

Title	Modification of cheese flavour through the use of surface microbiota
Authors	Bertuzzi, Andrea S.
Publication date	2017
Original Citation	Bertuzzi, A. 2017. Modification of cheese flavour through the use of surface microbiota. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-08-21 08:03:33
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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Modification of cheese flavour through the use of surface microbiota

A Thesis Presented to the National University of Ireland

For the Degree of Doctor of Philosophy

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study, leading to the award of PhD is entirely my own work and has not been submitted for another degree, either University College Cork, or elsewhere.

Signed_____

Student number: 113222509

Abstract

Surface microorganisms in surface-ripened cheese, combined with lactic acid bacteria, contribute to lipolysis and proteolysis, degradation of free fatty acids and free amino acids, and the metabolism of lactose, citrate and lactate, with a consequent biosynthesis of volatile flavour compounds. The intense metabolic activity of the yeasts and Gram-positive bacteria on the cheese surface is evident in surface-ripened cheese, where the resident surface microbial population is responsible for the characteristic strong flavour, typical for this cheese variety. Although cheese is a widely studied fermented food, the biochemical mechanisms which lead to the biosynthesis of volatile compounds, and the development of cheese flavour, are not completely clear and need to be explained. Therefore, the aim of the studies in this thesis was to investigate the development of flavour volatile compounds in cheese, produced by cheese microbiota, both as single strains in model systems, and when applied to cheese curd surface as simple or more complex culture mixes containing yeasts and Gram-positive bacteria.

Through the use of the metagenomic and enzymatic assays, it has been possible to investigate the potential flavour-forming ability of the cheese microorganisms, and correlate the results to volatile flavour compounds detected with gas-chromatography. Our results have shown how variations in the microbial population influence the flavour development in cheese during ripening. This research may be of industrial benefit for the manipulation of the microbiota and the production of fermented food with specific flavour characteristics.

Publications

Bertuzzi, A. S., Kilcawley, K. N., Sheehan, J. J., O’Sullivan, M. G., Kennedy, D., McSweeney, P. L. H., and Rea M. C. (2017). Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese. **Published in** *International Dairy Journal*, 72, 44-54.

Bertuzzi, A. S., Guinane, C. M, Crispie, F., Kilcawley, K. N., McSweeney, P. L. H., Rea, M. C. (2017). Genome Sequence of *Staphylococcus saprophyticus* DPC5671, a strain isolated from Cheddar cheese. **Published in** *Genome Announcements*. 5, e00193-17.

Bertuzzi, A. S., McSweeney, P. L. H., Rea, M. C., Kilcawley, K. N. Detection of volatile compounds of cheese and their contribution to the flavour profile of surface-ripened cheese (2018). **Published in** *Comprehensive Reviews in Food Science and Food Safety*, 17, 371-390.

Bertuzzi, A. S., Walsh, A. M., Sheehan, J. J., Cotter, P. D., Crispie, F., McSweeney, P. L. H., Kilcawley, K. N., Rea, M. C. Omics-based insights into flavour development and microbial succession within surface-ripened cheese. **Published in** *mSystem*, 3, e00211-17.

In my PhD research I have actively contributed with the development and optimization of Model System 1 to the following publication:

Stefanovic, E., Thierry, A., Maillard, M-B., **Bertuzzi, A. S.**, Rea, M. C., Fitzgerald, G., McAuliffe, O., Kilcawley, K. N (2017). Strains of the *Lactobacillus casei* group show diverse abilities for the production of flavour compounds in 2 model systems. **Published in** *Journal of Dairy Science*, 100, 6918-6929.

List of abbreviations

AEDA	Aroma extraction dilution analysis
AT	Aminotransferase
CAR	Carboxen
CAS	Chemical abstracts service
CEP	Cell external proteinase
CFE	Cell free extract
CFU	Colony-forming unit
CDS	Coding DNA sequence
CNS	Coagulase negative staphylococcus
Cryo-SEM	Cryo-scanning electron microscope
DI-SPME	Direct solid-phase microextraction
DPC	Dairy product centre
DVB	Divinylbenzene
EFSA	European food safety authority
FAA	Free amino acids
FDM	Fat in dry matter
FFA	Free fatty acid
FID	Flame ionization detector
FPD	Flame photometric detector
GC	Gas chromatography
GC×GC	Two-dimensional gas chromatography

GCMS	Gas chromatography-mass spectrometry
GC-O	Gas chromatography olfactometry
GDH	Glutamate dehydrogenase
HPLC	High pressure liquid chromatography
HS	Headspace
HS-SPME	Headspace solid-phase microextraction
HSSE	Headspace sorptive extraction
HSP	Heat shock protein
ITEX	In-tube extraction
YGC	Yeast extract glucose chloramphenicol agar
LAB	Lactic acid bacteria
LRI	Linear retention index
MIC	Minimum inhibitory concentration
MDS	Multidimensional scaling
MNFS	Moisture in non-fat substances
MS	Mass spectrometry
NCIMB	National collection industrial and marine bacteria
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
P&T	Purge and trap
PDMS	Polydimethylsiloxane
PFGE	Pulsed gel field electrophoresis
PLS	Partial least square regression

PFPD	Pulsed flame photometric detector
QqQ	Triple quadrupole
RDA	Ranking descriptive analysis
REF	Reference
SAFE	Solvent assisted flavour extraction
SBSE	Stir bar sorptive extraction
SD	Standard deviation
SDE	Simultaneous distillation extraction
SIM	Selected ion monitoring
SIS	Selected ion storage
S/M	Salt in moisture
SN	Soluble nitrogen
SPDE	Solid phase dynamic extraction
SPME	Solid-phase microextraction
TD	Thermal desorption
TIC	Total ion current
TN	Total nitrogen
TOF	Time of flight
TSA	Trypticase soy agar
TSB	Trypticase soy broth
urea-PAGE	Urea polyacrylamide gel electrophoresis

Chapter 1

Literature Review

Detection of volatile compounds of cheese and their contribution to the
flavour profile of surface-ripened cheese

This chapter has been published in *Comprehensive Reviews in Food Science and Food Safety* (2018), 17, 371-390, DOI: 10.1111/1541-4337.12332

1.1. Abstract

The volatiles responsible for the typical aroma of cheese are produced mainly by lipolytic, proteolytic pathways, and by the metabolism of lactose, lactate and citrate. The volatile profile of cheese may be determined using gas chromatography (GC), which includes the extraction, separation, and detection of volatiles. A wide range of extraction techniques is available, and technological improvements have been developed in GC separation and detection, that enhance our understanding of the role of individual key volatiles to cheese flavour. To date, for surface-ripened cheese, the main volatiles detected that contribute to flavour include acids, ketones, alcohols, and sulphur compounds. However, based on the limited number of studies undertaken and the approaches used, it appears that a significant degree of bias possibly exists that may have over- or under-estimated the impact of specific chemical classes involved in the flavour of these types of cheese.

1.2. Introduction

Flavour development of cheese is related to milk quality, processing operations and, most importantly, microbial activity. The cheese environment is characterized by a complexity of microbial populations, which contribute to numerous biochemical reactions, leading to the formation of cheese flavour. The resident microorganisms contribute to lipolysis and proteolysis, and metabolism of lactose, lactate and citrate, which are the pathways responsible for the formation of volatile compounds in cheese (Fox et al., 2017a; McSweeney, 2017).

The combination of volatile compounds and their interactions contributes primarily to the formation of aroma and taste, which together constitute cheese flavour (Zehentbauer and Reineccius, 2002; Niimi et al., 2015). Cheese flavour, in combination with the overall appearance of cheese and its consistency, is decisive for consumer selection and preference (Drake and Delahunty, 2017; Kilcawley, 2017). Consequently, the importance of studying cheese flavour is related principally to both the acceptance of a cheese within the marketplace and the perception of a cheese's flavour by the consumer (Drake and Delahunty, 2017). It is known that the flavour preference of consumers is motivated by the stimulation of human chemical senses, particularly those for odour (aroma) and taste (D'Acampora Zellner et al., 2008). Specifically, the perception of flavour is mainly driven by the combination of active volatile compounds perceived in the orthonasal, and/or retronasal cavity. Olfactory perception is a complex biological phenomenon triggered by certain volatile molecules, generally hydrophobic,

which interact with odorant receptors (G protein–coupled receptors) in the olfactory epithelium of the nasal cavity (Breer et al., 2006; Dunkel et al., 2014). A precise chemical characterization of the mixture of potential stimulants in food plays an important role in investigating olfactory perception and to evaluate food flavour.

The analysis of volatile compounds in food is commonly performed using gas chromatographic (GC) techniques, employing very sensitive technologies capable of detecting trace levels of volatile compounds. It is critical that GC detection is of high efficiency, especially as volatile compounds, which are present at trace levels, can be easily perceived during sensory analysis (low perception threshold) and can contribute significantly to the flavour profile of cheese (Hummel et al., 1997). The complete analysis of the volatile aroma components of cheese is a complex procedure which includes extraction/concentration techniques, chromatographic separation, and identification/quantification. However, it is necessary to concentrate the volatiles extracted from the food matrix, because the techniques available for the detection of volatiles are still less sensitive than the human nose, which is able to perceive odours at extremely low concentrations (parts per billion to parts per trillion) (Bartlett et al., 1997; Mackie et al., 1998). The techniques and analytical tools available can be adapted to optimize results for a particular cheese variety, such as surface-ripened cheese (Valero et al., 2001; Lecanu et al., 2002; Mondello et al., 2005; Salum et al., 2017).

Surface-ripened cheese varieties (e.g., Münster, Tilsit, Livarot, Limburger and Comté) are characterized by the growth of surface bacteria and yeasts on the cheese surface during ripening, which are responsible for the strong typical flavour and the characteristic “glistening” appearance (Desmasures et al., 2015; Fox et al., 2017b;

Mounier et al., 2017). This cheese type is characterized by an intense flavour, conferred by a wide variety of volatile compounds (acids, ketones, alcohols, esters and sulphur compounds), which can be detected in different ratios, in relation to the techniques and methods used for volatile analysis (Valero et al., 2001; Lecanu et al., 2002).

This review describes the formation of the main volatile classes that contribute to cheese flavour, and it examines the analytical methodologies used to detect volatile compounds in dairy products, highlighting the advantages and potential shortcomings of specific techniques. Subsequently, the flavour of surface-ripened cheese is described and the main volatiles, which contribute to the flavour of the surface-ripened cheese, are then presented, focusing on their flavour contribution and the methods used for their detection.

1.3. Flavour development in cheese

The biochemical processes which lead to the synthesis of volatile compounds are very complex (Ardö et al., 2017; Ganesan and Weimer, 2017; Kilcawley, 2017; McSweeney, 2017; McSweeney et al., 2017; Thierry et al., 2017). It is known that the volatile compounds identified in cheese are mainly the products of lipolysis, proteolysis, metabolism of residual lactose, lactate and citrate. They also include metabolism of free fatty acids (FFAs), and free amino acids (FAAs) (Fox et al., 2017a; McSweeney, 2017).

Lactose is the principal carbohydrate present in milk (~4.8% in raw milk). During cheese manufacture, the starter lactic acid bacteria (LAB) convert some lactose into lactate, which is responsible for the decrease in pH. Lactate can be further metabolized by LAB into formate, acetaldehyde, ethanol, and acetate. In surface-ripened cheese, the lactate is also converted by yeasts and molds into CO₂ and H₂O, while in Swiss-type cheese, propionic acid bacteria metabolize lactate into propionate, acetate, and CO₂ (McSweeney et al., 2017). After cheese manufacture, the residual citrate present in the curd can be metabolized by citrate-positive LAB into acetate and lactate, also producing other important flavour compounds such as acetoin, 2,3-butanediol, diacetyl, and 2-butanone (Singh et al., 2003; McSweeney et al., 2017).

The catabolism of FAAs produces mainly aldehydes, alcohols, carboxylic acids, amines, and sulphur compounds (Fig. 1.1) (Ganesan and Weimer, 2017; Kilcawley, 2017). Aromatic amino acids, branched-chain amino acids, methionine, and aspartic acid are converted into α -keto acids by a transamination reaction, catalysed by amino

acid aminotransferase. The resulting α -keto acids are then further degraded to branched-chain and aromatic aldehydes, acyl-CoA, hydroxy acids, and methanethiol (Smit et al., 2005; Ardö, 2006; Ganesan and Weimer, 2017). The transamination of valine, isoleucine, and leucine leads to the production of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, respectively (Singh et al., 2003; Ardö, 2006). Aspartic acid can be converted by transamination into oxaloacetate and further into acetoin, diacetyl, or 2,3-butanediol (Ardö, 2006). Recently, Peralta *et al.* (2014) observed that, for *Lactobacillus paracasei*, the transamination of aspartic acid has a fundamental role in the production of diacetyl. Previously, Morgan (1976) had observed that in *Lactococcus lactis* var. *multigenes* enzymatic pathways exist for the reduction of phenylalanine and methionine to phenylacetaldehyde and methional, respectively. However, aromatic aldehydes are produced mainly through spontaneous chemical oxidation of α -keto acids, derived from tryptophan and phenylalanine, producing benzaldehyde, or from tryptophan, generating nitrogen-containing compounds such as indoleacetate, indol-3-aldehyde, and skatole (Gao et al., 1997; Yvon and Rijnen, 2001).

Aldehydes can be converted to their corresponding alcohols by alcohol dehydrogenase, or oxidized to their corresponding carboxylic acids by aldehyde dehydrogenase (Yvon and Rijnen, 2001; Ganesan and Weimer, 2017; Kilcawley, 2017). The biosynthesis of primary and aromatic alcohols, and relative carboxylic acids, is mainly attributable to the metabolism of molds and yeast. *Geotrichum candidum* and yeasts isolated from Camembert, have the ability to produce alcohols and carboxylic acids, through the metabolism of FAAs (Yvon and Rijnen, 2001). The presence of alcohols, such as 2-methylpropanol, 2-methylbutanol, 3-methylbutanol, and

phenylethanol, together with the presence of carboxylic acids, such as 2-methylpropanoic, 2-methylbutanoic, 3-methylbutanoic, and phenylacetic acid, are indicators of the reduction of branched-chain and aromatic FAAs (Yvon and Rijnen, 2001; Singh et al., 2003).

The metabolism of FAAs by decarboxylation can produce amines, which are not associated with quality cheese, due to their adverse health implications and often poor flavour. Gram-positive bacteria, including LAB, are considered the principal producers of amines, due to their high decarboxylase activity. In some ripened cheese varieties (e.g. Parmesan, Cheddar, Feta, Pecorino), an extensive proteolysis can support the release of high amounts of FAAs, which are the fundamental substrates for the biosynthesis of amines (Linares et al., 2011; Marino et al., 2013; Benkerroum et al., 2016). The most important amines are histamine, tyramine, putrescine, cadaverine, and β -phenyl ethylamine, which are produced from the decarboxylation of histidine, tyrosine, ornithine, lysine, and β -phenylalanine, respectively (Linares et al., 2011; Marino et al., 2013).

In addition, catabolism of FAAs can be initiated by elimination reactions, catalysed by amino acid lyase, which cleave the side chain of amino acids. This pathway leads to the synthesis of phenol and indole from the metabolism of aromatic amino acids, and the production of methanethiol from methionine (Ardö, 2006; Ganesan and Weimer, 2017). *B. linens* (by methionine γ -lyase) and *L. lactis* (by cystathionine β - and γ -lyase) were reported to produce methanethiol, through elimination reactions, starting from methionine (Yvon and Rijnen, 2001). Yeasts, *Micrococcaceae*, and *Brevibacterium linens*, commonly found in surface-ripened cheese, have the ability to

cleave the side chains of tyrosine and tryptophan, releasing phenol and indole, respectively (Jollivet et al., 1992; Jollivet et al., 1994). For this reason, phenol is a flavour compound frequently detected in surface-ripened cheese (Urbach, 1997).

The further catabolism of methanethiol via oxidative reactions, leads to the production of dimethyldisulphide and dimethyltrisulphide. Sulphur compounds are particularly important contributors to the flavour of surface-ripened cheese, due to their very low odour perception, and their strong garlic and very ripe cheese odours (Sablé and Cottenceau, 1999; Curioni and Bosset, 2002). Numerous LAB species are capable of degrading methanethiol, producing dimethyldisulphide and dimethyltrisulphide. However, *Micrococcaceae*, *B. linens*, and *G. candidum*, commonly found on surface-ripened cheese, are considered the main producers of sulphur compounds, which are detected at high concentrations in these varieties (Jollivet et al., 1992; Jollivet et al., 1994).

