001250	Afu3g00650	amino	B	(Szilagyi et al., 2013)
OG-5A	AN1638	-	AO090023000645	ATEG_05313	AFL2G_04474	NFIA_107140	Afu4g09030	amino	B	(Saykhedkar et al., 2012)
OG-5B	AN4282	130008	AO090102000639	ATEG_07980	AFL2G_10011	NFIA_037760	Afu5g04330	amino	B	(Malavazi et al., 2006; Szilagyi et al., 2013)
OG-6	-	206384	AO090009000228	ATEG_03227	AFL2G_10434	NFIA_049620	Afu6g03260	aspartic	P	(Braaksma et al., 2010; Wang et al., 2008)
OG-7A	-	-	-	-	AFL2G_11784	-	-	aspartic	P	(de Groot et al., 2009)
OG-7B	AN8102	189966	AO090010000644	-	AFL2G_04852	NFIA_002160	Afu3g01220	aspartic	P	(de Groot et al., 2009; Wang et al., 2008)
OG-8	AN4422	213261	AO090023000872	ATEG_05510	AFL2G_04683	NFIA_109180	Afu4g07040	aspartic	P	(Guillemette et al., 2007)
OG-9	AN2903	37080	AO090003000693	ATEG_04676	AFL2G_02316	NFIA_065900	Afu3g11400	aspartic	P	(Hortschansky et al., 2007)
OG-10	-	40218	-	ATEG_04955	-	NFIA_065900	-	aspartic	P	(de Groot et al., 2009; Wang et al., 2008)
OG-11A	-	191956	AO090009000148	ATEG_05628	AFL2G_10504	NFIA_051920	Afu6g05350	aspartic	P	(Braaksma et al., 2010)
OG-11B	AN6487	53364	AO090701000002	ATEG_05628	AFL2G_05659	NFIA_051920	Afu6g05350	aspartic	P	(Braaksma et al., 2010)
OG-12	-	211797	-	-	-	NFIA_100060	-	aspartic	P	(de Groot et al., 2009)
OG-13	AN6888	201655	AO090120000474	ATEG_06182	AFL2G_08462	NFIA_073740	Afu5g13300	aspartic	P	(vanKuyk et al., 2000)
OG-14	AN6796	210782	AO0900012000695	ATEG_09753	AFL2G_03570	NFIA_040680	Afu5g01430	cysteine	E	
OG-15	AN7962	-	AO090001000135	ATEG_04941	AFL2G_07373	NFIA_102630	Afu4g13750	metallo	E ^d	(Emri et al., 2009; Katz et al., 2008)

Table 2. Putative proteases identified by proteomics in 7 *Aspergilli* on wheat bran and sugar beet pulp

Ortholog Groups	<i>A. nidulans</i>	<i>A. niger</i>	<i>A. oryzae</i>	<i>A. terreus</i>	<i>A. flavus</i>	<i>N. fisheri</i>	<i>A. fumigatus</i>	Protease group	Inh.	Ref.
OG-16	-	46803	AO090011000036	ATEG_07544	AFL2G_04842	NFIA_099860	Afu8g07080	metallo	E ^d	(Liang et al., 2009)
OG-17	-	-	AO090010000493	-	AFL2G_11655	-	-	metallo	E ^d	(te Biesebeke et al., 2005)
OG-18	-	-	AO090011000052	-	AFL2G_04856	-	-	metallo	E ^d	(te Biesebeke et al., 2005)
OG-19	ANI0540	-	AO0900023000980	ATEG_06810	-	NFIA_110010	Afu4g06140	metallo	E ^d	
OG-20	-	209872	AO090005000457	ATEG_06341	AFL2G_00447	NFIA_027170	Afu7g05930	metallo	E ^d	
OG-21	-	52703	-	-	AFL2G_03937	NFIA_033550	Afu2g01250	serine	P ^m	(Braaksma et al., 2010; Levin et al., 2007)
OG-22A	-	55493	AO0900026000083	-	AFL2G_07153	-	-	serine	P ^m	(Braaksma et al., 2010)
OG-22B	-	55133	-	-	-	-	-	serine	P ^m	(Braaksma et al., 2010)
OG-23	AN6273	52126	AO0900026000357	ATEG_01242	AFL2G_06902	NFIA_087760	Afu2g12630	serine	P ^m	(Oda et al., 2006)
OG-24	-	46979	AO0900020000351	ATEG_07509	AFL2G_10957	NFIA_047470	Afu6g00310	serine	P ^m	(de Oliveira et al., 2011; Morya et al., 2012)
OG-25	-	54734	-	-	-	-	-	serine	P ^m	(Guillemette et al., 2007)
OG-26	-	43917	-	ATEG_06406	-	NFIA_072360	Afu5g14610	serine	P ^m	
OG-27	AN2555	56161	AO090010000534	ATEG_09537	AFL2G_11692	NFIA_035860	Afu2g03510	serine	P ^m	(Braaksma et al., 2010; Sharon et al., 2009)
OG-28A	-	55665	AO090166000084	-	AFL2G_09418	NFIA_102320	Afu4g14000	serine	P ^m	(Zhu et al., 2012)
OG-28B	-	52700	-	-	-	-	-	serine	P ^m	(Zhu et al., 2012)
OG-29	AN5442	52603	AO090103000332	ATEG_03401	AFL2G_12064	NFIA_059500	Afu6g13540	serine	P ^m	(Guillemette et al., 2007)
OG-30	AN7231	56689	AO090102000079	ATEG_10012	AFL2G_09533	NFIA_092750	Afu2g17330	serine	P ^m	(Braaksma et al., 2010)
OG-31	ANI426	21460	AO090103000026	ATEG_00024	AFL2G_12331	NFIA_096830	Afu1g00420	serine	P ^m	(Blinkovsky et al., 1999)
OG-32	ANI0030	140344	AO0900020000517	ATEG_06546	AFL2G_10813	NFIA_078120	Afu5g09210	serine	P ^m	(de Groot et al., 2009)

Table 2. Putative proteases identified by proteomics in 7 *Aspergilli* on wheat bran and sugar beet pulp