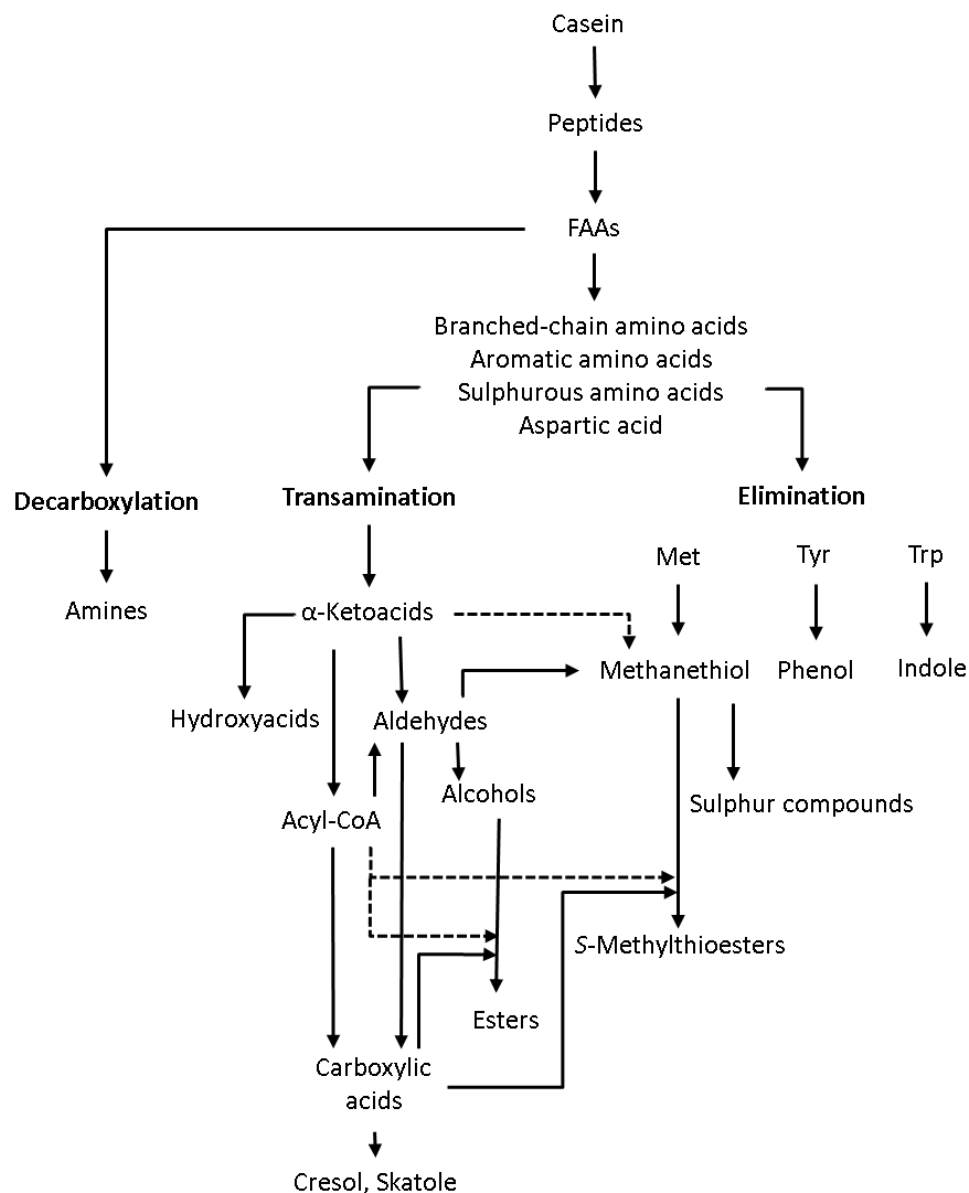


Figure 1.1. General pathways for catabolism of free amino acids in cheese, modified from Yvon and Rijnen (2001).

The lipolysis of the triglycerides by microbial and indigenous milk enzymes, and enzymes from rennet pastes, results in the development of medium- (carbon chain lengths ≤ 10), and long-chain (carbon chain lengths > 10) FFAs (Collins et al., 2003; Thierry et al., 2017). The enzymes catalyse the hydrolysis of triglycerides, with the

formation of FFAs, di- and mono-glycerides, and glycerol. The abundance of FFAs directly effects the flavour properties of cheese, and ranges from low levels in Edam cheese (total amount ~300 ppm) to very high levels in Blue veined cheese (total amount ~30,000 ppm) (Woo and Lindsay, 1984). The flavour contribution of FFAs in cheese is mainly influenced by the pH. The pH has an influence on the flavour, as the FFAs, at high pH levels, are less flavour active, and are perceived as soapy flavours as they are converted to non-volatile salts, while at low pH they exist in free form and are perceived as rancid at high concentrations (Singh et al., 2003; Alewijn, 2006).

FFAs contribute to the formation of the cheese flavour, not only directly, but also indirectly as they are precursors of methyl ketones, secondary alcohols, straight-chain aldehydes, lactones, esters, and S-thioesters (Fig. 1.2) (Collins et al., 2003; Smit et al., 2005; Thierry et al., 2017). FFAs can be oxidized to β -ketoacids, and then decarboxylated to corresponding methyl ketones, with one carbon less (Collins et al., 2003; Kilcawley, 2017; Thierry et al., 2017). The biosynthesis of methyl ketones, is mainly attributed to mold metabolism, e.g., *Penicillium roqueforti*, *Penicillium camemberti*, and *G. candidum*, and due to their typical odours and low perception thresholds, are most likely responsible for the characteristic flavour of surface-mold ripened, and blue-veined cheese (Sablé and Cottenceau, 1999; Curioni and Bosset, 2002; Collins et al., 2003). However, it is also postulated that ketones can be produced by heating milk, or directly from esterification of β -keto acids (Alewijn, 2006). Urbach (1997) suggested that some ketones may be formed directly from β -ketoacids in the GC inlet, possibly resulting in the over estimation of ketones in the volatile profile of cheese.

Ketones can be further converted by reductase to secondary alcohols (Collins et al., 2003; Kilcawley, 2017). This metabolic pathway is mainly attributed to molds (e.g., *Penicillium spp.*), which are considered responsible for the production of secondary alcohols (e.g., 2-pentanol, 2-heptanol, and 2-nonanol) in blue-veined cheese (Collins et al., 2003). However, secondary alcohols are thought not to strongly contribute to the cheese flavour (Singh et al., 2003), even if 2-heptanol was identified as a key odorant of Gorgonzola and Grana Padano cheese (Curioni and Bosset, 2002).

Although unsaturated fatty acids, both free and esterified, can be involved in non-enzymatic autooxidation reactions, these reactions do not commonly occur in cheese, as cheese is a very reducing environment. However, when this reaction occurs it produces straight-chain aldehydes, which are characterized by “green grass-like” aromas, producing compounds, such as propanal, hexanal, heptanal, octanal, nonanal, 2-decenal, and 2-undecenal, among others (Alewijn, 2006).

Esters are another group of volatile compounds which are involved indirectly in the metabolism of FFAs, whereby the esterification and alcoholysis reactions, lead to the biosynthesis of esters (Collins et al., 2003; Liu et al., 2004). Esterification is the formation of esters from alcohols and carboxylic acids by esterase, whereas alcoholysis is the production of esters from alcohols and acylglycerols, or acyl-CoA (derived from the metabolism of FFAs, FFAs, and/or carbohydrates), by acyltransferase. In alcoholysis, fatty acyl groups from acylglycerols and acyl-CoA derivatives, are directly transferred to alcohols and are the major mechanism of ester biosynthesis by LAB (Liu et al., 2004). Some esters are characterized by low perception thresholds and are generally appreciated for their sweet, fruity, and floral notes, and for their ability to

minimize the sharpness and bitterness of cheese flavour, associated with high levels of FFAs and amines (Sablé and Cottenceau, 1999; Curioni and Bosset, 2002; Liu et al., 2004).

FFAs, or fatty acyl groups, can react with methanethiol to produce S-methylthioesters. Similarly, in ester biosynthesis, S-methylthioesters originate from esterification or alcoholysis reactions (Liu et al., 2004). The biosynthesis of S-methylthioesters is strictly dependent on the availability of methanethiol, and for this reason is attributed firstly to those species which intensely contribute to methanethiol production (e.g., *Micrococcaceae*, *B. linens*, and *G. candidum*). S-Methylthioesters are frequently found in surface-mold ripened and blue-veined cheese, and are characterized by potent odours with low aroma threshold perception (Liu et al., 2004).

Hydroxylated FFAs represent the precursors of lactones. Hydroxylated FFAs are incorporated in milk fat triglycerides and are released by enzymatic lipolytic activities or by any heating process (Alewijn, 2006). Hydroxylated FFAs can also be produced by the activities of microbial enzymes (lipoxygenase and hydratase) with the catabolism of unsaturated fatty acids. Lactones are produced from hydroxylated FFAs, by a one-step transesterification reaction (Alewijn, 2006).

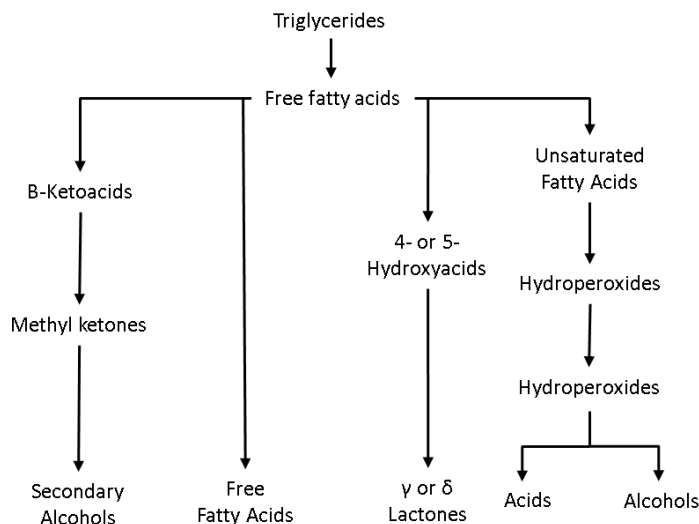


Figure 1.2. General pathways for the catabolism of free fatty acids in cheese, modified from McSweeney and Sousa (2000).

Some other volatile classes of compounds, such as phenols and terpenes, can be detected in several cheese varieties because they can be naturally present in milk before cheese manufacture. Phenolic compounds were detected in higher concentration in ewes' and goats' milk, compared to cows' milk, and were considered responsible for the typical phenolic flavour of ewes' milk cheese (Ha and Lindsay, 1991; Kilic and Lindsay, 2005). Phenolic compounds contribute positively to cheese flavour until a certain concentration is reached, but thereafter tend to give unpleasant notes, as their concentration increases. The presence of the phenolic compounds in milk is related to the process of conjugation, which is a detoxification mechanism that enables an animal to solubilize xenobiotics and excrete them, usually in urine. It seems that the abundance of phenolic compounds in ewes' milk is influenced by both feeding and breed (Kilic and Lindsay, 2005). Another class of compounds, often identified in dairy products are terpenes, which are volatiles mainly detected in artisan cheese manufactured in alpine

regions. These compounds primarily, if not solely, originate from the difference in a cow's feeding regime in alpine areas (Karoui and De Baerdemaeker, 2007). It is likely that the content of terpenes in cheese produced in the alpine region is high because of the diverse wild flora on which cows graze (Faulkner et al., 2017). However, Aprea *et al.* (2016) also reported an increase in terpene content of Montasio cheese over ripening, which could be attributed to the ability of LAB to modify and biosynthesize terpenoids (Belviso et al., 2011).

1.4. Extraction techniques of volatile compounds

As outlined above, there is a wide range of flavour compounds, predominantly volatile in nature, involved in the development of cheese flavour. As a result, there has been a wide array of extraction techniques employed to isolate and concentrate the volatiles from different cheese varieties since the advent of this type of technology. In this regard, the selection of the correct extraction technique is key, because it can significantly influence the type of chemical classes extracted and identified (Dumont and Adda, 1972; Lecanu et al., 2002; Bicchi et al., 2004). The extraction techniques most frequently used to isolate volatiles from cheese are distillation-extraction, simultaneous distillation-extraction (SDE), solvent assisted flavour extraction (SAFE), purge and trap (P&T), thermal desorption (TD), and solid-phase microextraction (SPME). More recently, newer extraction techniques, such as solid-phase dynamic extraction (SPDE), in-tube extraction (ITEX), stir bar sorptive extraction (SBSE) and headspace sorptive extraction (HSSE), have been adopted to detect volatile compounds in food matrices (Table 1.1).

Table 1.1. Extraction techniques available for the volatile analysis of food and dairy products.

Extraction technique	Sample Position	Operating Principle	Trapping Technology
Distillation/Solvent-Extraction	Flask	Flask under vacuum and heated to release the volatiles	Condenser (cold trap)
Solid Phase Microextraction	Sealed vial	Phase coated silica fiber exposed into the liquid phase or the headspace	Phase coated fiber
Stir Bar Extraction	Sealed vial	Coated magnetic stirring bar in contact with the liquid phase or the headspace	Coated magnetic stirring bar
Purge&Trap	U-shaped sparger	Inert gas is purged through the U-shaped glass sparger	Trap inside the unit
Thermal Desorption	Sampling chamber	Inert gas is purged through the sampling chamber	Tube trap
Solid Phase Dynamic Extraction	Sealed vial	Syringe with phase coated needle sucks volatiles from the headspace	Phase coated needle
In-Tube Extraction	Sealed vial	Syringe made by a needle with an above trap sucks volatiles from the headspace	Trap above the needle

1.4.1. Distillation/Solvent-extraction

Distillation-extraction techniques allow for the separation and concentration of volatile compounds from cheese samples through distillation. Distillation-extraction is usually performed under reduced pressure ($< 10^{-3}$ Pa), so-called “vacuum distillation”, to avoid thermal degradation of volatile compounds. The cheese sample is mixed with water, flushed with nitrogen and sealed. The resultant slurry is distilled under vacuum and the extract is collected by cooling traps (Fig. 1.3). The extract may be concentrated to remove the water by solvent-extraction. Usually, a solvent (acetonitrile, pentane, dichloromethane or diethyl ether) is added to the extract and a further vacuum distillation performed. The pH of the final extract can be adjusted to get different fractions (acidic, neutral, and alkaline) for GC injection. The adjustment of the polarity of the final extract causes the separation of the volatile compounds. The acidic fraction

contains primarily phenolic compounds and FFAs; the neutral fraction includes mainly alcohols, aldehydes, ketones, esters, and lactones; the alkaline fraction contains mainly nitrogen-containing compounds, sulphur compounds, and terpenes. The distillation-extraction technique was very common in the past, and numerous studies were performed on the analysis of the flavour of surface-ripened and Cheddar cheese (Libbey et al., 1963; Dumont et al., 1974a; Guichard et al., 1987). Groux and Moinas (1974) analysed the volatile compounds from Vacherin cheese, by using a “gas stripping” technique combined with vacuum distillation. In this technique, the cheese sample was homogenized with distilled water, and the volatiles extracted under reduced pressure, by purging the system with an inert gas (argon) at low temperatures.

It is possible to perform a distillation-extraction of the volatiles directly using a solvent. This extraction is called SDE, and is faster than a traditional distillation-extraction. It requires a lower temperature for the distillation, considering the solvent usually has a low boiling point (Dacre, 1955; Godefroot et al., 1981). This technique allows for the extraction of volatiles from the condensed solvent vapour (containing volatile compounds), which is separated from the condensed water vapour (containing non-volatile compounds) in relation to their density, with subsequent injection of the extract directly onto the GC column. SDE guarantees a high extraction rate and has been used for a variety of cheese types, such as Edam, Swiss, Parmesan, Roncal, Pecorino, Gouda, Emmental, Cheddar, and Danish Blue, and is an ideal extraction technique for components with low volatility (Aishima and Nakai, 1987; Dirinck and De Winne, 1999; Larráyoz et al., 2001; Alewijn et al., 2003).

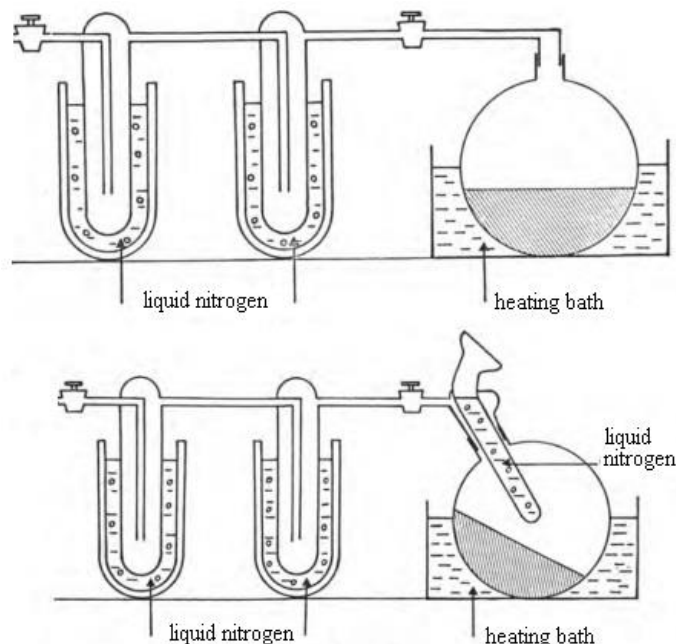


Figure 1.3. Distillation-extraction under vacuum (from Dumont et al., 1974a). The cheese is placed in a flask connected to a group of vacuum pumps. The vacuum is maintained during extraction through the use of valves located at the beginning and at the end of the series of traps. The flask containing the cheese sample is heated by immersion in a heating bath and the traps are cooled by liquid nitrogen to collect the extract. The procedure is performed with a “finger condenser” to complete the extraction.

SAFE is another extraction technique which allows the separation and concentration of volatiles by vacuum distillation. The distillation system consists of a vacuum pump and usually two cooling traps of liquid nitrogen. The cheese sample is mixed with a solvent, and the volatiles are collected by distillation with the solvent in the first trap, while the impurities and the water condense in the second trap. Subsequently the extract, containing volatiles and solvent, is concentrated by purging with nitrogen, before the injection onto the GC column (Engel et al., 1999). SAFE is frequently associated with GC-olfactometry analysis (Whetstine et al., 2005; Whetstine

et al., 2006; Zabaleta et al., 2016). In GC-olfactometry analysis of Cheddar cheese, Drake *et al.* (2010) compared SAFE to headspace-SPME (HS-SPME) with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber. In this study it was clear that some highly volatile compounds were detected by HS-SPME, and not by SAFE, while some higher molecular weight aroma-active volatiles were detected by SAFE, and not by HS-SPME. SAFE is a valid method which allows the extraction of volatile compounds without extensive preparation and it is particularly suitable for GC-olfactometry analysis, due to the preservation of the volatiles through extraction at low temperature. However, SAFE has some limits in the extraction of highly volatile compounds (McGorin, 2007; Thomsen et al., 2014; Kilcawley, 2017). Currently all distillation-extraction techniques, including SDE and SAFE, are not used as frequently as they were previously, due to the length of the procedure, the variable recovery rate of highly volatile compounds, the risk of thermal degradation of heat-labile volatile compounds (Mariaca and Bosset, 1997; Sides et al., 2000; Tunick, 2014). The solvent used in these types of extraction can also create interference in the GC chromatogram, producing artifacts. Other techniques are now more widespread, such as dynamic and passive extractions (discussed below), where the volatile compounds are captured by direct adsorption on to a solid phase, avoiding time-consuming steps of distillation-extraction techniques (Bosset and Gauch, 1993; Wampler, 1997; Tunick, 2014).

1.4.2. Passive extraction: Solid-Phase Microextraction, Stir Bar Sorptive Extraction and Headspace Sorptive Extraction

SPME is a passive extraction technique, commonly used for a wide variety of applications. SPME can be performed as direct-SPME (DI-SPME), by exposing a phase coated fiber, directly to a liquid sample, for the detection of semi- and non-volatile compounds, or as HS-SPME, by exposing a fiber in the headspace, for the detection of medium or highly volatile compounds (Mallia et al., 2005; Januszkiewicz et al., 2008) (Fig. 1.4). HS-SPME is a passive extraction method, which has become very popular for the detection of volatiles in cheese (Mondello et al., 2005; Condurso et al., 2008; Januszkiewicz et al., 2008; Yarlagadda et al., 2014; Bertuzzi et al., 2017).

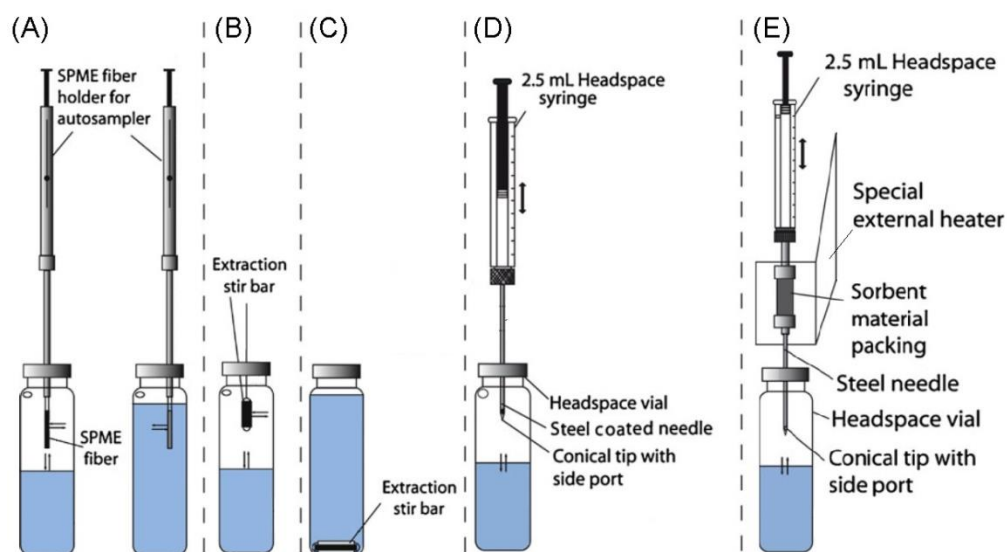


Figure 1.4. Solid-phase microextraction (direct and headspace) (A), headspace sorptive extraction (B), stir bar sorptive extraction (C), solid-phase dynamic extraction (D) and in-tube extraction (E) (from Laaks et al., 2012).

In HS-SPME, the coated fiber is exposed to the headspace above a sample in a sealed vial, without direct contact with the sample, because the volatile compounds are in equilibrium between the gas and liquid/solid phases. The cheese sample (grated or as a slurry) in the vial, is usually heated for a specified time (headspace-saturation time) to allow the volatiles to saturate the headspace. Subsequently, a coated silica fiber is inserted into the vial headspace for a precise time (equilibration/extraction time), at a specific temperature (equilibration/extraction temperature), to allow the volatile compounds to be captured by the polymer coating of the fiber (Pawliszyn, 1997). The sampling is commonly performed with a robotic auto-sampler, to reduce human error inherent in manual injections. After the sorption, the fiber is thermally desorbed at a precise temperature, and pre-conditioned at a higher temperature before the next extraction, to avoid the carryover of analytes to the next analysis.

The type of fiber coating used for the extraction is the main factor which influences the efficiency and discrimination of the extraction. A wide variety of fibers are available, and selection and choice of fiber is critical (Werkhoff et al., 2001; Mondello et al., 2005; Spietelun et al., 2010). The fiber selection has to consider the polarity and thickness of the coating, in relation to the nature of the analytes of interest. The CAR/PDMS fiber is a common matrix used for cheese analysis, and it is particularly sensitive for the detection of highly volatile sulphur compounds, such as carbonyl sulphide, hydrogen sulphide, or methanethiol (Lecanu et al., 2002; Lestremiau et al., 2003; Januszkiewicz et al., 2008). In this double phase fiber, the low molecular weight polar/apolar analytes are captured by the porous carbon structure of the CAR layer (Mondello et al., 2005). Salum *et al.* (2017) optimized HS-SPME for volatiles

from white-brined Turkish cheese, comparing the efficiency of two different fibers (DVB/CAR/PDMS and CAR/PDMS), for the analysis of 10 key compounds. CAR/PDMS fiber was the most suitable fiber for the extraction of volatile compounds in white-brined cheese, especially for those analytes with low molecular weights (isoamyl alcohol, ethyl lactate, and butanoic acid). Mondello *et al.* (2005) evaluated five different fibers (DVB/CAR/PDMS, CAR/PDMS, carbowax/DVB, polyacrylate, and PDMS), suggesting that DVB/CAR/PDMS was the most efficient, with the highest extraction capability for the detection of the analytes of goats' cheese (Fig. 1.5). In the triple phase fiber (DVB/CAR/PDMS), the larger compounds (less volatile), are captured by the porous DVB, while the smaller volatiles (highly volatile) pass through and are captured by the porous carboxen layer. However, the double-phase CAR/PDMS was efficient only for the extraction of low molecular weight polar/apolar analytes, with little affinity for compounds with high molecular weights.

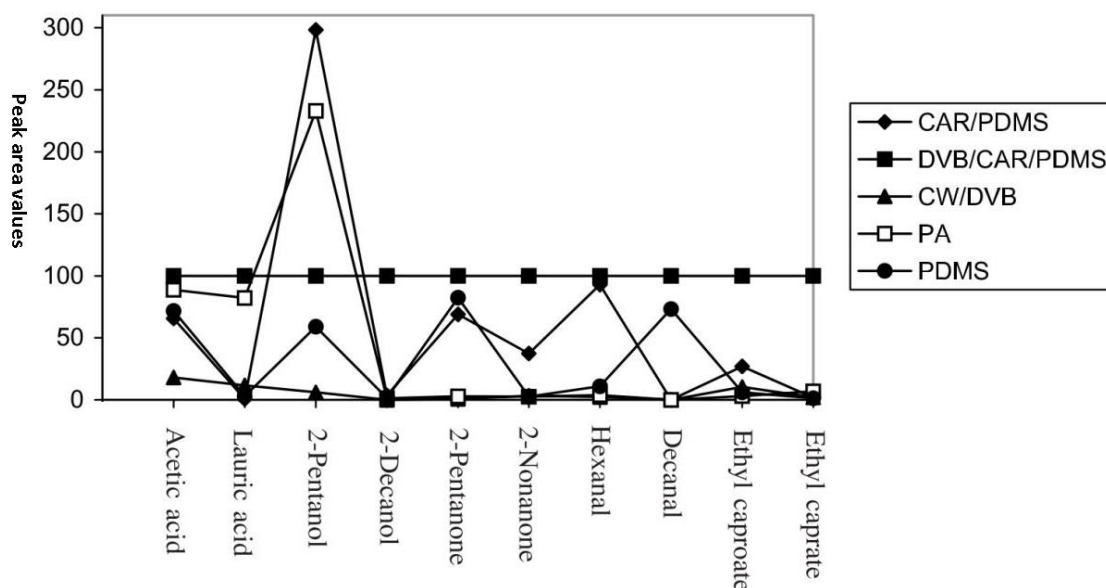


Figure 1.5. Optimization of HS-SPME for the analysis of goats' cheese (from Mondello et al., 2005). Extraction capability of the most common polymeric fibers for the detection of 10 key compounds.

Also, Risticvic and Pawliszyn (2013) analysed the efficiency of different SPME fibers on a mixture containing a wide range of volatility and polarity compounds, confirming that DVB/CAR/PDMS was the most sensitive fiber for analytes with molecular weight $<185 \text{ g mol}^{-1}$. Moreover, they studied the different interactions of specific analytes with the DVB/CAR/PDMS fiber. Due to the limited surface capacity of the SPME fiber, competition occurs between the analytes for the adsorption sites, driven by their chemical characteristics (molecular weight and polarity) and the distribution constant (K_f), which indicates the ratio of the distribution of the analyte, between the fiber coating and the sample. The analytes at high concentration, with higher K_f s, can displace analytes with smaller K_f s, adversely impacting on the ability to get a true reflection of the volatile profile of the sample. Risticvic and Pawliszyn (2013) reported

that interanalyte displacement was very limited by the use of DVB/CAR/PDMS fiber, and rarely occurred for compounds with small Kfs.

The extraction conditions, such as headspace-saturation time, extraction/equilibration temperature, extraction/equilibration time, sample volume, and sample agitation, can be controlled in relation to the nature of the sample, the analytes and the type of fiber coating used (Harmon, 2002; Spietelun et al., 2013). Mondello *et al.* (2005) optimized the conditions of HS-SPME associated with GC-FID and GCMS for the analysis of six local goats' cheeses. They compared different headspace-saturation times at the same temperature (60°C), suggesting 10 min as the optimal time. In general, lower molecular weight volatiles presented the highest peak areas after 5 min saturation time, while heavier compounds required a longer time (~10 min). Mondello *et al.* (2005) also showed that the optimal conditions were 60°C for the extraction/equilibration temperature, and 50 min for the extraction/equilibration time, which were the conditions shown to have the highest volatile extraction yield for both lower and heavier molecular weight volatiles. Burbank and Qian (2005) optimized the conditions of HS-SPME (with CAR/PDMS fiber), associated with GC-pulsed flame photometric detector, for the analysis of sulphur compounds in Cheddar cheese. They compared different extraction/equilibration times at the same temperature (50°C). The sulphur compounds detected increased their response over the course of 2 h, but 30 min was chosen as a good compromise between sensitivity and runtime efficiency for the instrument. Moreover, they compared different extraction/equilibration temperatures at the same equilibration time (30 min). The presence of methanethiol, dimethyldisulphide and dimethyltrisulphide did not change between 30°C and 70°C, while methional was

detected when the temperature was over 50°C, and dimethyl sulphone reached the maximum response at 55°C. Therefore 50°C was indicated as the optimal extraction/equilibration temperature. Salum *et al.* (2017) optimized the conditions for GC-FID analysis of white-brined Turkish cheese, for both fibers tested (DVB/CAR/PDMS and CAR/PDMS). DVB/CAR/PDMS fiber presented an optimal performance with an extraction/equilibration time of 86 min at 54.8°C, while CAR/PDMS fiber showed most effective extraction after 85 min, at 56.2°C.

HS-SPME has been shown to be highly sensitive for the identification of low concentrations of odorous compounds, such as sulphur compounds in different varieties of cheese (Frank *et al.*, 2004). Lecanu *et al.* (2002) highlighted that HS-SPME was more sensitive in detecting a wide range of volatile compounds in surface-ripened cheese which are important for odour perception (methanethiol, carbon disulphide, dimethyl sulphide, acetone, ethyl acetate, 2-butanone, 3-methylbutanal, and ethanol), that were not detected using vacuum-distillation extraction because of the overlap with a solvent peak, or losses during the extraction procedure. In the analysis of Parmigiano-Reggiano cheese, Bellesia *et al.* (2003) compared HS-SPME with P&T, without noticing any substantial difference in the recovery of volatiles from the same sample, confirming the reliability of the two methods.

Overall, SPME is an excellent technique because of automated sampling, high sensitivity and high throughput, without the use of extraction solvents (Harmon, 2002; Spietelun *et al.*, 2010; Spietelun *et al.*, 2013). The major drawback of SPME is the low entrapment capacity of the fiber coating; however, new fibers with thicker coatings have

become available, including SPME Arrow technology that has much greater capacity and thus sensitivity than the original fibers.

Another method successfully applied to food matrices is SBSE (Huang et al., 2009; Yu and Hu, 2009). The principle of SBSE is based on the sorption of volatile compounds in a sample (liquid or semi-liquid matrix), through the use of a magnetic stirring bar with a glass jacket, coated with a sorbent layer (Fig. 1.4). The stirring bar is in direct contact with the sample to allow the full sorption of compounds, and the sample is typically stirred for between 30 and 240 min, depending on to the size of the stirring bar. Once the volatiles are adsorbed, the stirring bar is rinsed in distilled water to remove non-volatiles and thermally desorbed similarly to TD (Baltussen et al., 1999). There are limited options for commercially available coatings, such as PDMS, ethylene glycol/silicone, and polyacrylate. However, newer technologies are now available to formulate special in-house coatings, made of organic polymers and modified polymers (Lancas et al., 2009; He et al., 2014). The fiber most commonly used for food analysis is PDMS fiber. SBSE performed with PDMS fiber was used for analysing flavour compounds in fresh and cooked milk, yogurt and cream cheese by Hoffmann and Heiden (2000), using a polar column for the separation. In the analysis of cream cheese, the compounds detected were mainly ketones, long-chain FFAs (C10-C16), lactones (δ -decalactone, δ -dodecalactone), and diethylphthalate. SBSE is a valid technique due to the high adsorption capacity, the minimal risk of thermal degradation of volatile compounds, and because the extraction does not require high temperatures (Lancas et al., 2009; Kilcawley, 2017). However, SBSE is not without its problems, with limited commercial availability of coatings and the difficulty of automating such a system (He

et al., 2014). In addition, SBSE may be less suitable for the analysis of cheese and dairy products, as the high fat and protein content may interfere with the extraction process (Licón et al., 2012).

HSSE represents a good alternative to SBSE, because it does not come into direct contact with the sample (Licón et al., 2012). HSSE is a technique recently adopted for cheese analysis with interesting results (Panseri et al., 2008; Licón et al., 2012; Licón et al., 2015). In HSSE, the coated stirring bar is placed in the headspace of a sealed vial containing the sample (Fig. 1.4). The cheese sample is finely grated or mixed with water, and the volatiles extracted similarly to HS-SPME, whereby the cheese sample is heated and stirred to ensure a uniform concentration of volatile compounds between the gas and liquid/solid phases. The time of extraction is longer than HS-SPME (usually ~1-4 hours) due to the volume of the coating material, to ensure maximum sorption of volatiles by the coating of the stirring bar (Panseri et al., 2008; Licón et al., 2012). However, the entrapment capacity is significantly higher than the original SPME fibers, which increases the sensitivity of the technique (Kilcawley, 2017). As in SBSE, after the sorption, the magnetic stirring bar is thermally desorbed. A PDMS stirring bar of 1 to 2 cm is preferred for cheese analysis (Panseri et al., 2008; Licón et al., 2012; Licón et al., 2015).

Licón *et al.* (2012) reported good linearity, recovery, precision and reproducibility of data, using HSSE for cheese analysis. This technique showed good recovery of ketones in ewes' milk cheese, and aldehydes in Bitto cheese (Panseri et al., 2008; Licón et al., 2012).

1.4.3. Dynamic extraction: Purge and Trap, Thermal Desorption, Solid-Phase Dynamic Extraction and In-Tube Extraction

In dynamic methods, such as P&T, TD, SPDE, and ITEX, the cheese sample is heated, and the volatile compounds are continuously removed and concentrated in a cold trap, or adsorbed onto an inert support before injection onto the GC capillary column.

TD is a common technique which has been used to differentiate the flavour compounds in different varieties of cheese (Villaseñor et al., 2000; Valero et al., 2001; Lawlor et al., 2002). Lawlor *et al.* (2002) used TD technique for hard-cheese types, such as Appenzeller, Dubliner, Emmentaler, Gabriel, Gruyère, Old Amsterdam, Raclette, and Tête de Moine. In TD, the cheese sample is heated in a sampling chamber to a desired temperature to release the volatile compounds. Using an inert carrier gas (helium or nitrogen), the stripped volatile compounds are transferred and trapped in a TD tube made of adsorbent trapping material, usually with low water affinity (e.g., Tenax TA) (Fig. 1.6). Subsequently, the TD tube is purged to ensure that water is removed and then thermally desorbed to transfer the volatile compounds to a cooled trap and finally to the GC inlet, by a flow of purging gas (helium or nitrogen) (Valero et al., 1997). A wide range of adsorbent and adsorbent trapping materials are available for the TD tube, based on the nature of the analytes. TD is an efficient technique, and the data emanating from this extraction are very representative, because a large amount of sample is analysed due to the large loading capacity of the sampling chamber.