Ortholog Groups	<i>A. nidulans</i>	<i>A. niger</i>	<i>A. oryzae</i>	<i>A. terreus</i>	<i>A. flavus</i>	<i>N. fisheri</i>	<i>A. fumigatus</i>	Protease group	Inh.	Ref.
OG-33	AN2237	192619	AO090701000220	ATEG_09343	AFL2G_05864	NFIA_079940	Afu5g07330	serine	P ^m	
OG-34	AN7159	211032	AO090011000235	ATEG_02150	AFL2G_05009	NFIA_029950	Afu4g03490	serine	P ^m	(Jin et al., 2007)
OG-35	-	-	AO090701000579	-	AFL2G_06196	-	-	serine	P ^m	
OG-36	-	-	-	-	-	NFIA_031000	Afu7g08350	serine	P ^m	
OG-37	AN2818	180130	AO090103000478	-	AFL2G_11938	-	-	serine	C	
OG-38	AN1182	208263	AO090038000317	ATEG_00287	AFL2G_07674	NFIA_014730	Afu1g10910	serine	P ^m	(Malavazi et al., 2006; Oakley & Morris, 1981)
OG-39	AN2366	-	AO090023000609	ATEG_05749	AFL2G_04440	-	-	t-serine	A	(Zhao et al., 2010)
OG-40	AN5558	203039	AO090003001036	ATEG_03900	AFL2G_01995	NFIA_104430	Afu4g11800	t-serine	A	(Katz et al., 1994; vanKuyk et al., 2000)
OG-41	AN0224	35620	AO090023000428	ATEG_05010	AFL2G_04274	NFIA_057190	Afu6g11500	t-serine	A	
OG-42	AN5129	181371	AO090012000995	ATEG_10178	AFL2G_03855	NFIA_081390	Afu1g07440	ubiquitin	U	(Araujo-Bazan et al., 2008; Freitas et al., 2011; Katz et al., 1994)
OG-43A	AN0687	207954	AO090012000528	ATEG_00551	AFL2G_03425	NFIA_012010	Afu1g13490	ubiquitin	U	(Liang et al., 2009)
OG-43B	AN2000	214265	AO090003001182	ATEG_00694	AFL2G_01863	NFIA_105720	Afu4g10350	ubiquitin	U	(Noventa-Jordao et al., 2000)
OG-44	AN7254	205183	AO090102000107	ATEG_10033	AFL2G_09558	NFIA_092420	Afu2g17110	ubiquitin	U	(Wartenberg et al., 2012)
OG-45	AN4016	52026	AO090003000947	ATEG_03809	AFL2G_02080	NFIA_020680	Afu1g04040	ubiquitin	U	(Lu et al., 2010)

B: Bestatin; P: Pepstatin; E: E-64 / L-cysteine; E^d: EDTA; P^m: AEBSE / DFP / PMSF; C: Calyculin A; A: Aprotinin / Antipain; U: Ubiquitinyl hydrolase 1; T-serine: trypsin-like serine.

The proteases found in both WB and SBP are marked pink, the ones only found in WB are marked yellow and the ones only found in SBP are marked blue. Putative non-extracellular proteins detected by proteomics are underlined. Orthologous proteases are clustered and mentioned in the first column. Absence of orthologs in each species are resembled by “-”.

While comparing proteomics-confirmed protein productivities to enzymology-identified protease activities, a strong correlation was found: WB generally induced more proteases than SBP with all tested *Aspergillus* species taking protein occurrence, abundance and enzyme activities all in consideration (Figure 2).

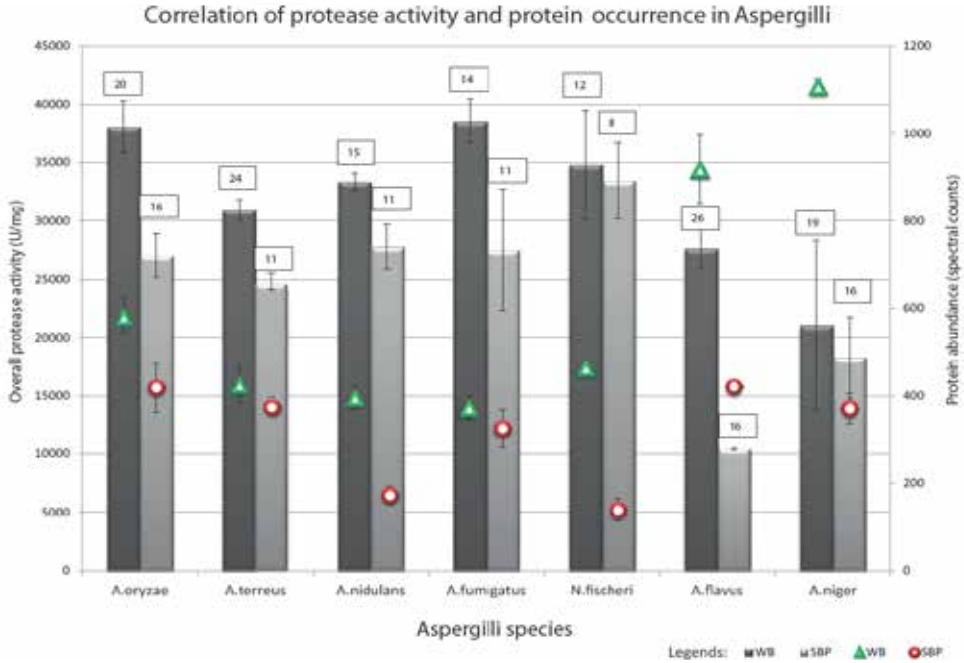


Figure 2. Correlation of protease occurrence, abundance and activity in *Aspergilli* on WB or SBP. While growing on 2 different crude substrates (on 30°C and sampled at 72h), the protease occurrence, abundance and the enzyme activity of seven tested *Aspergillus* strains show a general positive correlation. In WB more occurrences of proteases with higher abundance have been identified than in SBP, so as the enzyme activities. The protease abundance is presented in this figure by spectral counts, the amount of identified proteins which is presented in the figure by framed numbers. Legends: WB and SBP: protease activity in wheat bran and sugar beet pulp, respectively; WB^p and SBP^p: Protease abundance in wheat bran and sugar beet pulp, respectively.

Intriguingly, contradictions were also found when delving deeper into the protease production profile of individual species. For example, *A. terreus* has the second largest amount of proteases identified in WB (24) whereas only 11 proteins were found in SBP. However, the protease activity in SBP was only around 20% less than in WB (Supplementary Table 4). In *N. fisheri* a lower than average number of proteins was detected by proteomics, but the protease activity was amongst the

highest of all species. In *A. fumigatus* only 3 additional proteins (15% extra quantity by spectral counts) were identified in WB compared to SBP, but the overall protease activity in WB was 40% higher. These findings demonstrate that the total protease activity does not only depend on the total production of proteases. This is likely due to the fact that the enzyme assays measure the combined activity of the available proteases. As they have different specific activities, the total activity is not equal to the sum of the protein amount. For instance, high abundance of a protease with a low specific activity may affect the overall protease activity less than moderate abundance of a protease with a high specific activity.

Closer examination of the produced protease activity using protease inhibitors

In order to elucidate the extracellular protease composition in more detail, a series of inhibitor specificity tests was performed. Most of the proteases that were identified in *Aspergilli* could be classified into the following major groups: amino, aspartic, metallo and serine. Based on literature, the main inhibitor of each group was Bestatin (Muskardin et al., 1994; Schaller et al., 1995), Pepstatin (Marciniszyn et al., 1976, 1977; Umezawa et al., 1970), Ethylenediaminetetraacetic acid (EDTA) (Auld, 1995; Thompson et al., 2012) and phenylmethanesulfonylfluoride (PMSF) (James, 1978), respectively (Table 2, Supplementary Table 4). These inhibitors were added to the supernatants and protease activities were compared to those without inhibitors (Supplementary Table 5).

In general, for all samples protease activity was found to be inhibited predominantly by PMSF (43.48-67.12% decrease of activity). Lesser inhibition of activity was detected with EDTA (1.56-40.05 %), Bestatin (1.29-14.92 %) and Pepstatin (2.24-28.40 %) (Figure 1B).