P&T is another dynamic extraction method commonly used in the determination of volatile compounds in cheese. P&T is a good technique for the detection of highly volatile compounds with lower boiling points, such as alcoholic compounds (Mallia et

al., 2005, Thomsen et al., 2014). Bosset and Gauch (1993) used the P&T method to analyse different cheese varieties, such as Parmigiano, Mahón, Fontina, Comté, Beaufort, and Appenzeller, with numerous compounds detected, especially alcohols. There was a predominance of alcohols among the compounds detected when volatile analysis of Spanish ewes' milk cheese was performed with P&T (Gómez-Ruiz et al., 2002; Fernández-García et al., 2004; Barron et al., 2007). Using P&T technique, the cheese sample is usually homogenized with water, placed in a U-shaped glass sparger and heated. Subsequently an inert gas (nitrogen or helium) is purged through the sample, to transfer the volatile compounds to an inert support of trapping material, which is thermally desorbed and concentrated once again in a cold trap (cryofocusing) before injection onto the GC-column (Fig. 1.6) (Bellar et al., 1974). A wide range of trapping materials is available for this technique. P&T is a solvent-free and efficient technique, especially for the detection of highly volatile compounds (Mallia et al., 2005).

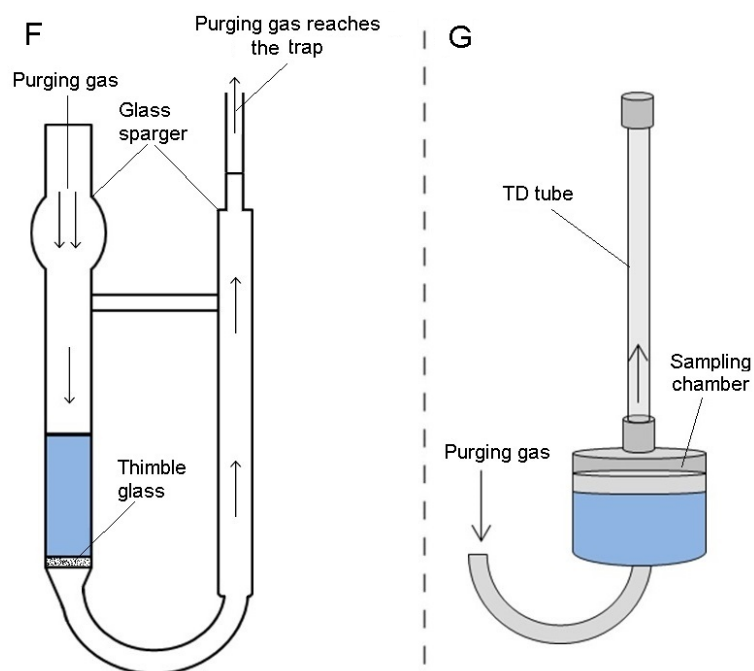


Figure 1.6. Purge and trap (F), and thermal desorption (G).

However, the high levels of moisture in some types of cheese can interfere with P&T and TD extractions, and even damage the MS-detector (Pillonel et al., 2002). The moisture in the cheese samples can cause condensation in the TD tubes, impeding the good desorption of volatiles, while in P&T the moisture can be purged from the sample to the trap, and injected onto the GC column, contributing to the production of artifacts (Valero et al., 1997; Kilcawley, 2017). Nowadays, numerous strategies are available to eliminate the moisture in the desorption process in cheese analysis. For example, before desorption, the solid trap can be flushed with an inert dry gas to remove part of the water (dry purge technique) (Canac-Arteaga et al., 1999a). Another solution is to install a cold water trap (condenser at $\sim -10^{\circ}\text{C}$) located before the trap to condense the residual water from the sample (condensation technique) (Canac-Arteaga et al., 1999b). Other approaches include mixing hygroscopic salts (e.g., sodium sulphate, sodium chloride, and potassium carbonate) with the sample (Valero et al., 1997; Canac-Arteaga et al., 2000; Villaseñor et al., 2000), or placement of cartridges of hygroscopic salts (e.g., sodium carbonate, magnesium sulphate, and calcium chloride) in front of the trap to eliminate water (Guillot et al., 2000). A drying tube made of permeable material such as Nafion has also been used to facilitate diffusion and to remove water from the sample (Pankow, 1991). Despite various strategies being adopted, these methods are not totally efficient due to partial removal of moisture and/or the losses of analytes (Pillonel et al., 2002). More modern systems contain built-in moisture-control units, but moisture must still be managed for the effectiveness of these techniques.

SPDE is another dynamic technique that is now becoming commonly used (Bicchi et al., 2004; Gamero et al., 2013). The innovative technology of SPDE involves

a steel needle, coated internally with a sorbent material, which extracts volatiles from the headspace. The needle is inserted inside the sample vial, and the plunger of the syringe is moved up and down several times to draw the volatiles inside the needle. Subsequently, the volatiles are thermally desorbed and a purging gas (helium or nitrogen) passes through the syringe with a direct injection onto the GC column (Lipinski, 2001) (Fig. 1.4). Depending on the nature of the analytes, different needles, coated with various sorbent materials, are commercially available. To maximize the recovery in SPDE, the choice of coating materials and extraction conditions are critical. In flavour research, the most common sorbent materials used for the SPDE needles are PDMS and CAR/PDMS. During sampling, the major factors influencing the end result of SPDE are the sampling temperature and the number of extraction strokes, while the flow rate of the draw and eject steps were found not to be critical (Kamphoff et al., 2007; Van Durme et al., 2007). For the analysis of volatiles in roasted coffee, Bicchi *et al.* (2004) compared SPDE to HS-SPME, maintaining the same extraction conditions and trapping material (PDMS) for both techniques. SPDE resulted in higher concentration factors, compared to HS-SPME for almost all analytes detected. Bicchi *et al.* (2004) suggested that the difference between the two methods was due to the fact that the extraction volume of the coating material of the needle in SPDE was higher than the volume of coating fiber of HS-SPME. However, different results were reported by Gamero *et al.* (2013), who stated that HS-SPDE (with PDMS/active carbon needle) had the least sensitivity, compared to HS-SPME (with DVB/CAR/PDMS fiber), DI-SPME (with DVB/CAR/PDMS fiber), and SBSE (with PDMS bar coating) for the analysis of volatiles in wine. The results presented in this study are not directly comparable to each

other, because different adsorbent material and extraction times were selected to compare different extraction techniques. Overall, in these studies, the efficiency of HS-SPDE was variable, and this extraction method should be further evaluated to understand its full potential in flavour analysis.

ITEX is a technique very similar to SPDE. It consists of a unique setup of a needle, a microtrap made of sorbent material, and a headspace syringe surrounded by an external heater (Fig. 1.4). During extraction, the sample is heated under controlled conditions and the needle is inserted into the headspace of the sample vial. Similarly to SPDE, the plunger of the syringe is moved up and down several times, to draw and concentrate the volatiles dynamically into the microtrap. The analytes are thermally desorbed in the GC injector with a purging gas (helium or nitrogen) which flows through the syringe. Different coating materials are available for different analytes. As with SPDE, the number of extraction strokes performed during the sorption step, is the most important parameter influencing the results (Laaks et al., 2015).

Together with the other dynamic extractions, SPDE and ITEX use large adsorption volumes and these techniques can be easily automated using a robotic auto-sampling system. Moreover, in SPDE and ITEX, the desorption step can be performed directly onto the injection port of the GC column. To date, very little research has been published on the use of ITEX or SPDE in food analysis.

1.4.4. Effect of salt and pH on the headspace extraction

The sample preparation, through the modification of pH levels or salt content can influence the yield of extraction of volatiles in the headspace. The practice of adding salt to the sample, or “salting out”, is considered an efficient and low-cost method to increase the extraction rate of certain analytes. The added salt dissociates to its ions, reducing the solubility of specific analytes and forces these to move into the headspace. “Salting-out” facilitates the extraction of more-polar and lower molecular weight analytes, but it is not effective for high molecular weight analytes, which may adhere to the glass vial (Shirey, 2000). The most commonly used salt is sodium chloride, even though recent studies have highlighted new applications using bivalent or multivalent salts (Pinho et al., 2002; Fiorini et al., 2015). This technique showed positive effects on the extraction of isopropylamine, isopropanol (Shirey, 2000), carboxylic acids (Harmon, 2002; Fiorini et al., 2015), phenols (Buchholz and Pawliszyn, 1994), aromatic hydrocarbons (Djozan and Assadi, 1997), terpenoids (García et al., 1996), amines (Müller et al., 1997), and Maillard reaction products (Coleman, 1996). Some adjustments may greatly influence the “salting-out” effect, as the headspace volume has to be kept as small as possible to increase the concentration of analytes. Also, stirring of the sample was observed to influence the extraction rate of specific analytes. The extraction/equilibration time of less volatile compounds was reduced by stirring, while having only a minimal effect on highly volatile compounds (Buchholz and Pawliszyn, 1994; Pillonel et al., 2002).

Moreover, variation in pH can influence the extraction rate of specific analytes in the headspace, particularly those weakly acidic or basic compounds, such as amines and phenols, which should be maintained in an undissociated form (Spietelun et al., 2013). The modification of pH facilitates the dissociation of the polar analytes, which become more easily extractable. Compounds with low pKa (acid dissociation constant) are dissociated at acidic pH, while compounds with high pKb (basic dissociation constant) are dissociated at alkaline pH. Shirey (2000) showed that acetic acid was best extracted at pH 2, while isopropylamine and propionitrile were best extracted at pH 11. In this study, it was not anticipated that the pH would have an effect on the extraction of non-polar compounds; however, isopropanol and acetone were more efficiently extracted at high pH levels.

The modification pH and “salting out” are two methods recommended only for headspace extractions. In a direct immersion extraction, the adsorbent coating material cannot be resistant to strong alkaline/acidic pH, or at high salt concentration (Spietelun et al., 2013). Prior to performing these techniques, it is recommended that a sample be completely homogenized with the salting agent, or with the basic/acidic solution, to ensure that the salt concentration, or the pH level, is uniform inside the matrix. Generally, in the analysis of cheese flavour, it is not a common practice to add salt, or modify pH, because there is no need to increase the extraction rate of certain chemical classes (non-targeted-analysis). However, acidification and the “salting out” effect are procedures used in the targeted-analysis of FFAs of cheese (Fiorini et al., 2015, Pinho et al., 2002, Gonzalez-Cordova and Vallejo-Cordoba, 2001). Recently, Fiorini *et al.* (2015) analysed the volatile FFAs of cheese with HS-SPME (with a DVB/CAR/PDMS),

showing that the use of a mixture of multivalent salts, $(\text{NH}_4)_2\text{SO}_4/\text{NaH}_2\text{PO}_4$, improved the extraction of short-chain FFAs (carbon chain lengths ≤ 5), while the monovalent salt NaCl performed better for medium-chain FFAs (C8-C10).

1.5. Gas chromatography

In flavour research, GC is the most commonly used analytical methodology to separate and identify volatile compounds. GC techniques use a mobile phase, which is an inert carrier gas (such as helium, hydrogen, nitrogen, and argon) to carry the extracted volatile compounds through a GC column. In volatile analysis, GC columns are heated in an oven following a set temperature program. The most volatile compounds elute first and usually within the first 30 min; subsequently, the column is heated to just below its maximum temperature to remove any volatile or lower volatile components, so that the column is uncontaminated for the next sample. Inside the GC column, the volatile compounds form weak electrostatic interactions with the stationary phase, based on the polarity of the compounds. The polarity of the stationary phase varies, usually depending on the analytes to be separated. Depending on the analyte volatility and degree of interaction with the stationary phase, the analytes elute from the column at different set times, during a fixed or isothermal temperature ramp. The volatile compounds are subsequently identified using a specific detector.

The factors which influence the performance of a GC system are inlet liners, type of injection (on-column, split, and splitless), type of column (stationary phase, length, diameter, and thickness), carrier gas (flow rate, pressure), and oven temperature (using cryotrap, ramping rates) (Kilcawley, 2017). The selection of the GC column is the most influential factor for the detection of the compounds (Imhof and Bosset, 1994). In the past, the analyses were performed on cheese using a packed GC column (Dumont et al., 1974a; Groux and Moinas, 1974), but fused silica open tubular capillary columns

have become universally used; they have a much longer stationary phase, and thus offer better separation potential. GC capillary columns can have polar stationary phases (e.g., polyethyleneglycol, nitroterephthalic acid modified polyethyleneglycol), resulting in very efficient separation of polar compounds such as acids and some alcohols. Alternatively, columns can have non-polar stationary phases (e.g., PDMS, phenyl-methylpholsiloxane), for the separation of compounds with low polarity such as aldehydes, ketones, esters, amines hydrocarbons, and phenols. Non-polar columns tend to be much more stable than polar columns. However, in recent years, columns with intermediate stationary phases (e.g., phenyl-methylpholsiloxane, cyanopropylphenyl-methylpolysiloxane, cyanopropylphenyl-dimethylpolysiloxane) are becoming more widely used, as they are more suitable for the detection of a wider range of polar and non-polar volatile compounds.

Imhof and Bosset (1994) compared different stationary phases for the GC analysis of volatiles of Swiss Emmental cheese. They stated that a GC capillary column, coated with 4 μm layer of 100% PDMS, had a better resolution, higher loading capacity, higher number of volatiles detected, than columns made of polyethyleneglycol and modified PDMS columns.

However, in a food matrix such as cheese, both polar and non-polar compounds are present in considerable amounts; therefore it is advantageous to carry out the analysis with GC columns of different polarities or through a two-dimensional GC system.

1.5.1. Two-dimensional gas chromatography

In many cases one-dimensional GC (using one column) cannot effectively separate co-eluting compounds. However, depending on the nature of the co-eluting compounds, these can still be identified and quantified successfully using advanced data processing deconvolution software. However, sometimes multiple compounds, or structurally very similar compounds, may co-elute, making identification and quantification more difficult or even impossible. Two-dimensional GC (GC×GC) is a powerful gas chromatographic technique that was developed to improve separation of co-eluting compounds in complex sample matrices. In GC×GC technology, the effluent passes through a first GC column and is then entirely (comprehensive GC×GC) or partially (heart-cutting GC×GC) transferred through a thermal, or flow modulator, to a second GC column, usually shorter and with a different polarity, and at the end reaches the detector (Fig. 1.7). The sensitivity of the GC×GC systems is very high, because the volatiles which come from the first column are usually cryofocused in a trap and then released in the second GC column in a narrow band, generating a sharper signal (Qian et al., 2007; Kilcawley, 2017). The two GC columns, with different stationary phases, work independently and with different temperature settings, and thus have different interactions with the volatile compounds. The second column may also be set up in a separate column oven to provide more independent control of the temperature settings. Usually, the first GC column is selected to separate the compounds in relation to volatility, while the second GC column is used for the separation based mainly on differences in polarity (or hydrogen bonding with the stationary phase) (Pursch et al.,

2002; Qian et al., 2007). The data from each GC column can be combined to have a resultant two-dimensional chromatogram. In flavour analysis GC×GC is now widely applied to different food matrices, such as wine, fruit, coffee, and nuts (Mommers et al., 2013; Samykanno et al., 2013; Dugo et al., 2014; Manzano et al., 2014). GC×GC can be used with single quadrupole mass spectrometry but is possibly best used with time-of-flight mass spectrometry as the acquisition rate is often higher thus providing more sensitivity.

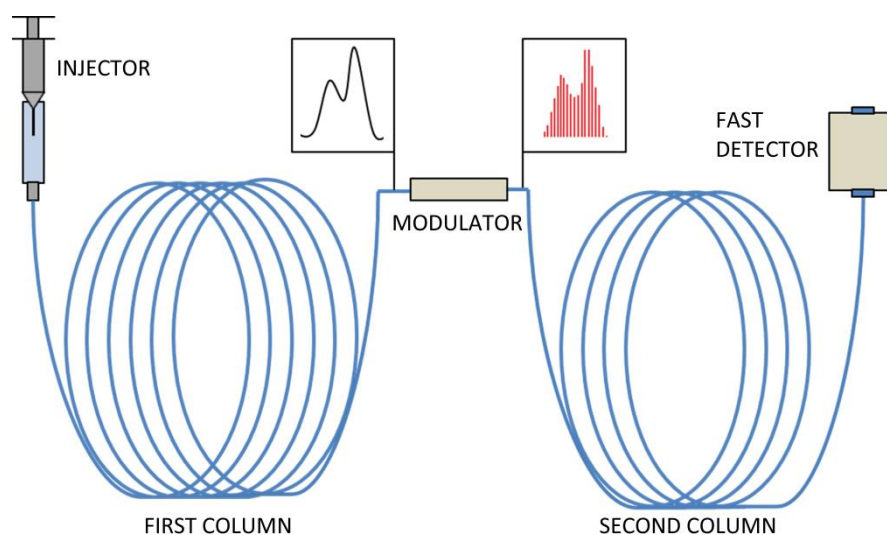


Figure 1.7. Two dimensional gas chromatography system (GC×GC).

Up to now, only a limited number of studies have been carried out using GC×GC for cheese analysis. For the detection of the volatiles of Cheddar cheese, Arora *et al.* (1995) used a GC×GC system, using a non-polar column, followed by a polar column, with a P&T extraction, while Gogus *et al.* (2006) selected a non-polar column followed by a column of intermediate polarity, using a TD extraction. This technology increased the separation and identification power of the analysis of volatile components in cheese.

It is clear that GC×GC has better sensitivity than those technologies with a single column. It is to be expected that GC×GC will be more widely applied to dairy products in the future to improve their flavour characterization.

1.6. Detection systems

After GC column separation it is necessary to identify and quantify the volatile compounds. The detection systems used are multiple and sophisticated and the selection of the correct technology depends on the product being analysed and the range of analytes of interest. The detectors most commonly used in dairy research for the identification and quantification are: the mass spectrometry (MS) detector, flame ionization detector (FID), flame photometric detector (FPD), and pulsed flame photometric detector (PFPD). It is also possible to perform an olfactory analysis with the human nose, which is called GC-olfactometry (GC-O).

1.6.1. Flame ionization and flame detectors

In the past, flame ionization detectors (FID) were most frequently used for the detection of volatiles in food, but now have been surpassed by MS detectors. FID has been the most commonly used detector in GC analysis and are very popular for the detection of flavour compounds in cheese (Bergamini et al., 2010; Peralta et al., 2014; Wolf et al., 2016). In FID, the column effluent is combusted by an air-hydrogen flame, causing ionization of compounds and producing a current which is detected and converted to a signal. FID is widely used due to low-cost, long-term reliability, and universal applicability for compounds with carbon-hydrogen bonds in their chemical structure (Mariaca and Bosset, 1997; Rahman et al., 2015).

FPD and PFPD are characterized by the combustion of compounds which produce a flame emission passing across a photomultiplier window. Thereafter, the

flame emission of the compounds can be electronically separated and elaborated as a signal. In the PFPD, the flaming process is not continuous, and some classes of sulphur- or phosphorous-containing compounds are separated because they have a delay in the flame emission. These classes of compounds produce a flame emission also during the post pulsed flame time, due to lower chemical bond energies. For this reason, PFPD has more selectivity and sensitivity for sulphur, phosphorus and nitrogen than FPD, where the flaming is continuous. PFPD is low-cost, easy-to-use, and highly selective for sulphur or phosphorous compounds. The main advantage of PFPD for dairy products is that it is very sensitive for the detection of sulphur compounds, which are major contributors to the strong flavour associated with many cheese varieties, especially surface-ripened cheese (Mariaca and Bosset, 1997; Jing and Amirav, 1998). Thus this detector is used for studies mainly focused on the detection of volatile sulphur compounds in cheese (Parliment et al., 1982; O'Brien et al., 2017). Burbank and Qian (2005) optimized HS-SPME with CAR/PDMS fiber, associated with PFPD, to investigate the volatile sulphur compounds of Cheddar cheese. These authors found a predominance of certain types of compounds such as dimethyl sulphide, methanethiol, hydrogen sulphide, when analysing Cheddar cheese of various stages of maturity.

1.6.2. MS detectors

The principle of MS is based on the ionization of a molecule and the resolution of the ionized molecule based on mass-to-charge (m/z) ratios in an electrostatic field (Croissant et al., 2011). Every sample analysed with GCMS produces a total ion chromatogram which then has to be analysed and interpreted. It is important to have a

good quality chromatogram to facilitate the identification of the compounds. However, the resulting chromatogram may present a complex background which can interfere with the analysis. Specific software is now used to deconvolute the chromatogram to eliminate background (noise). During the chromatographic analysis each peak is identified by comparing the mass spectra to commercial or internally developed libraries in order to associate every peak to a specific compound. It is also possible to use a formula based on retention time in comparison to a set of specific standards to aid sample identification. The LRI of each compound analysed is compared with data in an internal or/and external database to confirm the identification. In the case of isothermal analysis (constant oven temperature throughout the GC run), the LRI could be calculated in agreement with Kovats (1965), as shown below:

$$LRI_A = 100N + 100 \frac{\log t_{R(A)} - \log t_{R(N)}}{\log t_{R(N+1)} - \log t_{R(N)}}$$

when a temperature program is applied to the GC analysis (heating the oven at a controlled rate throughout the GC run) the LRI is calculated according with Van den Dool and Kratz (1963) as follows:

$$LRI_A = 100N + 100 \frac{t_{R(A)} - t_{R(N)}}{t_{R(N+1)} - t_{R(N)}}$$

where LRI_A is the linear retention index of the compound A, $t_{R(A)}$ is the retention time of the compound A, $t_{R(N)}$ and $t_{R(N+1)}$ is the retention time of the alkane with carbon number N and N+1 respectively. N is the carbon number of the alkane which elutes earlier than

the compound analysed. For the detection of specific analytes (targeted-analysis) the use of standards which indicate the compounds to identify is a more practical method.

The quantification of the spectra is performed by calculating the peak area in the chromatogram for each compound identified. Different software packages are commercially available for deconvolution, identification, and quantification.

The most commonly used MS detectors are quadrupole mass analysers whereby an electric field is produced by applying a voltage across four hyperbolic rods. Each adjacent rod has the opposite charge compared to its nearest counterpart. When DC voltage is applied and the voltage signs are rapidly reversed, varying electrical fields are produced, enabling ions with specific mass-to-charge ratios to pass (Rahman et al., 2015).

MS detector systems can be used in full-scan mode (total ion current, TIC) to identify compounds via their fragmentation profile, comparing the mass spectra to a library which allows the identification of the compounds in relation to the similarity with reference spectra. Alternatively, MS detectors can be used in selected ion storage (SIS) or selected ion monitoring (SIM) mode to detect only a small number of ions and quantify specific compounds with high selectivity and sensitivity (e.g., sulphur compounds) (Mariaca and Bosset, 1997).

Recently, triple quadrupole mass spectrometers have been used for confirmatory analysis. This detector uses two quadrupoles, two mass filter quadrupoles at either end of a collision mass quadrupole (fragmentator). Despite its high efficiency, this type of MS detector is not commonly employed for cheese analysis due to the low mass of volatile compounds in cheese which would be further fragmented with the triple

quadrupole (QqQ) (Kilcawley, 2017). However, a time of flight (TOF) detector has been used in association with GC×GC in Cheddar cheese (Gogus et al., 2006). This detector is based on an ion's charge-to-mass ratio versus time. An electrical field of known strength is used to accelerate ions. Ions of the same charge, i.e., equal kinetic energy, are accelerated through this electrical field at the same rate. The velocity of the ion depends on its mass-to-charge ratio. By calculating the amount of time it has taken for an ion to reach the detector at known distance, the mass-to-charge ratio of the ion can be estimated (heavier particles travel at lower speeds) (Rahman et al., 2015). The high cost of this technology is the main reason why TOF MS has not been as widely used as quadrupole MS in cheese flavour research.

1.7. Gas chromatography olfactometry

In this technique the human nose is used as a detector. A sniffing port is connected directly to the end of the GC column and used to perform olfactory analysis. With respect to cheese and food in general, not all volatiles, even if present in high quantities, affect the flavour profile, while others detected in small quantities can be extremely significant (Delahunty et al., 2006; D'Acampora Zellner et al., 2008). Through the GC-O technique it is possible to discriminate between the odour-active and non-odour active compounds and report the duration and the intensity of the corresponding odour activity. Moreover, GC-O enables the association of an odour-active compound with an individual sensory descriptor for aroma characterization.

In GC-O, experienced assessors sniff the effluent of the column to detect the presence of odour-active compounds via a specifically designed odour port (Fig. 1.8). The effluent of the column is split into two portions; one goes to the GC-O port and the other one to a detector (usually MS or FID); in this way, it is possible to relate the odours sniffed by GC-O port with the volatile compounds identified by the detector at the same time. Different methodologies varying in complexity are available to interpret data obtained by GC-O. In the “direction frequency” method, the compounds detected more frequently by a panel of assessors are considered the most relevant and the corresponding odour durations are measured. Thus, it is possible to build an aromagram where the breadth of the peaks is related to the odour duration, while the height is related to the number of odour detections. In other methods called “dilution to threshold” such as “aroma extraction dilution analysis” (AEDA) and CharmAnalysis™,

the sample is diluted (usually by factor of 2-3) to reach the odour threshold of the compounds in air. In AEDA, it is not necessary to register the odour duration or the intensity, as it only considers the presence of odour-active compounds at different dilutions. Thus, AEDA measures the maximum dilution at which it is still possible to perceive an odour-active compound, and this value is called the “flavour dilution factor”. With CharmAnalysis™, the Charm value is calculated for each odour-active compound through an algorithm. This algorithm considers the dilution factor, the number of coincident odour responses detected at a single retention index and the odour duration (Delahunty et al., 2006; D’Acampora Zellner et al., 2008). Another method called “direct intensity” is used to classify the intensity of the odour-active compounds using a scale of measurement. The data are elaborated in an aromagram taking into account the intensity and duration of the odours perceived.

GC-O has been widely applied to different varieties of cheese enabling the association between volatile compounds and aroma notes (Thomsen et al., 2012; Fuchsmann et al., 2015; Zabaleta et al., 2016). However, GC-O was also recently used together with GC×GC. The combination of GC-O with GC×GC-TOF considerably increased the identification of odour-active compounds in different food matrices (Cordero et al., 2015).

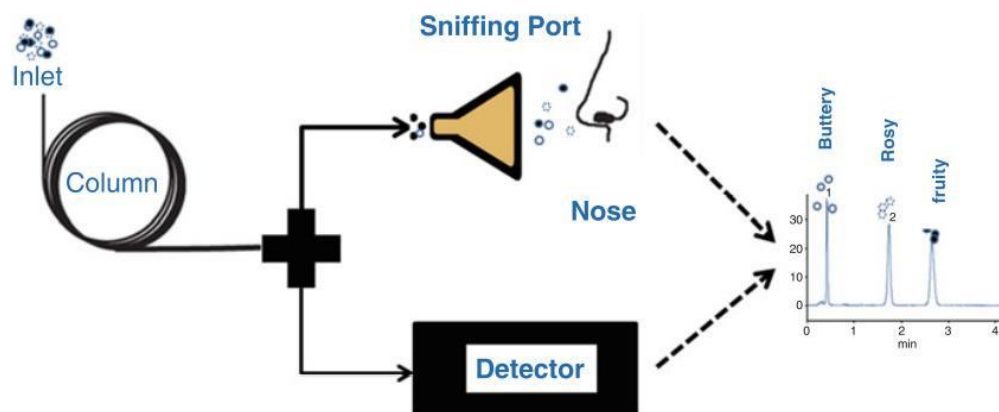


Figure 1.8. Gas chromatography olfactory system (GC-O) (from Kilcawley, 2017).

1.8. Volatiles in surface-ripened cheese

The right selection of the techniques and analytical tools, for the extraction and separation of volatile compounds, is important for the flavour characterization of the various cheese types, including surface-ripened cheese. Surface-ripened cheese belongs to the category of rennet-coagulated cheese, and is a very heterogeneous group. They can be soft (e.g., Münster and Reblochon), semi-hard (e.g., Tilsit, Brick, Port Salut, Livarot, and Limburger) or hard (e.g., Gruyère and Comté), with a variable period of ripening (Cogan et al., 2014; Mounier et al., 2017; Fox et al., 2017b).

The sensory properties of surface-ripened cheese are very variable and have been described using a myriad of descriptive terms including oily, rancid (Chaumes), pepper undertones (Appenzeller) and pungent, silage and sweaty/sour odour with a rancid, mushroom, oily, smoky, silage, bitter, and burnt-after taste flavours (Tête de Moine and Raclette) (Lawlor and Delahunty, 2000; Lawlor et al., 2002). In an in-depth analysis of 152 European cheeses, Koppel and Chambers (2012) defined the general aroma profiles of surface-ripened cheese, soft and semi-hard, as musty, moldy, sour, salty, and buttery.

Even if flavour studies on surface-ripened cheese are limited, different extraction techniques and stationary phases were used for the volatile analysis of several surface-ripened cheeses, influencing their volatile profiles (Fig. 1.9), and the volatile compounds most commonly identified were mainly aldehydes, alcohols, carboxylic acids, methyl ketones, ethyl esters, sulphur compounds, and aromatic hydrocarbons (Table 2).

Aldehydes have mainly been identified in some Swiss and French surface-ripened cheese (Table 2). Hexanal and 3-methylbutanal were the aldehydes most commonly detected. Hexanal is characterized by green, slightly fruity, lemon, and herbal notes, while 3-methylbutanal is related to malty, powerful, cheese, green, and dark chocolate notes (Curioni and Bosset, 2002; Thomsen et al., 2012; Kilcawley, 2017). Benzaldehyde was also frequently detected, and it was usually associated with bitter almond and sweet cherry flavours (Singh et al., 2003; Smit et al., 2005). In surface-ripened cheese, aldehydes were detected at high percentages using non-polar/medium-polar stationary phase especially with TD and P&T extractions (Valero et al., 2001; Lawlor et al., 2002; Bonaïti et al., 2005) (Fig. 1.9).

Generally in surface-ripened cheese, alcohols are the volatile compounds detected in highest numbers, when compared with other classes of compounds (Table 2). The primary alcohols mostly detected were ethanol (dry dust, alcohol notes), 1-propanol (sweet and wine-like notes), and 3-methylbutanol (fresh cheese, breath taking, alcoholic, fruity, grainy, and solvent-like notes), while the most common secondary alcohol identified was 2-butanol (sweet, fruity fusel oil, and wine-like) (Kilcawley, 2017). In surface-ripened cheese, a high percentage of alcohols were identified, using intermediate stationary phase, mainly with vacuum-distillation and P&T extractions (Dumont et al., 1974a, b; Bosset and Gauch, 1993) (Fig. 1.9).

Carboxylic acids, such as butanoic, 2-methylbutanoic, hexanoic, and octanoic acid, have not been frequently identified in surface-ripened cheese (Table 1.2), because the GC columns used for volatile compound detection are usually non-polar columns, which are less suitable for polar compounds (Croissant et al., 2011). Moreover, at the

pH levels of surface-ripened cheese ($\text{pH} \sim 6$), carboxylic acids are present as non-volatile salts, reducing their flavour impact (Singh et al., 2003). When identified in surface-ripened cheese, carboxylic acids (Table 2) had strong and unpleasant odours, described as sweaty, rancid, and cheesy (Kilcawley, 2017). As expected, in surface-ripened cheese the use of medium-polar/polar stationary phases greatly influenced the detection of carboxylic acids, which were extracted with various techniques (TD and HS-SPME) (Valero et al., 2001; Fuchsmann et al., 2015) (Fig. 1.9).

Some methyl ketones, such as 2-butanone, 2-pentanone, 2-nonanone, and 2-heptanone, have commonly been identified in a wide range of surface-ripened cheese varieties (Table 2). Sweet, ether-like, slightly nauseating notes were associated with 2-butanone; orange peel, sweet, and fruity notes, with 2-pentanone; malty, rotten fruit, hot milk, green, and earthy notes, with 2-nonanone (Kilcawley, 2017). Blue cheese notes were mainly attributed to 2-heptanone (Curioni and Bosset, 2002; Singh et al., 2003). In surface-ripened cheese, a high percentage of ketones were detected, using a non-polar stationary phase with vacuum-distillation, and TD extractions (Parliment et al., 1982; Lawlor et al., 2002) (Fig. 1.9).

Esters have an interesting impact on the development of surface-ripened cheese flavour, due to their sweet, fruity, and floral notes (Urbach, 1997; Niimi et al., 2015). Ethyl acetate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate represent the major esters commonly identified in many varieties of surface-ripened cheese (Table 1.2). Higher percentages of esters were detected with vacuum-distillation, using both non-polar, or polar stationary phase for the separation (Dumont et al., 1974a; Guichard et al., 1987) (Fig. 1.9).

Another group of volatile compounds widely detected in surface-ripened cheese, and considered one of the key compounds for flavour, are the sulphur compounds (Table 2), especially dimethyltrisulphide and dimethyldisulphide (Sablé and Cottenceau, 1999; Curioni and Bosset, 2002). Dimethyltrisulphide is generally associated with odour notes of vegetable, sulphurous, garlic, putrid and cabbage-like aromas, while dimethyldisulphide is related to green, sour, and onion notes (Kilcawley, 2017). In surface-ripened cheese, the detection of sulphur compounds was performed effectively using a non-polar stationary phase, with vacuum-distillation, and P&T extractions (Parliment et al., 1982; Bonaïti et al., 2005). However, Fuchsmann *et al.* (2015) detected high numbers of sulphur compounds using a polar stationary phase, and HS-SPME, with a MS/PFPD detector (Fig. 1.9).

Aromatic hydrocarbons, such as indole and toluene, were identified mainly in soft and semi-soft French surface-ripened cheese (Table 1.2). Indole is considered one of the compounds responsible for unclean-utensil, rose-like off-flavours, while toluene is associated with nutty, and rancid odours (Yvon and Rijnen, 2001; Delgado et al., 2011), but they are also known to have a high odour threshold. In surface-ripened cheese, the detection of aromatic hydrocarbons was not particularly affected by the polarity of the stationary phase, but was mainly influenced by the use of the vacuum-distillation extraction, which is considered a reliable technique for the detection of high molecular weight-compounds (Dumont et al., 1974a, b) (Fig. 1.9).

Other compounds, which were often identified in surface-ripened cheese, are the terpenes. A range of studies looking at a variety of surface-ripened cheese such as Vacherin, Comté, Beaufort, and Tête de Moine, found that a broad spectrum of terpenes

was present (Dumont et al., 1974b; Guichard et al., 1987; Bosset and Gauch, 1993). Terpenes, as aromatic hydrocarbons, are high molecular weight-compounds and were predictably extracted in high percentages by vacuum-distillation and SDE (Dumont et al., 1974b; Valero et al., 2001; Guichard et al., 1987) (Fig. 1.9).