For PMSF inhibition the ratio of decrease was similar in all species (55±12%), even though *A. niger* has the lowest overall protease activity and *N. fischeri* one of the highest (Figure 1 and Supplementary Table 5). No significant difference of PMSF inhibited activities was found between WB and SBP in *A. oryzae*, *A. nidulans*, *A. terreus*, *N. fischeri* and *A. niger*. Although the occurrence and abundance of serine proteases were different in the samples, PMSF inhibited around half of the protease activity in all samples (Fig 1B, Supplementary Table 5). Nevertheless, some of the prevalently produced serine protease clusters may be responsible for at least half of total enzyme activity in these species regardless of carbon source differences. Examples could be OG-30 that contains the lysosomal Pro-Xaa carboxypeptidase ProtA (56689)

(Braaksma et al., 2010; Levin et al., 2007), OG-31 that contains the dipeptidyl peptidase II (214460) (Braaksma et al., 2010), OG-33 that contains the carboxypeptidase CpyI (AO090701000220) (Blinkovsky et al., 1999) and OG-34 that contains the tripeptidyl-peptidase TppA (AO090011000235) (Jin et al., 2007) (Table 2).

In contrast, *A. fumigatus* and *A. flavus* showed noticeable inhibition differences depending on the growth substrate. In *A. flavus* inhibition of serine proteins on WB showed a 50% higher effect than that on SBP. The opposite was observed for *A. fumigatus* where SBP seemed to promote more serine-protease activity than WB (Figure 1B and Figure 3).

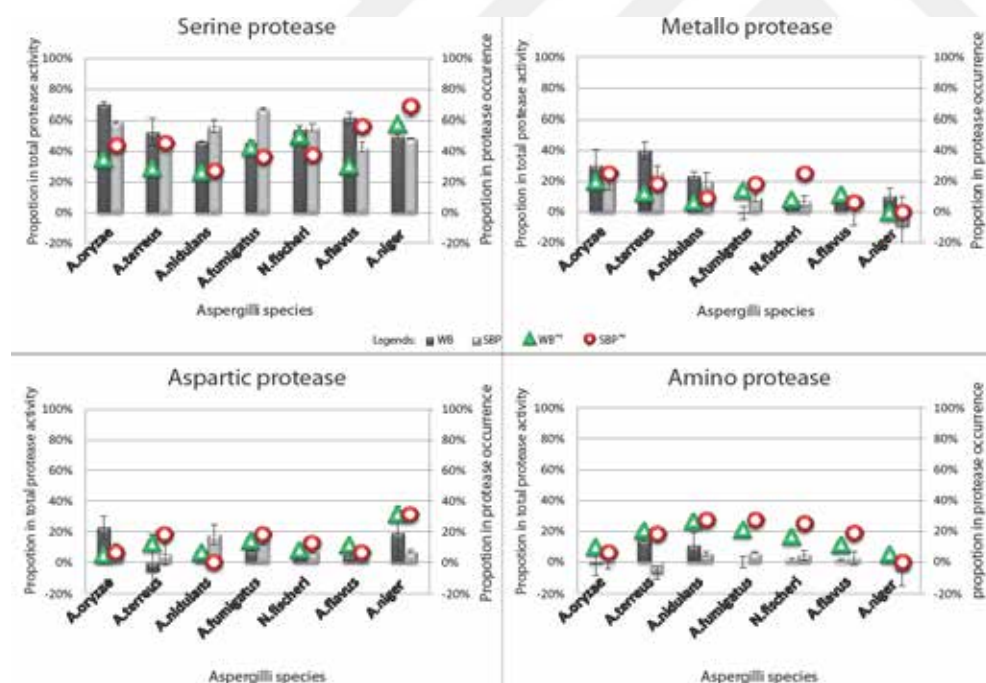


Figure 3. Correlation for each subgroup of protease by occurrence and activity in *Aspergilli* on WB or SBP (growing on 30°C and sampled at 72h). The protein occurrence is presented by the percentage of serine, metallo, aspartic and amino proteases in all proteomics-identified proteases, respectively. The proportion of serine, metallo, aspartic and amino proteases is presented by the percentage of inhibited enzyme activity by adding PMSF, EDTA, pepstatin and bestatin (PercentageDroppedActivity= $[1 - (\text{activity after adding inhibitor} / \text{original activity without inhibitor})] \times 100\%$ as in Figure 2). The occurrence of corresponding protease in the spectrum was calculated by AmountSpecificProtease/AmountTotalProtease%. Legends: WB and SBP: Proportion of specific protease activity in the spectrum while growing on wheat bran and sugar beet pulp, respectively; WB^{PV} and SBP^{PV}: percentage of protease occurrence in wheat bran and sugar beet pulp, respectively.

EDTA was the second best inhibitor, but a large variation of inhibited activity was detected (1.56–40.05%). *A. terreus* and *A. oryzae* showed the highest activity inhibition in WB samples. *A. nidulans* also showed a significant inhibition effect (~20%), though only a small difference between WB and SBP was detected. The enzyme activity in these species did not show strong correlation to their protease profiles. In *A. terreus* minor amounts of metallo proteases were identified by the proteomics experiments. The spectral counts in WB were comparatively lower than in SBP, even though the activity inhibition was much higher in WB than in SBP, suggesting higher specific activity of metallo proteases present in WB. Alkaline protease AN7962 (Emri et al., 2009) was the only metallo protease detected in *A. nidulans* cultures by proteomics. The spectral counts of this protein in WB were two-fold higher than in SBP. EDTA showed an equal effect on this protein with both substrates. In *A. oryzae* the main metallo proteases identified were neutral protease I (AO090011000036), neutral protease II (AO090010000493) and the leucine aminopeptidase (AO090011000052) (Liang et al., 2009). They showed higher total abundance in WB than SBP, and the inhibition effects confirmed this.

Among all species, *A. oryzae* and *A. niger* showed the highest activity inhibition when pepstatin was added to the supernatant produced with WB, indicating the presence of aspartic proteins in these species. In *A. niger* Aspergillopepsin A (PepA, 201655) (vanKuyk et al., 2000) was found to be the most dominant protease, with a four-fold higher abundance in WB than in SBP.

Bestatin mainly inhibits the activity of amino-protease/peptidases. Adding Bestatin to the supernatants showed minor inhibition of protease activities (<10%). This was intriguing because a rather high presence of amino proteases was identified in *A. terreus*, *A. nidulans*, *A. fumigatus*, *A. flavus* and *N. fischeri* by proteomics. The putative aminopeptidase OG-4 (Schinko et al., 2010; Szilagyi et al., 2013) (AN8445, AO090003000354, ATEG_09137, AFL2G_02631, NFIA_001250, and Afu3g00650) was the most abundant amino protease regardless of the carbon source in all species except *A. niger* (Figure 3).

Discussion

We have performed sets of heterogeneous tests on *Aspergillus* species using two complex substrates as carbon sources, aiming to construct a snapshot of fungal life that reflects the variation in protease productivity in different species. In contrast to

commonly reported genome-scale protease analysis results (Lu et al., 2010; Nierman et al., 2005; Oda et al., 2006), besides comparative genomics and proteomics we also included the analysis of enzymatic measurements, which provided further elucidation on the composition of extracellular protease spectra.