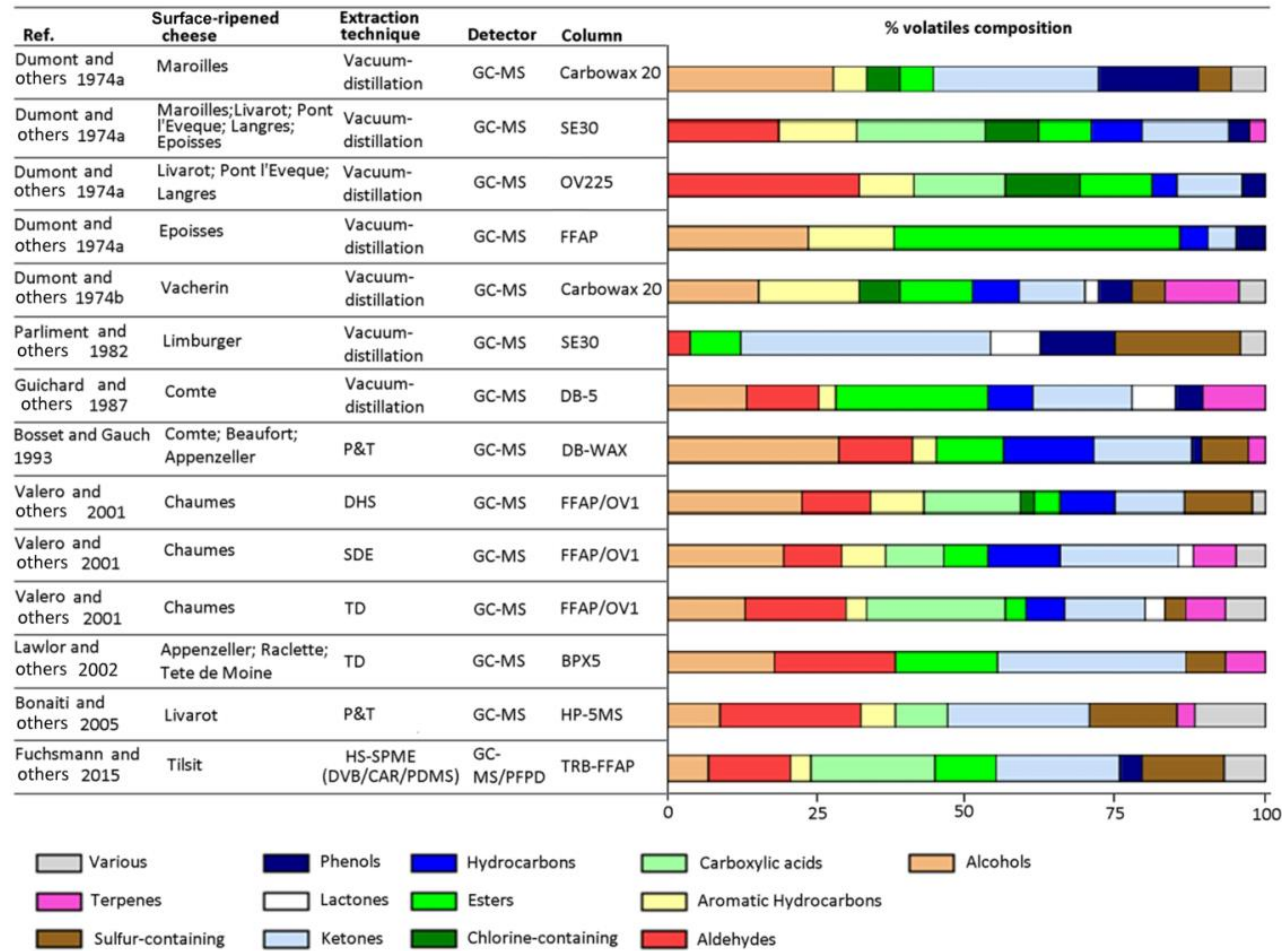


Figure 1.9. Percentages of volatile compounds detected in different surface-ripened cheese varieties.

Table 1.2. Principal volatile compounds most frequently detected in surface-ripened cheese varieties, and related extraction techniques utilized, namely vacuum-distillation, DHS (dynamic headspace extraction), TD (thermal desorption), HS-SPME (headspace-solid-phase microextraction), P&T (purge and trap), and SDE (simultaneous distillation-extraction).

Compounds	Surface-ripened cheese	Extraction technique	Ref.
Butanoic acid	Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; TD; HS-SPME	Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
3-Methylbutanoic acid	Limburger; Livarot; Tilsit	Vacuum-distillation; P&T; HS-SPME	Parliment et al., 1982; Bonaïti et al., 2005; Fuchsmann et al., 2015
Hexanoic acid	Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; TD; HS-SPME	Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
Octanoic acid	Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; SDE; TD; HS-SPME	Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
3-Methylbutanal	Comté; Beaufort; Appenzeller; Chaumes; Tilsit	P&T; DHS; TD; HS-SPME	Bosset and Gauch, 1993; Valero et al., 2001; Fuchsmann et al., 2015
Hexanal	Comté; Chaumes; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; SDE; TD	Guichard et al., 1987; Valero et al., 2001; Lawlor et al., 2002
Benzaldehyde	Comté ; Raclette; Tête de Moine ; Livarot; Tilsit	Vacuum-distillation; P&T; TD; HS-SPME	Guichard et al., 1987; Bosset and Gauch, 1993; Lawlor et al., 2002; Bonaïti et al., 2005; Fuchsmann et al., 2015
2-Butanone	Vacherin; Maroilles; Livarot; Pont l'Eveque; Comté; Beaufort; Appenzeller; Raclette; Tête de Moine ; Tilsit	Vacuum-distillation; P&T; TD; HS-SPME	Groux et al., 1974; Dumont et al., 1974a; Bosset and Gauch, 1993; Lawlor et al., 2002; Fuchsmann et al., 2015
2-Pentanone	Vacherin; Maroilles; Limburger; Chaumes; Appenzeller; Raclette; Tête de Moine ; Livarot	Vacuum-distillation; DHS; TD; P&T	Groux et al., 1974; Dumont et al., 1974a; Parliment et al., 1982; Valero et al., 2001; Lawlor et al., 2002; Bonaïti et al., 2005
2-Heptanone	Maroilles; Pont l'Eveque; Langres; Vacherin; Livarot; Limburger; Comté ; Beaufort; Appenzeller; Chaumes; Raclette; Tête de Moine	Vacuum-distillation; DHS; SDE; TD; P&T	Groux et al., 1974; Dumont et al., 1974a, b; Parliment et al., 1982; Guichard et al., 1987; Bosset and Gauch, 1993; Valero et al., 2001; Lawlor et al., 2002; Bonaïti et al., 2005;
2-Octanone	Limburger; Comté; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; TD	Parliment et al., 1982; Guichard et al., 1987; Lawlor et al., 2002

2-Nonanone	Maroilles; Pont l'Eveque; Langres; Epoisses; Vacherin; Livarot; Limburger; Comté; Beaufort; Appenzeller; Chaumes; Raclette; Tête de Moine	Vacuum-distillation; DHS; SDE; TD; P&T	Groux et al., 1974; Dumont et al., 1974a, b; Parliment et al., 1982; Guichard et al., 1987; Bosset and Gauch, 1993; Valero et al., 2001; Lawlor et al., 2002; Bonaïti et al., 2005
2-Undecanone	Maroilles; Epoisses; Vacherin; Limburger; Comté; Livarot	Vacuum-distillation; P&T	Groux et al., 1974; Dumont et al., 1974a, b; Parliment et al., 1982; Guichard et al., 1987; Bonaïti et al., 2005
Acetophenone	Maroilles; Livarot; Pont l'Eveque; Langres; Limburger; Comté	Vacuum-distillation	Dumont et al., 1974a; Parliment et al., 1982; Guichard et al., 1987
Ethanol	Vacherin; Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; P&T; TD	Groux et al., 1974; Dumont et al., 1974a; Bosset and Gauch, 1993; Lawlor et al., 2002;
1-Propanol	Maroilles; Pont l'Eveque; Langres; Epoisses; Vacherin; Livarot; Comté; Beaufort; Appenzeller	Vacuum-distillation; P&T	Groux et al., 1974; Dumont et al., 1974a, b; Bosset et al., 1993
2-Propanol	Pont l'Eveque; Langres; Comté; Appenzeller; Chaumes; Livarot	Vacuum-distillation; TD; P&T	Dumont et al., 1974a; Bosset and Gauch, 1993; Valero et al., 2001; Bonaïti et al., 2005
2-Methylpropanol	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Appenzeller	Vacuum-distillation; P&T	Dumont et al., 1974a; Bosset et al., 1993
1-Butanol	Pont l'Eveque; Epoisses; Comté; Beaufort; Appenzeller	Vacuum-distillation; P&T	Dumont et al., 1974a; Bosset et al., 1993
2-Butanol	Maroilles; Livarot; Pont l'Eveque; Langres; Vacherin; Comté; Beaufort; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; P&T; TD	Dumont et al., 1974a, b; Bosset and Gauch, 1993; Lawlor et al., 2002
2-Methylbutanol	Comté; Beaufort; Appenzeller; Tête de Moine	P&T; TD	Bosset and Gauch 1993; Lawlor et al., 2002
3-Methylbutanol	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Vacherin; Comté; Beaufort; Appenzeller; Chaumes	Vacuum-distillation; P&T; DHS; TD	Dumont et al., 1974a, b; Bosset and Gauch, 1993; Valero et al., 2001
2-Pentanol	Vacherin; Maroilles; Pont l'Eveque; Comté; Beaufort; Appenzeller; Chaumes; Raclette	Vacuum-distillation; P&T; DHS; TD	Groux et al., 1974; Dumont et al., 1974a; Bosset and Gauch, 1993; Valero et al., 2001; Lawlor et al., 2002
Hexanol	Livarot; Pont l'Eveque; Comté; Chaumes	Vacuum-distillation; P&T; TD	Dumont et al., 1974a; Guichard et al., 1987; Bosset and Gauch, 1993; Valero et al., 2001
2-Heptanol	Maroilles; Pont l'Eveque; Langres; Vacherin; Comté; Appenzeller; Chaumes	Vacuum-distillation; P&T; DHS; SDE	Groux et al., 1974; Dumont et al., 1974a, b; Guichard et al., 1987; Bosset and Gauch, 1993; Valero et al., 2001
2-Nonanol	Livarot; Pont l'Eveque; Langres; Vacherin; Comté	Vacuum-distillation	Dumont et al., 1974a, b; Guichard et al., 1987

Phenol	Maroilles; Livarot; Pont l'Eveque; Vacherin; Limburger; Chaumes	Vacuum-distillation; DHS; SDE; TD	Dumont et al., 1974a, b; Parliment et al., 1982; Valero et al., 2001
2-Phenylethanol	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Limburger; Comté; Chaumes	Vacuum-distillation; TD	Dumont et al., 1974a; Parliment et al., 1982; Guichard et al., 1987; Valero et al., 2001
Cresol	Livarot; Pont l'Eveque; Langres; Limburger; Comté	Vacuum-distillation	Dumont et al., 1974a; Parliment et al., 1982; Guichard et al., 1987
Ethyl acetate	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Appenzeller; Tête de Moine	Vacuum-distillation; P&T; TD	Dumont et al., 1974a; Bosset and Gauch, 1993; Lawlor et al., 2002
Ethyl butanoate	Langres; Epoisses; Comté; Chaumes; Appenzeller; Raclette; Tête de Moine ; Tilsit	Vacuum-distillation; TD; HS-SPME	Dumont et al., 1974a; Guichard et al., 1987; Valero et al., 2001; Lawlor et al., 2002; Fuchsmann et al., 2015
Ethyl hexanoate	Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Chaumes; Tête de Moine ; Tilsit	Vacuum-distillation; P&T; TD; HS-SPME	Dumont et al., 1974a ; Guichard et al., 1987; Bosset and Gauch, 1993; Valero et al., 2001; Lawlor et al., 2002; Fuchsmann et al., 2015
Ethyl octanoate	Livarot; Pont l'Eveque; Langres, Epoisses; Limburger; Chaumes; Tilsit	Vacuum-distillation; SDE; HS-SPME	Dumont et al., 1974a; Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
Methyl thioacetate	Pont l'Eveque; Epoisses; Limburger; Beaufort; Chaumes	Vacuum-distillation; P&T; DHS	Dumont et al., 1974a; Parliment et al., 1982; Bosset and Gauch, 1993; Valero et al., 2001
Dimethyl disulphide	Maroilles; Pont l'Eveque; Langres; Epoisses; Vacherin; Livarot; Limburger; Chaumes; Raclette; Tête de Moine	Vacuum-distillation; DHS; SDE; TD; P&T	Dumont et al., 1974a, b; Parliment et al., 1982; Valero et al., 2001; Lawlor et al., 2002; Bonaïti et al., 2005
Dimethyl trisulphide	Maroilles; Pont l'Eveque; Langres; Livarot; Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; TD; P&T; HS-SPME	Dumont et al., 1974a; Parliment et al., 1982; Valero et al., 2001; Lawlor et al., 2002; Bonaïti et al., 2005; Fuchsmann et al., 2015
Indole	Maroilles; Livarot; Pont l'Eveque; Langres; Vacherin; Limburger; Chaumes	Vacuum-distillation; SDE; TD	Dumont et al., 1974a, b; Parliment et al., 1982; Valero et al., 2001
Toluene	Pont l'Eveque; Langres; Epoisses; Vacherin; Livarot; Chaumes	Vacuum-distillation; DHS; SDE; TD; P&T	Dumont et al., 1974a, b; Valero et al., 2001; Bonaïti et al., 2005
Benzene	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Chaumes	Vacuum-distillation; TD	Dumont et al., 1974b; Valero et al., 2001
Naphthalene	Maroilles; Livarot; Langres; Epoisses; Comté	Vacuum-distillation	Dumont et al., 1974a; Guichard et al., 1987

1.9. Conclusion

Numerous extraction techniques are available for the analysis of cheese volatiles. However, the techniques available all have some limitations. The extraction technique, the nature of the extraction polymeric material, the GC column and the detector, greatly influence the sensitivity and reliability of the detection of specific analytes; evident from studies performed on surface-ripened cheese, where the volatiles detected were predictable based on the characteristics of the extraction techniques and the column stationary phase used. At present, the various analytical approaches have to be considered as complementary techniques in order to get a complete volatile profile. Further work is required to fully elucidate the flavour characteristics of surface-ripened cheese.

1.10. Acknowledgements

Thanks to my supervisor Dr Kieran Kilcawley for the suggestions for writing this review.

1.11. References

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Chapter 2

Flavour-forming ability of Gram-positive bacteria isolated from surface-
ripened cheese

2.1. Abstract

Surface-ripened cheese is characterized by the growth of complex microbiota (smear-bacteria and yeasts) on the cheese surface, and a distinctive flavour. Gram-positive bacteria are mainly responsible for the biosynthesis of volatile compounds, and thus the development of cheese flavour during ripening. In this study, 11 Gram-positive bacteria were characterized for specific enzymatic activities known to be important in cheese flavour development, such as glutamate dehydrogenase, aromatic aminotransferase, esterase (on p-nitrophenol butyrate) and lipase (on tributyrin) activities. Their flavour-forming ability was screened using two distinct cheese model systems (MS1 and MS2). In MS1, stationary phase cells were inoculated into a free amino acid based media at pH >6; in MS2, the strains were inoculated during exponential phase into a cheese curd based media at a pH <6, and incubated under controlled conditions. Volatiles were characterized by headspace-solid phase microextraction gas chromatography mass spectrometry (HS-SPME GCMS) and correlated with the bacterial strain metabolomic and enzymatic activities. Although HS-SPME GCMS analysis revealed numerous compounds within both model systems, significantly more volatiles were associated with the bacterial strains in MS1, suggesting this method provides a better screening approach for the flavour-forming ability of smear bacteria, possibly due to more readily available substrates (FAAs and peptides) in MS1, compared to the more complex curd-based substrate in MS2.

2.2. Introduction

Flavour development of cheese is related to milk quality, processing operations and, most importantly, microbial activity. The role of the bacterial population in the ripening of fermented food, such as surface-ripened cheese, has a strong impact on the final flavour and appearance (Bertuzzi et al., 2017). Surface-ripened cheese is characterized by the growth of a complex microbial population on the surface, which is responsible for the distinctive strong flavour and the typical “glistening” appearance. The microbial environment of surface-ripened cheese is very heterogeneous and characterized by the cohabitation of different yeast and bacterial species, which metabolize the available substrates on the cheese surface (Cogan et al., 2014; Larpin et al., 2011; Mounier et al., 2005; Rea et al., 2007). It is clear that variations in the microbial population on the cheese surface influences the biosynthesis of specific volatile compounds, which differentiate the individual surface-ripened cheese varieties (e.g., Münster, Tilsit, Livarot, Limburger and Comté) (Bertuzzi et al., 2017; Bonaïti et al., 2005; Fuchsmann et al., 2015).

Cheese is a product with high levels of fat and protein, with traces of carbohydrates, which are used as substrates by the resident microbial population to produce flavour compounds (Kilcawley, 2017; McSweeney, 2017). The proteolytic processes cause the release of free amino acids (FAAs), and their subsequent metabolism (Ganesan and Weimer, 2017). The aromatic, branched-chain and sulphur amino acids, are converted to branched-chain and aromatic aldehydes, carboxylic acids

or alcohols, phenols, indole, and sulphur compounds, while aspartic acid can be degraded into acetoin, diacetyl, or 2,3-butanediol (Ardö, 2006; Ganesan and Weimer, 2017; Peralta et al., 2014). Numerous enzymes are involved in the catabolism of FAAs, resulting in the biosynthesis of flavour compounds (e.g. decarboxylases, aminotransferases, deaminases, lyases and dehydratases) (Ardö, 2006; Ganesan and Weimer, 2017). Catabolism of FAAs commonly starts with the transfer, by aminotransferase (AT) activity, of the amino group from FAAs to 2-oxoglutarate, producing the relative α -keto acid, and L-glutamate, which is converted again by glutamate dehydrogenase (GDH) to 2-oxoglutarate (Ardö, 2006). Microbial enzymes are also involved in lipolytic processes. The carboxylesterases responsible for the catabolism of triacylglycerols in cheese and the release of FFAs, are lipases, which are active against water-insoluble long chain triacylglycerols (carbon chain lengths >10), and esterases, which are able to hydrolyse water-soluble short acyl chain esters (carbon chain lengths ≤ 10) (Chahiniana and Sarda, 2009). Free fatty acids (FFAs) contribute to the formation of the cheese flavour, not only directly, but also indirectly as they are precursors of methyl ketones, secondary alcohols, straight-chain aldehydes, lactones, esters, and S-thioesters (Thierry et al., 2017).

Numerous types of cheese models have been developed to study the biosynthesis of volatile compounds by specific bacterial strains (Deetae et al., 2007; Pogačić et al., 2015; Stefanovic et al., 2017a; Van de Bunt et al., 2014). These cheese models were used as practical, low-cost and rapid methods to screen the volatile-forming capacities of cells, through the use of gas-chromatographic techniques.

In this study, 11 Gram-positive bacterial strains, from different taxonomic groups, and isolated from surface-ripened cheese, were screened for their enzymatic potential for aromatic AT, GDH, esterase (on p-nitrophenol butyrate), lipase (on tributyrin) activities, and for their abilities to produce volatile compounds in two different model cheese systems, characterized by different cell growth phases, model composition, and incubation time.

2.3. Material and methods

2.3.1. Strains and conditions of growth

The origins of the strains used in this study are outlined in Table 2.1. The strains were stored at -80°C for long term storage, and before use, were streaked onto trypticase soy agar (TSA; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated at 30°C, for a specific incubation time, as specified in Table 2.1. After growth on the agar, a sterile 5 µL loop was used to pick up colonies and inoculate them into trypticase soy broth (TSB; Becton, Dickinson and Company) and the cultures were incubated at 120 rpm shaking, at 30°C, as specified in Table 2.1.

Table 2.1. Strains used in this study and related growth conditions.

Strains	Identification code	Source	Incubation time in TSA	Incubation time ins TSB
<i>Brevibacterium aurantiacum</i>	DPC5683	Irish surface-ripened cheese	96h	72h
<i>Brevibacterium linens</i>	B11	Commercial smear strain	96h	72h
<i>Brevibacterium linens</i>	DPC5699	Surface-ripened cheese	96h	72h
<i>Corynebacterium casei</i>	DPC5298	Surface-ripened cheese	72h	48h
<i>Corynebacterium flavescens</i>	NCIMB 8707	Cheese	72h	48h
<i>Corynebacterium variabile</i>	DPC5313	Irish surface-ripened cheese	72h	48h
<i>Staphylococcus equorum</i>	DPC4416	Comte cheese	24h	24h
<i>Staphylococcus saprophyticus</i>	DPC5671	Cheddar cheese	24h	24h
<i>Arthrobacter arilaitensis</i>	DPC6297	Irish surface-ripened cheese	72h	48h
<i>Microbacterium gubbeenense</i>	DPC5286	Irish surface-ripened cheese	96h	72h
<i>Micrococcus luteus</i>	DPC6921	Cheese	72h	48h

2.3.2. Tributyrin agar assay

Tributyrin agar (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) was prepared as described by the manufacturer, with the following modifications. After autoclaving, 1% tributyrin (v/v) (Sigma Aldrich) was added to the liquid hot agar (~60°C), and the

mixture was homogenized by using Ultra Turrax Ika (Imlab, Boutersem, Belgium) for 20 sec, and poured in petri dishes and left to set. To test the lipase activity, the bacterial strains were grown in 10 mL of TSB as previously described, and 5 μ l of broth culture were placed on the surface of the tributyrin agar. The plates were incubated at 30°C aerobically and the activity was screened after 48 h. The appearance of a halo around the bacterial growth indicated that the strain was lipase positive. *Yarrowia lipolytica* DPC6266 was used as a positive control. Tributyrin results are shown in Fig. 2.1, and a positive result scored on the basis of the size of the halo of clearing (+: ≥ 1 mm; ++: ≥ 2 mm; +++: ≥ 3 mm). The test assay was performed in triplicate.

2.3.3. Preparation of cell free extract

After the growth of the bacterial strains in 10 mL of TSB, the cells were centrifuged ($8000 \times g$, 10 min, 4°C) and washed twice, with 50 mmol L⁻¹ sodium-phosphate buffer pH 7.5, and resuspended in the same buffer to a final volume of 2 mL. The cell suspension was sonicated by Soniprep 150 (MSE, Lower Sydenham London, UK), with a cycle of 5 min (45 sec sonication, 15 sec rest). The sonication process was performed keeping the samples cool in an ice cold bath throughout. The sonicated preparation was centrifuged with a bench top centrifuge (Eppendorf, Hamburg Germany) for $16,000 \times g$, for 5 min, and the cell free extract (CFE) used for the enzymatic assays. The negative control for all assays was CFE heated at 98°C for 15 min. The concentration of protein in the CFE was determined using Qubit® Protein Assay Kit (Cambio Ltd, Cambridge, UK).

2.3.4. Aminotransferase activity

Aromatic AT activity was determined by the detection of the conversion of phenylalanine to phenylpyruvate, using a UV transparent black 96 well microplate (Greiner Bio-One, Kremsmünster, Austria) as previously described by Brandsma *et al.* (2008), with modifications made by Stefanovic *et al.* (2017b). To measure the AT activity, 100 μ l of CFE was added to 150 μ l of the final reaction mixture containing 20 mmol L⁻¹ L-phenylalanine, 10 mmol L⁻¹ α -ketoglutarate, 0.5 mmol L⁻¹ sodium EDTA, 0.05 mmol L⁻¹ pyridoxal-5'-phosphate, all dissolved in 25 mmol L⁻¹ borate buffer pH 8.5. The plate was incubated at 30°C for 12 hours and the absorbance was measured at 290_{nm} post incubation, using a plate reader (Synergy HT, Bio-Tek Multi Detection Plate Reader). A standard curve obtained with standards, ranging from 5 to 450 nmol, of sodium-phenylpyruvate, was used for the conversion of data to μ mol of phenylpyruvate released per mg of protein. All strains were analysed in triplicate.

2.3.5. Glutamate dehydrogenase activity

Glutamate dehydrogenase activity (GDH) was measured using the method of Kieronczyk *et al.* (2003), as described by Stefanovic *et al.* (2017b). The GDH kit was modified by the addition of bacterial CFE to the kit reagents provided with Megazyme L-Glutamic Acid Kit assay (K-GLUT®, Megazyme International Ireland Ltd), allowing for the quantitative determination of GDH activity of the bacterial strains, using a transparent 96 well microplate (Sarstedt Lim., Wexford, Ireland). Modification of the original assay conditions were as follows: 100 μ l of CFE was added to the final reaction

mixture containing 10 μl of diaphorase, 40 μl of TEA buffer, 20 μl of glutamic acid solution (0.1 mg ml^{-1}), and 20 μl of INT-NAD⁺ solution (all reagents were supplied with the K-GLUT® Kit). After 1h of incubation at 37°C, the absorbance was determined at 492_{nm}. The total quantity of enzyme that resulted in an increase of absorbance of 0.01 per 1 min corresponded to one unit (U) of activity. The results were expressed as the number of units of activity per mg of protein. All strains were analysed in triplicate.

2.3.6. Esterase activity

To determine esterase activity, the conversion of p-nitrophenol butyrate to p-nitrophenol and butyric acid was measured using a transparent 96 well microplate (Sarstedt Lim.). The assay mixture was composed of a buffer (100 mmol l⁻¹ sodium phosphate, 150 mmol l⁻¹ sodium chloride, 0.5% v/v triton X-100, at pH 7) and a substrate (50 mmol l⁻¹ p-nitrophenol butyrate in acetonitrile). In each well, 50 μl of buffer, 50 μl of CFE and 10 μl of substrate were mixed and absorbance was measured with a plate reader, after 1 hour of incubation at 37°C at 400_{nm}. The amount of p-nitrophenol released was determined from a standard curve obtained for a set of standards ranging from 0 to 500 nmol of p-nitrophenol. The activity was expressed as μmol of p-nitrophenol released per mg of protein.

2.3.7. Model system 1

Model system 1 (MS1) was based on the procedure outlined by Van de Bunt *et al.* (2014), following the modifications made by Stefanovic *et al.* (2017a). In this study a solution of NZ amine (Sigma-Aldrich) was used as the base ingredient for the cheese model medium. This AA-rich medium was used to determine the diversity of strains by their ability to metabolize AA, because the products of AA catabolism are generally seen as highly important food flavor contributors. The cell suspensions (1 mL) were added to 9 mL of cheese model system medium, which was incubated at 30°C for 24h. Before incubation the cells were counted by serially diluting the cultures on TSA (at 30°C, following the Table 2.1), and after the incubation were counted again, and the pH was also measured. Samples were stored at -20°C, for headspace-solid phase microextraction gas chromatography mass spectrometry (HS-SPME GCMS) volatile analysis. The un-inoculated model system was used as a control. Test strains and the controls were prepared in triplicate.

2.3.8. Model system 2

For the preparation of the model system 2 (MS2), a method previously outlined by Pogačić *et al.* (2015) was used with some modifications. Non-salted curd of Cheddar cheese (<2 h post manufacture) was cut and stored wrapped in aluminium foil, in vacuum bags (Südpack Verpackungen, Ochsenhausen, Germany) at -20°C. Before use, the defrosted curd was blended on a high speed cycle using a food processor (Russell Hobbs, Failsworth, UK), and 10 g of unsalted blended curd, were added to 20 g of a

solution of bactotripton (Becton, Dickinson and Company, City West, Dublin, Ireland) (1.2 g L^{-1}) and NaCl (18 g L^{-1}) inside a glass tube and autoclaved at 110°C , 15 min. Before the use, 300 μL of a sterile lactose solution (120 g L^{-1}) was added to each tube (final concentration of lactose $\sim 1.2 \text{ g L}^{-1}$). Strains were grown in 10 mL of TSB, as previously described (Table 2.1), and when cultures reached $\text{OD}_{600\text{nm}}$ of ~ 1 , were washed and resuspended in 10 mL of NaCl solution 0.5 % (w/v), and 300 μL inoculated in each tube containing model system medium. The cell numbers at the start of the experiment were determined (cfu mL^{-1}) by serially diluting the cultures and spread plating the dilutions on TSA and incubated as described in Table 2.1. After 15 days incubation at 30°C , the pH of the sample was measured and the cells were counted again. Samples were stored at -20°C , for HS-SPME GCMS volatile analysis. A sample of the uninoculated model system was used as a control. Both the test strains and the controls were prepared in triplicate.

2.3.9. Volatile analysis

The samples taken from the model systems were stored at -20°C and were defrosted at room temperature before the analysis. HS-SPME GCMS analysis was carried out as outlined by Bertuzzi *et al.* (2017) (see “Volatile analysis” p 138-139), and the chromatogram was analysed using R-3.2.2 (Team, 2014). All analyses were performed in triplicate.

2.3.10. Statistical analysis

The data related to the pH measurement and cell enumeration, were tested with one-way analysis of variance (ANOVA) using SAS 9.4 (SAS, Dublin, Ireland) (level of significance at $P<0.05$). The volatile compounds detected with HS-SPME GCMS were analysed with principal component analysis (PCA) with Unscrambler (The Unscrambler X 10.3, Camo Software, Oslo, Norway). The significant association between volatiles and bacterial strains was determined by the selection of the volatile compounds with the highest component coefficients for the first, second and third principal components. The selected volatile compounds were analysed with one-way (ANOVA), using SAS 9.4, to observe if they were significantly higher for the strains, compared to the controls (level of significance at $P<0.05$).

2.4. Results

2.4.1. Enzymatic assays

Initially, CFE of the strains were screened for their enzymatic capability for some key enzymes involved in flavour development and the activity of the strains varied depending on the genus. All strains demonstrated some GDH activity when compared to the control, but *A. arilaitensis* DPC6297 and *C. flavescentis* NCIMB 8707 showed the greatest activities, which were significantly higher ($P<0.05$) than the control (Fig. 2.1). *M. luteus* DPC6921 showed significantly greater esterase activity (on p-nitrophenol butyrate) ($P<0.05$) compared to the control, and *A. arilaitensis* DPC6297 displayed significantly greater aromatic AT activity, compared to the control ($P<0.05$) (Fig. 2.1). The tributyrin agar test demonstrated very high activity (+++) for *S. saprophyticus* DPC5671, medium activity (++) for *B. linens* DPC5699 and *B. aurantiacum* DPC5683 and low activity (+) for *S. equorum* DPC4416, *B. linens* Bl1, *C. variabile* DPC5313, and *A. arilaitensis* DPC6297 (+) (Fig. 2.1).

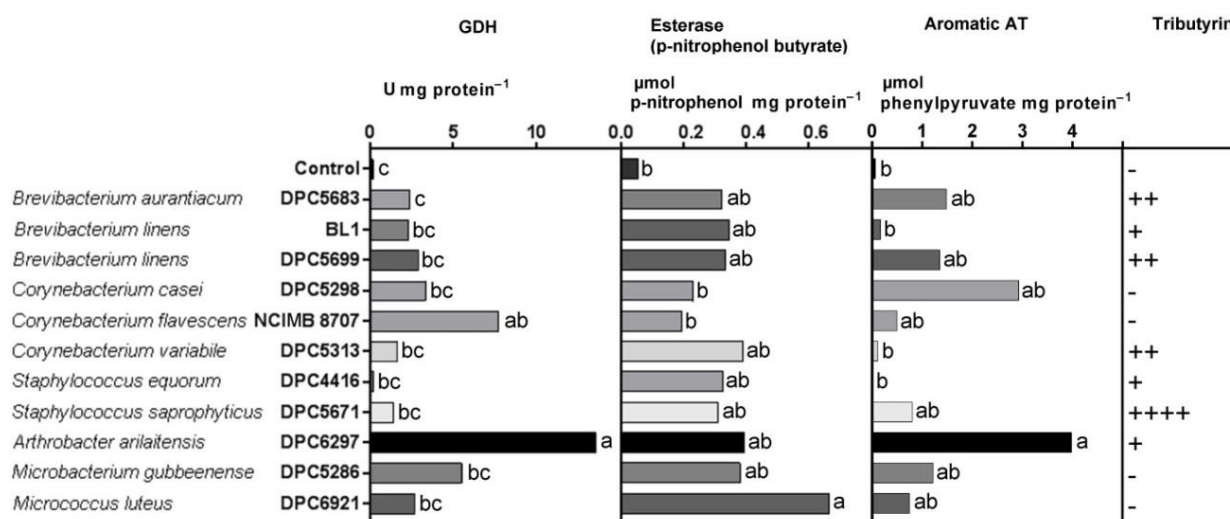


Figure 2.1. Means of three replicates of GDH (expressed in U mg protein⁻¹), esterase (on p-nitrophenol butyrate) (expressed in μmol of p-nitrophenol mg protein⁻¹) and aromatic AT (expressed in μmol of phenylpyruvate mg protein⁻¹) activities in CFE. The significant differences ($P < 0.05$) are indicated with a, b, c. Tributyrin hydrolysis: negative: -; halo size: $\geq 1\text{mm}$: +; $\geq 2\text{mm}$: ++; $\geq 3\text{mm}$: +++.

2.4.2. Model system 1 (MS1)

To determine whether there was any change in cell numbers during the incubation period, (24h) the cells were counted prior to and post incubation. In all cases there was no increase in cell numbers, but there was a significant decrease ($P < 0.05$) (≤ 1 log reduction) for *S. equorum* DPC4416, *A. arilaitensis* DPC6297 and *M. luteus* DPC6921 (Table 2.2). In addition, there was a significant reduction in pH ($P < 0.05$) when compared to the control for all strains, except for the three *Brevibacterium* strains and *C. flavescens* (Table 2.2). Considering all 73 volatile compounds detected in MS1, only 35 were significantly associated ($P < 0.05$) with specific bacterial strains (Table 2.3 and Table 2.4). Specifically, they were 3 acids (acetic acid, butanoic acid, 3-methylbutanoic acid), 8 ketones (acetoin, 6-methyl-2-heptanone, 6-methyl-5-heptene-2-one, 2-

hexanone, 2-heptanone, 2-nonaone, 2-tridecanone, 2-pentadecanone), 5 esters (isobutyl acetate, isopentyl acetate, isopentyl isovalerate, 2-furanmethyl acetate, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate), 3 pyrazines (2,5-dimethylpyrazine, trimethylpyrazine, 2-ethyl-5-methyl-pyrazine), 10 alcohols (3-methyl-1-butanol, 1-pentanol, hexanol, 2-ethylhexanol,

octanol, 2-hexyl-1-octanol, nonanol, 2-undecanol, benzyl alcohol, phenylethyl alcohol), 2 sulphurs (dimethyl sulphide, methyl thiolacetate) and other compounds (1,3-bis-1,1-dimethylethyl-benzene, 1-octene, furfural, p-xylene). The PCA plot (Fig. 2.2), based on the volatiles detected in MS1, describes 47%, 31% and 13% of the total variability with the first, second and third principal component, respectively.