By comparative genomics, a rather even distribution (around 3%) of putative proteases was detected in *Aspergillus* genomes despite genome size variations (Table 1). Among species, the proportions of proteins in each specific subgroup were also consistent, namely $25 \pm 1\%$ serine, $18 \pm 1\%$ metallo, $8 \pm 1\%$ amino and 5% aspartic proteases. Further ortholog clustering revealed only a very low number of extracellular “orphan” genes (9 putatively extracellular genes that have no homologs in the other six species included in this analysis). In fact, more than 60% of the extracellular putative proteases clusters were found to be ubiquitous by containing at least one gene per species. Moreover, the major extracellular protease regulator PrtT was also found to have a single presence per *Aspergillus* genome (except for *A. nidulans*) (Chen et al., 2014; Hagag et al., 2012; Mattern et al., 1992; Punt et al., 2008; Sharon et al., 2009). This might have brought assumptions that in during evolution, moderate divergence of protease genes has occurred in this genus since most of the encoding genes were well conserved at sequence level and only a small number of species-specific genes was identified. If this hypothesis applies, the production rate of extracellular proteases in all *Aspergillus* species should follow the distribution of encoded genes and have similar influence of the regulator prtT, meaning even protein count and quantitative measurement should be detected by proteomics. However, large variations in protein occurrence and abundance were found, indicating more profound mechanisms might be playing important roles.

For example, *A. flavus* and *N. fisheri* contain almost identical percentages of putative extracellular proteases in the genomes, but when cultivated on the same carbon sources a double amount of proteases and even higher abundance were identified in *A. flavus*. Should the protease productivity in *Aspergilli* follow the distribution of protease encoding genes, the production of each specific subgroup of protease would be consistent among categories and species. Indeed at least one semi-ubiquitous protease ortholog group of genes were identified for each sub-category of proteases on at least one of the substrates, such as OG-4 (lap2 amino protease, AN8445) (Szilagyi et al., 2013), OG-9 (pepE aspartic protease, AN2903) (Hortschansky et al., 2007), OG-16 (neutral metallo protease I, AO090011000036) (Liang et al., 2009)

and OG-30 (ProtA serine protease, 56689) (Braaksma et al., 2010). Moreover, a larger number of serine proteases were identified in all species, which correlates with the serine protease encoding genes being the largest subgroup of proteases in *Aspergilli*. However when quantitative measurements (abundance) were taken into account this correlation was absent because the most abundant individual proteases were never in the serine group, neither did the sum of abundances of the total serine group per species make this the dominant group (Supplementary table 4). In *A. flavus* (AFL2G_02631), *A. fumigatus* (Afu3g00650), and *A. oryzae* (AO090003000354) the most abundant protease belong to the amino protease group, while in *A. nidulans* (AN7962) and *A. terreus* (ATEG_04941) the most abundant proteases were metallo proteases. In the other species aspartic proteases (201655 and NFIA_073740) were more abundant. Taking *A. niger* as an example, the highest amount of serine proteases were indeed identified in the supernatant. However, based on comparative genomics the second most abundant group should be the metallo proteases, but no metallo protease was detected by proteomics on either substrate, which could possibly indicate that some of the proteases of the other classes also require metal ions (Dodia et al., 2008; Stadtman, 1990). The second most abundant group detected in *A. niger* were the aspartic proteases, including *pepA* (213261) (Guillemette et al., 2007), *opsA* (211797) (de Groot et al., 2009) and *opsB* (53364) (Braaksma et al., 2010). This demonstrated that even on the same substrate protease occurrence and abundance in *Aspergillus* species can differ significantly.

Although in industrial applications the productivity of proteases usually refers to the production rate of proteases per time per unit, in this study we aim to construct a snapshot of *Aspergilli* life style which reflects the protease production mechanisms, therefore the productivity measurements of proteases did not only include the occurrence or abundance of proteins but also the enzyme activities.

Summarizing the comparison results of genomics, proteomics and enzymology tests, a general trend was detected. WB induced higher total protease activity, richer proteomics profiles and more protein abundance than SBP. This strongly suggests that in *Aspergilli*, carbon source difference is the most important factor that influences protease productivity (see Supplementary Table 8 for monosaccharide composition of WB and SBP and (Eklund et al., 2014) for the composition of amino acids). This was further confirmed by the fact that using glucose (minimal medium +3% glucose) or glucose plus casein (minimal medium +1% glucose+1% casein) only low protease

induction could be detected in *A. nidulans* while sampled at the same time point as the WB or SBP cultures (data not shown).

While outside the scope of this study, it should be mentioned that it has been frequently reported that proteases are largely produced upon environment-induced cell lysis/damage (Malavazi et al., 2006), especially with sugar or nitrogen depletion (Jarai & Buxton, 1994; Nitsche et al., 2012; Schinko et al., 2010; Szilagyi et al., 2013). In our analysis, WB-based substrates showed higher protease activity as well as profiles than SBP-based substrates. This may indicate that WB cultivation resulted in a faster growth rate and earlier sugar depletion, and has therefore promoted an earlier production of proteases (Emri et al., 2006; Szilagyi et al., 2013). To further reveal the mechanisms behind *Aspergillus* protease productivity, aspects such as sugar consumption and fungal growth rate should be taken into account in future studies.

Besides amino, aspartic, metallo and serine proteases, a certain amount of ubiquitin and trypsin proteases were also detected by proteomics. The specificity of these proteases was not tested due to the unavailability of inhibitor kits. Although very low abundance was found for these proteins, these proteins may also take part in the total extracellular protease activity in *Aspergilli*.

Other factors may also cause variability between individual *Aspergillus* species. pH has been reported to be one of these factors (Poulsen et al., 2012; St Leger et al., 1999; te Biesebeke et al., 2005) and some of the data of this study supports this assumption. For example, AN6888 (*pepA*) has been reported to be an acidic protease (vanKuyk et al., 2000) and was not detected in *A. nidulans* (pH 7 on WB and 8 on SBP). In contrast, the ortholog of this protein in *A. niger* (201655) had high abundance (pH = 5-6) (Braaksma et al., 2010; Mattern et al., 1992; Morya et al., 2012; Nitsche et al., 2012).

Finally, even though 6 well known protein SCL predictors were employed in order to guarantee the accuracy of extracellular protease prediction, improvements could still be made for secretome prediction. Among all six used tools the prediction rate varied largely. The WoLF-PSORT prediction fitted best with the proteomics results, while Multi-LOC was most different from this (data not shown). Interestingly, although with low area abundance 25 proteases were detected extracellularly by proteomics that lack a translocation signal peptide. Most of them were found in

A. flavus and *A. terreus* (7 proteins each species), 3 were found in *A. oryzae* and the rest disseminated among the other species. If this was not caused by cell lysis or leakage, these proteins can be considered as indications of alternative secretion systems in *Aspergilli*. Hardly any of these proteins were correctly predicted by the SCL predictors we used. Hence, this study may also be of value as a testing or training set to improve currently existing prediction methods.

Conclusions

We have performed a series of *in silico* and biological experiments to gain understanding of protease production in *Aspergilli*. According to the results of comparative genomics *Aspergillus* species contain a similar proportion of protease encoding genes with serine proteases as the biggest group. The proteomics and enzymatic experiments generally confirm this composition, as serine proteases indeed make up the largest subcategory in the protease spectrum across the species. Furthermore, taking carbon source differences into account, wheat bran resulted in a higher induction of proteases than sugar beet pulp. An interesting variation of total protease activity, composition of the protease spectrum, and their abundance were observed between the species. The broadest set of proteases was found in *A. flavus*, while the highest overall protease abundance was found in *A. niger*, and the highest protease activity was detected for *A. fumigatus* in wheat bran and for *N. fischeri* in sugar beet pulp. It is very likely that even cultivated in an identical environment, the tested *Aspergillus* species were experiencing different physiology when sampled at the same time point. Concerning the high protein sequence conservation level (1E-20, sequence coverage 85%) among clustered proteases, it is likely that the variation of protease productivity is caused by more complicated mechanisms such as gene regulation related to environmental changes by carbon source differences (Hartmann et al., 2011; Katz et al., 2008; te Biesebeke et al., 2005) but not by enzymatic differences between the orthologous proteases themselves.

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Supplementary Tables

Supplementary Table 1,

File name: Supplementary_table_1.xls

File format: .xls

Title of data: The putative protease clusters in seven Aspergilli species.

Description of data: The subcellular localization prediction, protease family classification, Pfam domain prediction and functional annotation of each protein are listed. The proteins found in the original AspGD are marked bold.

Supplementary Table 2,

File name: Supplementary_table_2.xls

File format: .xls

Title of data: The “Orphan” proteases in 7 Aspergilli

Description of data: The “Orphan” proteases in 7 Aspergilli , together with their subcellular localization prediction, protease family classification, Pfam domain prediction and functional annotation.

Supplementary Table 3,

File name: Supplementary_table_3.xls

File format: xls

Title of data: The subcellular localization prediction on putative proteases.

Description of data: Using majority vote the compartment of the proteins was predicted by 6 different tools.

Supplementary Table 4,

File name: Supplementary_table_4.xls

File format: .xls

Title of data: The proteomics identification of proteases.

Description of data: the proteomics identification of protease in *Aspergilli* with SBP and WB respectively. Both ion count and spectral counts are provided.

Supplementary Table 5,

File name: Supplementary_table_5.xls

File format: .xls

Title of data: The original protease activity measurements without and with inhibitors.

Description of data: Two batches of biological replicates were measured with triplicated technical replicates of each sample, the triplicates were corrected by blanco and the mean/standard deviation values are listed in the table.

Supplementary Table 6,

File name: Supplementary_table_6.xls

File format: .xls

Title of data: the pH optimization pilot test with protease activity measurements

Description of data: The protease activity was measured under pH 7.2 (recommended by manufacturer), 4, 6 and 8. pH 6 was used to perform further experiments for less bias was caused.

Supplementary Table 7,

File name: Supplementary_table_7.xls

File format: .xls

Title of data: the time series enzyme activity measurements of *A. nidulans* on day 2, 3 and 4.

Description of data: The protease activity and inhibition tests were measured under pH 7.2 (recommended by manufacturer), on 48, 72 and 96 h post-inoculation.

Supplementary Table 8,

File name: Supplementary_table_8.xls

File format: .xls

Title of data: The monosaccharide composition of wheat bran and sugar beet pulp.



CHAPTER 7

General Discussion and Future Perspectives

General Discussion

This PhD thesis describes the study of a raw milk cheese ripened with the aid of a rich microbiota and also points out the protease and lipase enzyme amounts secreted by individual strains belong to this microbial community on a laboratory version of this cheese. With these results, further insight has been generated into the dynamics of the cheese ageing process, and reflects their biochemical consequences with regard to peptide, fatty acid and volatile profiles of cheese, which are the key aspects of the sensory characteristics in a cheese variety. Furthermore, this thesis compares the enzyme sources of *Aspergillus* spp. to the strains isolated from this cheese variety which are mainly consist of *Penicillium* spp.

Complex microbiota of Divle Cave cheese

This PhD thesis primarily focused on the assessment of the microbial communities and temporal evolution of them in a raw milk cheese (Chapter 2, Ozturkoglu-Budak et al., 2016a) and targeted to estimate the predominant strains contributing to the cheese ripening process, based on their proteolytic and lipolytic activities (Chapter 5).

The sensory characteristics of traditional cheeses cannot be dissociated from the diversity of their microbiota and microbial activities. In this thesis we determined a complex microbiota with species that belong to the classes of Bacilli (9), Gammaproteobacteria (3) and Actinobacteria (11), yeasts (5), and filamentous fungi (18). Besides, the wide diversity determined in cheese microbiota, particularly the diversity among filamentous fungi was more remarkable considering that 15 different species of *Penicillium* were identified. The increase of pH in the second half of the ripening process favored the growth of fungi and Actinobacteria and interestingly, no LAB were isolated except for *L. paraplantarum*. During the progress of ripening, the broad microbial diversity decreased in the cheese core, which is likely due to the effect of an anaerobic medium and the evolution of the chemical composition inside of the cheese (Chapter 3, Chapter 4). In contrast to lower counts determined inside of the cheese, particularly yeast and filamentous fungi on the outside of the cheese showed a gradual increase throughout the whole process and are detected at high levels at the end of ripening (Chapter 3). This result also confirmed the high alcohols, ketones, and esters determined as the major volatile groups during volatile profiling analyses (Chapter 4, Ozturkoglu-Budak et al., 2016b), which are probably produced

by the abundant yeast and filamentous fungi in Divle cheese. Another strong effect on the cheese flavor caused by the presence of a highly abundant and diverse fungal community was thought to be the formation of methyl ketones by their enzymatic activities (Molimard & Spinnler, 1996). They are produced by the oxidation of fatty acids and related secondary alcohols produced from FFA, and play an important role in determining the characteristic flavor components in Blue cheese (Gallois & Langlois, 1990). Since methyl ketones have typical odors detectable at a low perception threshold, similar to the results of this thesis these compounds were also reported as significant contributors to the aroma of Camembert in raw ewes' milk cheese (Di Cagno et al., 2003; Izco & Torre, 2000) and Blue cheeses (Hayaloglu et al., 2007).

Another interesting finding in this thesis is that no pathogenic microorganisms were determined in the cheese at the end (120th day) of ripening. Although we determined total coliforms, staphylococci and *Enterobacteriaceae* until the 90th day of ripening, they disappeared afterwards (Chapter 3). Therefore, it could be considered that the microbiological safety of Divle Cave cheese was ensured through the 90-days ripening process, even though it is produced from raw ewes' milk. This result demonstrated that the microbiological risk of cheese can be reduced by proper ripening processing. The ability of traditional cheeses to combat pathogens is related to native antipathogenic strains or microbial consortia rather than natural non-microbial inhibitors from milk (Montel et al., 2014). The inhibition seems to be associated with qualitative and quantitative composition of the above mentioned strains and the effects of their metabolites on other microorganisms. Particularly LAB, the dominant indigenous microorganisms of raw milk cheese produce pathogen-inhibiting substances such as bacteriocins, organic acids and hydrogen peroxide (Yoon et al., 2016).

Contributors of ripening and flavor development

In addition to their role in inhibition of food-borne pathogens, the naturally existing microbial community also develops the flavor and sensory characteristics of a specific cheese variety, by its enzyme profiles along with the native enzymes of milk and rennet. Analyses of SPME-GC-MS demonstrated a total of 110 volatile compounds derived from Divle Cave cheese during production and ripening comprising 13 acids, 18 alcohols, 12 ketones, 15 esters, 12 terpenes, 7 aldehydes,

18 hydrocarbons and 15 miscellaneous compounds (Chapter 4). PCA was applied to the total concentrations of these volatile groups to identify if there is a significant variation among cheeses produced in different dairy farms and also to determine the effect of production and ripening durations on the volatile profiles of cheese at that stage. This analysis demonstrated that a variable cheese medium and dynamic conditions were present in the structure of cheese during production and ripening, but the subtle differences derived from traditional production techniques are eliminated during the long ripening period (Chapter 4).

According to volatile profile analyses, carboxylic acids, secondary alcohols, methyl ketones, and ethyl esters were detected both frequently and at high concentration at different stages of the ripening process. High alcohol amount in the beginning of production until the 60th day of ripening could originate from the microbial populations of LAB, enterococci, and yeast derived from raw milk (Bontinis et al., 2012). However, when ripening progressed a decrease was observed in alcohol amounts, which could be the result of the esterification reaction between alcohols and fatty acids (Delgado et al., 2010). This observation correlates with the increasing ester amounts towards the end of ripening in Divle Cave cheese. In addition, high fat content of ewe's milk (4.58 %), corresponding to high fat content of cheese (16.5-19.5 %) during ripening, might have promoted the presence of high amount of lipolysis products showed in Chapter 4. Carboxylic acids are not only aroma components, but can also serve as precursors for the formation of esters, methyl ketones, alcohols and lactones (Collins et al., 2003). Due to their low detection thresholds, particularly short- and medium-chain carboxylic acids are thought to contribute significantly to the aroma profile of cheeses (Tavaria et al., 2004). Therefore in this study, lipolysis was hypothesized as the main biochemical reaction as a producer pathway of carboxylic fatty acids, which can be accepted as the initiator compound for the flavor development.

To answer this question, lipolysis and proteolysis levels were determined at specific points beginning from raw milk until the end of ripening by using GC and HPLC, respectively. Within this scope, accumulated FFA and small peptides are considered as representative of the degree of lipolysis and proteolysis. As expected, the lipolysis and proteolysis degree generally increased during the whole ripening period and a substantial accumulation of FFA and peptides was observed at the end of ripening.

This was confirmed by PCA of the peptide profiles, which clearly demonstrated that the samples grouped according to their age.

The FFA and peptide accumulations were due to the contributions of the secondary microbiota producing the catabolic enzymes that we isolated from cheese (Chapter 3), as well as native proteases, lipases and esterases present in raw milk. The action of proteases cause the degradation of hydrophobic peptides and release of low molecular weight peptides and amino acids (Gonzales De Llano et al., 1995), as is also demonstrated in this thesis in Chapter 3. The potential contributor strains to ripening were selected according to the protease and lipase production of individual strains on sterilized Divle Cave cheese, prepared from irradiated cheese. Yeasts, fungal and bacterial strains were inoculated on these culture mediums individually and incubated for growth and enzyme production at conditions adapted from authentic conditions. Lipases were mainly secreted by yeast and fungal strains (Chapter 5) and *Y. lipolytica* was identified as the best contributor to cheese ripening with regard to lipase production, particularly early in the ripening process. Fungal strains of *M. racemosus*, *P. commune*, *P. bifforme*, *P. brevicompactum* and *P. cavernicola* were the best lipase producing strains after *Y. lipolytica*. These results are in agreement with the data shown by (Desmaures, 2014), in which mold-ripened cheeses are characterized by an intense lipolysis as a result of the extracellular lipases particularly secreted from *Penicillium camemberti*, *P. roqueforti*, and *G. candidum*. In addition, most of the *Penicillium* spp. were also responsible for proteolysis during ripening. *P. brevicompactum* had the highest protease production followed by *P. corylophilum* and *P. chrysogenum*.

Among the bacterial strains, indigenous and secondary microbiota including *M. luteus*, *B. antiquum*, *B. stratosphericus*, *P. proteolytica* and *P. glacincola* were considered to be important for the ripening process. Unexpectedly, we detected lower proteolytic and lipolytic activities of LAB. This may be caused by the fact that these are intracellular enzymes in these bacteria that are not released into the cheese matrix without cell lysis (El Soda et al., 2000). The majority of the proteases secreted by most yeasts and filamentous fungi were found to belong to aspartic (acid) proteases. Milk clotting enzymes also belong to this group of enzymes (Claverie-Martin & Vega-Hernández, 2007). This finding indicates the importance of proteases produced

by yeast and fungal strains for cheese ripening. In contrast, inhibition studies revealed that the bacterial proteases mainly belonged to the serine protease class (Chapter 5).

FFA content determined by lipolysis analyses (Chapter 3) revealed similarities with the carboxylic acid content of volatile profile (Chapter 4) results, in which SCFA such as butyric (C4:0) and pentanoic (C5:0) acids were detected at the end of ripening. Among FA, SCFA (C4:0-C8:0) and MCFA (C10:0-C14:0) are known to contribute significantly to the aroma profile of cheeses due to their low detection thresholds (Kraggerud et al., 2008; Pinho et al., 2003; Tavaría et al., 2004; Zerfiridis et al., 1984). In particular the more volatile SCFA were reported to directly affect the aroma of cheese in many aged cheeses (Curioni & Bosset, 2002). Therefore, our similar results reiterate the significant contribution of these compounds to the aroma of Divle Cave cheese. Interestingly, the MCFA amount was higher in Divle Cave cheese than SCFA, despite the broad volatile diversity. The reason for this could be esterification of SCFA with alcohols (Delgado et al., 2010). A positive outcome of this is the prevention of rancid taste which can stem from excessive butyric acid accumulation.

Towards the end of ripening, the breakdown of accumulated branched-chain amino-acids leads to the production of flavor compounds including aldehydes, alcohols and acids (McSweeney, 2004). The enzymatic conversion of amino acids such as leucine, isoleucine and valine is catalyzed by aminotransferases (AT) and they formed the carboxylic acids of 3-methyl butanoic (isovaleric acid), 2-methyl butanoic and 2-methyl propanoic acid (isobutyric acid), respectively (Ardö, 2006; Smit et al., 2000). 3-methylbutanoic acid was the most abundant branched-chain fatty acid in Divle Cave cheese (Chapter 4). 3-methylbutanoic acid gives rancid, cheese-like and sweet flavor to sheep cheeses (Bergamini et al., 2010; Marilley & Casey, 2004; Yvon & Rijnen, 2001). 2-methyl propanoic acid (isobutyric acid) gives a very similar aromatic note to Camembert and Cheddar cheeses with the aroma of butyric acid, which is a short-chain fatty acid arising mainly from lipolysis (Yvon & Rijnen, 2001). The high amount of branched chain acids synthesized from amino acids are associated with severe proteolysis (Carbonell et al., 2002), which is similar to our finding in Divle Cave cheese in Chapter 3. Moreover, this excessive degradation of proteins in long ripened cheeses causes the formation of bitter peptides. This bitterness in cheese was described as a necessary consequence of proteolysis in

previous reports (Lemieux & Simard, 1991). Therefore, a better understanding of the origin of these compounds is needed in order to control and accelerate the formation of cheese flavor.

The potential of genomic analysis to further analyze the *Penicillium* proteases based on a reference study of *Aspergillus* proteases

State-of-the-art genomic methodologies combined with biochemistry can provide a more detailed insight into the diversity of proteases and other enzymes produced by fungi. As the genomes of the *Penicillium* strains isolated from Divle cheese are not available, we performed a reference study on 7 fully sequenced *Aspergilli* in relation to their protease content and compared this to the production of proteases by these strains in Chapter 6. *Aspergillus* is a sister genus of *Penicillium* and therefore justified as a reference group for this study. Several species of *Aspergillus* have been reported to produce a variety of proteases (Conesa et al., 2001). The extracellular enzyme productions of seven *Aspergillus* species were tested during growth on wheat bran and sugar beet pulp. A combined analysis of comparative genomics, proteomics and enzymology tests was performed (Chapter 6) to compare the protease productivities of these *Aspergilli*. Similar to bacterial strains isolated from Divle Cave cheese, all *Aspergilli* strains were inhibited predominantly by PMSF; therefore it was considered that under these growth conditions, serine proteases encompass the largest group in their produced protease spectrum. Wheat bran showed a better induction of proteases than sugar beet pulp.

In future studies, we would like to obtain genome sequences for the strains isolated from Divle cheese and use this for a proteomic analysis during growth on irradiated cheese, as well as for proteomic analysis of samples taken from the natural cheese at different stages of ripening. This will enable us to identify the proteases and lipases involved in the ripening process and select enzymes that could be specifically produced and added to cheese to stimulate the ripening process. From an economical point of view, utilization of pure microbial strain as an adjunct in cheese production could be a more feasible alternative than usage of a purified enzyme, due to the high cost of enzyme isolation and purification.

Concluding remarks

As a general result, with this thesis study, we provide data for characterization of an artisanal cheese made from raw ewes' milk which is of great importance for the establishment of the Protected Designation of Origin (PDO) for Divle Cave cheese.

The obtained data establish a link between specific microbial species, protease and lipase activities of this community and their effects on cheese flavor, which particularly emphasize on the main biochemical events of proteolysis and lipolysis during ripening. Therefore, it highlights that the native and secondary microbiota and their enzymes are associated with more complex volatile profiles and higher scores for some sensorial attributes.

Moreover, these results are highly valuable for the eventual standardized manufacture of this cheese under controlled conditions and on an industrial scale while maintaining the traditional characteristics of this cheese. In addition, to isolate the new spontaneous microbiota such as NSLAB and secondary cultures from raw milk has crucial importance to restore the lost territory in diversity of cheese-related microbiota and to create a geographical ownership (Dutton, 2014). Several ongoing projects planning to isolate microorganisms from raw milk, to culture them and then to put them back into pasteurized milk for not losing the traditional cheeses belong to a specific territory (Dutton, 2014). However, selection of wild strains for usage as starter or adjunct culture is a laborious project in which many aspects should be considered. Although wild strains are known to be less sensitive to bacteriophage attack (Smid et al., 2014), testing their resistance to bacteriophages remains a requirement. In recent years, cheese producers have been mainly interested in non-starter bacteria due to problems associated with the activity loss of starter cultures arising from daily transfers. As a consequence in of practises in cheese technology, previously used starter cultures were determined to have lost their production of the α -keto acid decarboxylase enzymes which are needed to obtain a rich and complex cheese flavor (Ardö, 2006). A detailed study of the microbial community of complex and undefined cheese starter cultures could be a solution to this problem (Smid et al., 2014).

Future research should also apply new omics methods on milk, cheese and environmental samples for describing the microbiota. This will increase our

knowledge of the source, nature and metabolic activities of different populations in the cheese microbiome during the cheese production process. Especially using metagenome sequencing will provide significant evidence for taxonomical and functional characterization of the microbiota (Erkuş et al., 2013). These new methods will also lead to a better understanding of the numerous interactions between different microorganisms in cheese (Escobar-Zepeda et al., 2016).

It is clear that there are many artisanal cheeses hosting diverse microorganisms waiting to be discovered. To understand the composition of the microbiota and their role in cheese ripening is highly important. Co-cultivation studies might provide an insight into the interactions between the strains, with the aim of understanding, controlling and predicting the cheese production and ripening processes.

This study has a potential to guide not only new researchers but also starter culture and cheese producers. Furthermore, in case one of these strains will be used as an adjunct in a different type of cheese, the data of this thesis will strongly contribute to prediction of the additional enzymatic capacity of the microbiota as a whole.

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Samenvatting

Samenvatting

Divle Cave kaas is gemaakt van rauwe schapenmelk en wordt gerijpt met behulp van een rijke microbiota. Individuele stammen van deze microbiota scheiden een breed scala aan proteases en lipases uit. De studie beschreven in dit proefschrift richt zich voornamelijk op het definiëren van de diversiteit en de evolutie van de complexe microbiota tijdens de traditionele rijping van de Divle Cave kaas. De protease en lipase activiteiten van bepaalde stammen wordt beschreven. De dominante micro-organismen die het meeste bijdragen aan de traditionele productie en rijping processen van Divle Cave kaas worden bestudeerd met betrekking tot de mate van eiwit en vet afbraak, en de vorming van vrijkomende vluchtige verbindingen te analyseren.

De moleculaire identificatie van bacteriën, filamenteuze schimmels en gisten die aanwezig zijn in het midden en aan het eind van de rijping van Divle Cave kaas is beschreven in **Hoofdstuk 2**. Een complexe microbiota met een brede diversiteit werd waargenomen, inclusief een aantal unieke en intrinsieke micro-organismen, zowel aan de buitenkant als in de kaas. Dit omvatte species van Bacilli, Gammaproteobacteriën en Actinobacteriën, gisten en filamenteuze schimmels. Met name de diversiteit van filamenteuze schimmels was opmerkelijk, met 15 verschillende species van *Penicillium*. Gedurende het rijpingsproces nam de microbiële diversiteit af in de kern van de kaas, waarschijnlijk als gevolg van de anaerobe condities en de ontwikkeling van de chemische samenstelling in de kaas. De verhoogde pH in de tweede helft van het rijpingsproces bevorderde de groei van schimmels en Actinobacteriën en onderdrukte de groei van coliforme bacteriën.

Hoofdstuk 3 beschrijft de rol van de aanwezige microbiële gemeenschap in de tijdsafhankelijke ontwikkeling van proteolyse en lipolyse profielen gedurende productie en rijpening. In het algemeen namen de lipolyse en proteolyse toe gedurende de hele rijping periode met relatief hoge niveaus aan het einde van de rijping. Dit kan verklaard worden door de endogene en secundaire microbiota samen met de enzymen die reeds aanwezig waren in de rauwe melk. Ophoping van FFA en kleine peptiden zijn representatief voor de mate van lipolyse en proteolyse.

De natuurlijk aanwezige microbiële gemeenschap onderdrukt niet alleen de groei van andere micro-organismen, maar is ook verantwoordelijk voor de ontwikkeling

van de smaak en aroma karakteristieken van een specifieke kaas variëteit, door middel van hun enzym profiel dat de natuurlijk aanwezige enzymen in melk complementeert. In **Hoofdstuk 4** wordt de tijdsafhankelijke ontwikkeling van de chemische eigenschappen en de vluchtige verbindingen gedurende de rijping van Divle Cave kaas beschreven. 110 vluchtige verbindingen werden waargenomen, waaronder vooral carboxyl zuren, alcoholen, ketonen, esters en terpenen. De aanwezigheid en concentratie van deze vluchtige verbindingen varieerde in de verschillende fasen van de rijping. In deze studie werden ook de meest voorkomende sleutel aroma componenten geïdentificeerd, die waarschijnlijk belangrijk zijn voor de karakteristieke geur en smaak van deze ambachtelijke kaas van rauwe schaapsmelk.

Een belangrijk aspect is het identificeren van die micro-organismen die verantwoordelijke zijn voor de proteolyse en lipolyse. **Hoofdstuk 5** beschrijft de protease en lipase productie van verschillende gisten, filamenteuze schimmels en bacteriën, die geïsoleerd waren Divle Cave kaas. De resultaten van deze studie bevestigen de hoge frequentie van stammen met een goede enzym productie in Divle Cave kaas en hebben tot de identificatie van de beste protease en lipase producerende stammen geleid. Om te bepalen tot welke klasse de proteases hoorden, werd gebruik gemaakt van klasse-specifieke protease inhibitoren.

Hoofdstuk 6 demonstreert de waarde van genomische analyses voor de beschrijving van proteolytische profielen van schimmels. De protease productiviteit van verschillende *Aspergilli* is bepaald door een combinatie van comparatieve genomics, proteomics en enzymologie testen. Hiervoor werden de extracellulaire protease activiteiten van zeven *Aspergillus* species getest na groei op tarwe zemelen en suikerbieten pulp. De inductie van protease activiteit op tarwe zemelen was hoger dan op suikerbieten pulp. Protease assays met en zonder klasse-specifieke inhibitoren werden gebruikt om de activiteit over de verschillende protease klassen te verdelen. Door middel van proteomics werden de proteases verantwoordelijk voor de activiteit geïdentificeerd. De hoogste inhibitie van protease activiteit van de *Aspergilli* gebeurde door PMSF, wat aangaf dat onder deze groei condities met name serine proteases geproduceerd werden.

De resultaten van dit proefschrift zullen uiteindelijk een nauwkeurige voorspelling van de dominante soorten mogelijk maken, waarmee op een gecontroleerde wijze geselecteerde stammen toegevoegd kunnen worden. Door een gestandaardiseerde en gecontroleerde kaasproductie en rijping wordt het productieproces verbeterd met behoud van het traditionele karakter van de kaas.

De verkregen resultaten kunnen ook bijdragen aan de innovatie van de industriële kaasproductie door middel van het toevoegen van nieuwe microbiota die bijdraagt aan een nieuw of verbeterd karakter van de kaas. Vanuit economisch oogpunt zou het gebruik van microbiële reïnculturen als additief in de kaasproductie een beter alternatief zijn dan het gebruik van een gezuiverd enzym, vanwege de hoge kosten van de productie van zulke enzymen.

Curriculum Vitae

Sebnem Ozturkoglu Budak was born in Adana, Turkey on March 18th 1978. She attended Ankara University in 1997 and graduated with a BSc diploma in Food Engineering in 2001. She obtained her degree of Master of Science in Food Engineering from Middle East Technical University (Ankara, Turkey) in 2004. During her main Master research training, she investigated the growth of *Listeria* spp. during White cheese production and storage under the project called “Behavior and control of *Listeria innocua* during manufacture and storage of Turkish White Cheese”. In September 2010, she started her PhD in Fungal Physiology in conjunction with Utrecht University, and CBS-KNAW Fungal Biodiversity Center, The Netherlands. The PhD project was carried out at CBS-KNAW Fungal Biodiversity Center under the supervision of Prof. Dr. ir. Ronald P. de Vries and Dr. Peter Bron from NIZO Food Reserch, The Netherlands.

List of Publication

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S. Ozturkoglu-Budak, C. Akal, A. Yetisemiyen. 2016. Effect of dried nut fortification on functional, physicochemical, textural and microbiological properties of yogurt. *Journal of Dairy Science* (Accepted for publication).

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S. Ozturkoglu Budak, I. Aksahin. 2016. Multivariate characterization of fresh tomatoes and tomato-based products based on mineral contents including major-trace elements and heavy metals. *Journal of Food and Nutrition Research* 56: 11-21.

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Abstracts and Presentations

S. Ozturkoglu Budak, N. Turkmen. The IDF Parallel Symposia Conference 2016. April 11-13, 2016. Dublin, Ireland (Poster presentation)

S. Ozturkoglu Budak. VI International Conference on Environmental, Industrial and Applied Microbiology. October 28-30, 2015, Barcelona, Spain (Virtual presentation)

S. Ozturkoglu Budak, M. Figge, J. Haubracken, R. P de Vries. 7th International Symposium on Sheep, Goat and other non-Cow Milk, March 23-25, 2015, Limassol, South Cyprus (Oral presentation)

S. Ozturkoglu Budak, Ceren Akal, Atila Yetişemiyen. 2nd International Congress on Food Technology, November 23-25, 2014, Antalya, Turkey (Poster presentation)

S. Ozturkoglu Budak, J. Haubracken, M. Figge, R. P de Vries. 8th NIZO Dairy Programme. September 11-13, 2013, Papendal, Arnhem, Netherlands. (Poster presentation)

S. Ozturkoglu Budak, İnci Akşahin. International Food Congress-Novel Approaches in Food Industry, May 26-29, 2011, İzmir, Turkey (Oral presentation)

S. Ozturkoglu Budak, H.Z. Şenyuva, John Gilbert, Vinai Pitiyont. 3rd Congress of European Microbiologists – FEMS 2009, June 28- July 2, 2009, Gothenburg, Sweden.

N. Gurel, **S. Ozturkoglu Budak**, H. Z Senyuva. 5th Black Sea Basin Conference on Analytical Chemistry, September, 23-26 2009, Ordu, Turkey (Poster presentation)

S. Ozturkoglu Budak, H.Z, Senyuva, J. Gilbert. XII. International Congress of Mycology. August, 5-9, 2008, İstanbul, Turkey (Oral presentation)

H.Z. Şenyuva, J. Gilbert, **S. Ozturkoglu**, S. Özcan, D. Çimen. 122nd AOAC Annual Meeting & Exposition. September 21-25, 2008, USA (Poster presentation)

H.Z. Senyuva J. Gilbert, **S. Ozturkoglu**. 3rd International Symposium on Recent Advances in Food Analysis. November 7-9, 2007, Prague, Czech Republic. (Poster presentation)

S. Ozturkoglu, Senyuva H.Z, Gilbert John. 2nd International Congress on Food and Nutrition, TÜBİTAK- Marmara Research Center, October, 24-26 2007, Istanbul, Turkey (Poster presentation)

S. Ozturkoglu, Yaralı A, Öztan A. 26th World Congress and Exhibition of The International Society for Fat Research, Modern Aspects of Fats and Oils. September, 25-28 2006, Prague, Czech Republic. (Poster presentation)

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