Table 2.2. Cell enumeration presented in log cfu mL⁻¹, in MS1 and MS2, before and after incubation¹. pH values, in MS1 and MS2, after the incubation².

Species	Identification code	MS1			MS2		
		0 (h)	24 (h)	pH (24h)	0 (day)	15 (day)	pH (15day)
Control		ND	ND	6.65±0.01 ^{AB}	ND	ND	5.76±0.01 ^A
<i>Brevibacterium aurantiacum</i>	DPC5683	9.92±0.07	10.17±0.14	6.59±0.05 ^{BCD}	6.05±0.02	7.45±0.41	5.69±0.07 ^{ABC}
<i>Brevibacterium linens</i>	BI1	9.64±0.22	9.71±0.14	6.59±0.05 ^{BC}	6.18±0.08	7.19±0.09	5.66±0.03 ^{ABC}
<i>Brevibacterium linens</i>	DPC5699	9.59±0.26	9.23±0.14	6.71±0.02 ^A	6.29±0.19	8.86±0.69	5.72±0.07 ^{AB}
<i>Corynebacterium casei</i>	DPC5298	9.49±0.11	9.54±0.12	6.51±0.06 ^{CDE}	6.22±0.11	7.00±0.10	5.65±0.01 ^{ABC}
<i>Corynebacterium flavescens</i>	NCIMB 8707	10.38±0.22	10.30±0.29	6.55±0.04 ^{BCD}	6.90±0.17	8.51±0.12	5.72±0.02 ^{ABC}
<i>Corynebacterium variabile</i>	DPC5313	9.85±0.07	10.17±0.14	6.46±0.04 ^{DE}	6.9±0.14	7.83±0.15	5.68±0.02 ^{ABC}
<i>Staphylococcus equorum</i>	DPC4416	9.82±0.11	9.13±0.10	6.49±0.02 ^{CDE}	6.31±0.28	6.81±0.47	5.70±0.02 ^{ABC}
<i>Staphylococcus saprophyticus</i>	DPC5671	9.94±0.04	9.92±0.32	6.47±0.01 ^{DE}	6.06±0.05	8.32±0.71	5.52±0.01 ^D
<i>Arthrobacter arilaitensis</i>	DPC6297	8.94±0.14	8.23±0.19	6.52±0.04 ^F	6.09±0.10	6.65±0.60	5.60±0.02 ^{CD}
<i>Microbacterium gubbeenense</i>	DPC5286	9.68±0.08	9.79±0.06	6.57±0.07 ^{CDE}	6.57±0.38	7.13±0.06	5.64±0.01 ^{BC}
<i>Micrococcus luteus</i>	DPC6921	10.06±0.27	9.51±0.06	6.44±0.05 ^{EF}	6.29±0.04	7.93±0.32	5.34±0.07 ^E

¹Results are showed as means ± SD of triplicate experiments. Strains that showed significant difference ($P<0.05$) in CFU mL⁻¹ after the incubation time are reported in bold. ND= not detected.

² A-F Results of pH values sharing the same letter in the column corresponding to MS1 or MS2 do not significantly differ ($P<0.05$)

associated ($P < 0.05$) with specific bacterial strains (Table 2.3 and Table 2.5). Specifically, they were 1 acid (acetic acid), 1 ketone (2-heptanone), 1 lactone (δ -decalactone), 2 esters (ethyl acetate, isobutyl acetate), 6 alcohols (2-methyl-1-butanol, 2-heptanol, 2-nonanol, benzyl alcohol, octanol, phenylethyl alcohol), 1 sulphur (dimethyl disulphide), and other compounds (E-2-nonenal, furfural, indole). The PCA plot (Fig. 2.3), based on the volatiles detected in MS2, describes 47%, 37%, and 4%, of the total variability with first, second and third principal components respectively.

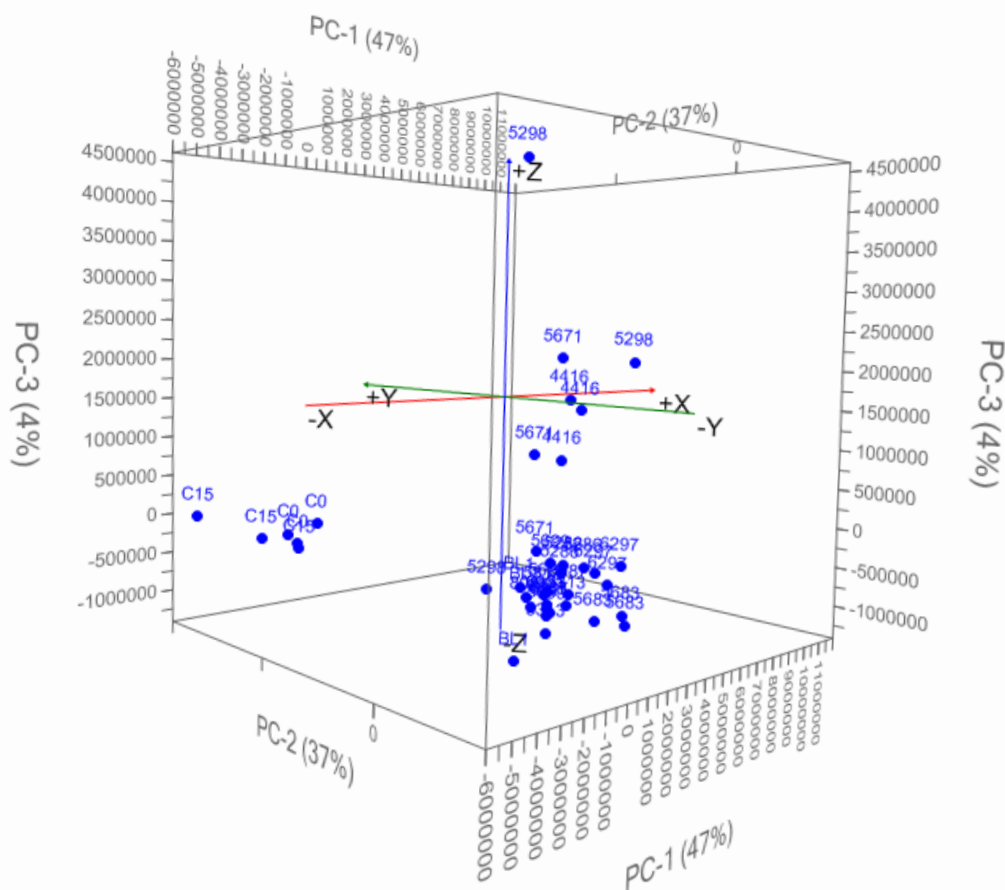


Figure 2.3. Principal component analysis (PCA) plot of MS2, shows the separation between the volatile profiles of the controls (C15 and C0), and the bacterial strains, showing a development of volatile compounds during the 15 days of incubation.

Table 2.3. Volatile compounds significantly ($P<0.05$) associated¹ with the bacterial strains in the two model systems (MS1 and MS2).

Strains	MS1	MS2
<i>Brevibacterium aurantiacum</i> DPC5683	2-Ethylhexanol, p-Xylene, Methyl thiolacetate, Dimethyl sulphide	2-Heptanone, 2-Methyl-1-butanol, Phenylethyl alcohol, Ethyl acetate, Isobutyl acetate, Acetic acid, δ -Decalactone, Furfural
<i>Brevibacterium linens</i> B11	6-Methyl-2-heptanone, 2-Tridecanone, 2-Ethylhexanol, 3-Methyl-1-butanol, Dimethyl sulphide, 2,5-Dimethylpyrazine, 1-Octene, Furfural	2-Heptanol, Benzyl alcohol, Isobutyl acetate
<i>Brevibacterium linens</i> DPC5699	2-Ethylhexanol, Benzyl alcohol, Dimethyl sulphide, Methyl thiolacetate	Phenylethyl alcohol, 2-Nonanol
<i>Corynebacterium casei</i> DPC5298	2-Hexanone, 2-Heptanone, 2-Tridecanone, 1-Pentanol, Hexanol	2-Heptanone, δ -Decalactone
<i>Corynebacterium flavescens</i> NCIMB 8707	2-Pentadecanone, 3-Methyl-1-butanol, 2-Ethylhexanol, Benzyl alcohol, Phenylethyl alcohol, Dimethyl sulphide, Methyl thiolacetate, Isobutyl acetate Isopentyl acetate, 2-Furanmethyl acetate, 3-Methyl-butanoic acid, Butanoic acid, p-Xylene, 2,5-Dimethylpyrazine, 1-Octene, 3-Hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate	2-Heptanol, 2-Nonanol, Phenylethyl alcohol
<i>Corynebacterium variabile</i> DPC5313	6-Methyl-2-heptanone, 2-Tridecanone, 2-Ethylhexanol, Benzyl alcohol, Dimethyl sulphide, p-Xylene, 1-Octene	Octanol, 2-Nonanol, Acetic acid

<i>Staphylococcus equorum</i> DPC4416	6-Methyl-5-heptene-2-one, 6-Methyl-2-heptanone, 2-Nonaone 2-Ethylhexanol, Methyl thiolacetate, 3-Methyl-butanoic acid, Acetoin, p-Xylene, Acetic acid, 1,3-Bis(1,1-dimethylethyl)-benzene	2-Methyl-1-butanol
<i>Staphylococcus saprophyticus</i> DPC5671	6-Methyl-2-heptanone, 2-Tridecanone, 2-Pentadecanone, 2-Ethylhexanol, 3-Methyl-1-butanol, Benzyl alcohol, Dimethyl sulphide, Acetic acid, Acetoin, 2,5-Dimethylpyrazine, 2-Ethyl-5-methyl-pyrazine	2-Heptanol, Acetic acid
<i>Arthrobacter arilaitensis</i> DPC6297	6-Methyl-2-heptanone, 2-Tridecanone, 2-Ethylhexanol, Benzyl alcohol, Trimethyl-pyrazine	2-Heptanone, δ -Decalactone, Indole
<i>Microbacterium gubbeenense</i> DPC5286	2-Hexanone, 6-Methyl-2-heptanone, 2-Tridecanone, 2-Ethylhexanol, 3-Methyl-1-butanol, Nonanol, Isobutyl acetate, Isopentyl isovalerate	2-Heptanone, δ -Decalactone, 2-Heptanol, Isobutyl acetate, Dimethyl disulphide, (E)2-Nonenal
<i>Micrococcus luteus</i> DPC6921	2-Tridecanone, 2-Pentadecanone, 2-Ethylhexanol, Octanol, 2-Undecanol, 2-Hexyl-1-octanol, Benzyl alcohol, Dimethyl sulphide, 3-Methyl-1-butanol, Isopentyl acetate, Acetic acid, p-Xylene, 2,5-Dimethylpyrazine	

¹ The significant association between volatiles and bacterial strains was determined by the selection of the volatile compounds with the highest component coefficients for the first, second or third principal components. The selected volatile compounds were analysed with one-way (ANOVA), to observe if they were significant higher ($P < 0.05$) for the strains, compared to the controls.

2.5. Discussion

Two approaches were taken to determine the suitability of model systems to screen the flavour potential of a range of genera associated with surface ripened cheese. MS1 was used to study the flavour producing capability of strains in the stationary phase of bacterial growth using a simple substrate, composed mainly of high concentrations of FAAs and small peptides (NZ amine). MS2 was used to study the strains in an exponential phase within a complex substrate, mainly composed of cheese curd (fat and protein). Results of the model systems demonstrated that the growth of the bacterial strains was influenced by the growing phase of the cells inoculated, and by the type of nutrients added. This was consistent with other studies where synthetic models did not show an increase in the number of cells (Kieronczyk et al., 2001; Stefanovic et al., 2017a), while in cheese based model systems there was a significant growth ($P<0.05$) during the incubation (Milesi et al., 2008).

The pH varied in both model systems and was significantly lower ($P<0.05$) at the end of the incubation for specific strains, compared to the control (Table 2.2). The pH was mainly influenced by the acidic (e.g. carboxylic acids, and phenolic compounds), or basic compounds (e.g. nitrogen-containing compounds and sulphur compounds) derived from the bacterial metabolism of the model system components; however, in MS2, the pH was also related to the metabolism of the lactose (added after the autoclaving) and lactate, which were not present within MS1. The higher pH of MS1 (starting pH ~6.65) likely supported the metabolism of the bacterial strains, compared to

the lower starting pH in MS2 (starting pH ~5.7), simulating the condition of the exterior of surface-ripened cheese. Most of the Gram-positive bacteria (not acidophilic) are not able to convert lactose into lactate (with lactate dehydrogenase), and grow on the cheese surface when the lactate is metabolized by the yeasts, and the pH increases (>6) (Bertuzzi et al., 2017; Mounier et al., 2006).

It is likely that the pH levels, and the composition of MS1, provided better conditions for the metabolism of the substrates by the selected bacterial strains, with a consequent biosynthesis of a more diverse range of volatile compounds (Fig. 2.4).

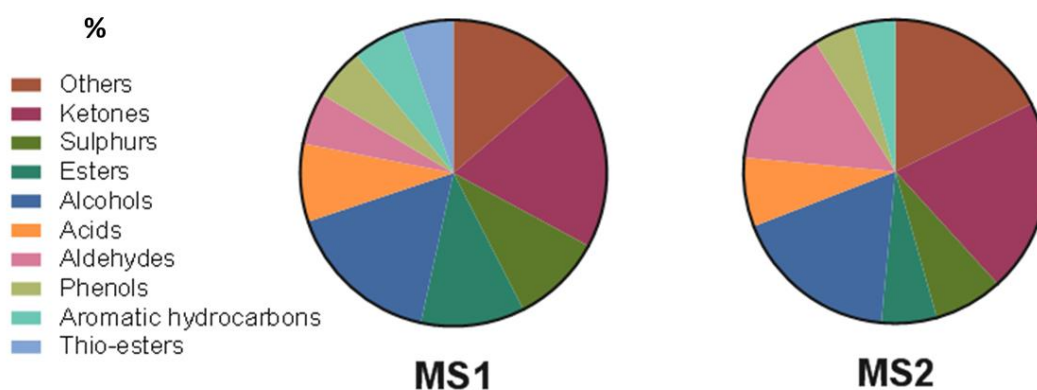


Figure 2.4. Percentages (%) of the class of compounds identified in MS1 and MS2.

Only *B. aurantiacum* DPC5683 was significantly associated ($P < 0.05$), with higher numbers of volatiles in MS2 compared to MS1. However, in both model systems, there was a separation between the volatile profiles of the controls, and of the strains, as shown in the two PCA plots (Fig. 2.2; Fig. 2.3), suggesting that the strains were metabolically active under both conditions. Numerous compounds, such as ketones,

lactones and secondary alcohols, were detected within both two model systems (Table 2.4 and Table 2.5).

Table 2.4. Volatiles detected with HS-SPME GCMS in MS1, showing the relative identification chemical abstracts service (CAS) number and the linear retention index (LRI).

MS1		
Compounds	CAS	LRI
Acetone	67641	<500
Dimethyl sulphide	75183	501
Carbon disulphide	75150	530
2-Methyl-1-propanol	78831	620
Acetic acid	64197	630
2-Methyl-cyclobutanone	1517153	661
2-Methylbutanal	96173	662
Pentanal	110623	700
Methyl thiolacetate	1534083	702
Propanoic acid	79094	708
Acetoin	513860	718
3-Methyl-1-butanol	123513	734
2-Methyl-1-butanol	137326	735
Dimethyl disulphide	624920	744
Isobutyl acetate	110190	767
Toluene	108883	767
1-Pentanol	71410	775
Ethyl isobutyrate	97621	778
1-Octene	111660	783
2-Hexanone	591786	788
Butanoic acid	107926	791
Butyl acetate	123864	812
Hexanal	66251	813
Furfural	98011	836
3-Methyl-butanoic acid	503742	846
2-Methyl-butanoic acid	116530	849
S-Methyl 2-methylpropanethioate/Butanethioic acid, S-methyl ester	42075423/ 2432511	851
Ethyl 2-methylbutanoate	7452791	855
2-Furanmethanol	98000	858
Hexanol	111273	864
p-Xylene	106423	869
Isopentyl acetate	123922	875
2-Heptanone	110430	893
Cyclohexanone	108941	900
2-Heptanol	543497	901
Methional	3268493	910

2,5-Dimethylpyrazine	123320	917
α -Pinene	80568	939
6-Methyl-2-heptanone	928687	950
Benzaldehyde	100527	970
Dimethyl trisulfide	3658808	979
1-Octen-3-ol	3391864	982
6-Methyl-5-heptene-2-one	110930	986
2-Pentylfuran	3777693	992
2-Furanmethyl acetate	623176	992
Trimethyl-pyrazine	14667551	1005
2-Ethyl-5-methyl-pyrazine	13360640	1011
2-Ethylhexanol	104767	1030
D-Limonene	5989275	1035
Benzyl alcohol	100516	1040
Benzeneacetaldehyde	122781	1052
p-Cresol	106445	1065
Octanol	111875	1072
Acetophenone	98862	1074
2-Nonanone	821556	1091
S-Methyl hexanethioate	1534083	1098
Isopentyl isovalerate	659701	1099
2-Nonanol	628999	1101
Phenylethyl alcohol	60128	1124
Octanoic acid	124072	1161
Nonanol	143088	1177
Methyl furfuryl disulphide	57500002	1224
Dimethyl tetrasulfide	5756241	1236
Benzene, 1,3-bis(1,1-dimethylethyl)-	1014604	1255
2-Undecanone	112129	1293
2-Undecanol	1653301	1298
Indole	120729	1305
Methyl phenylthiolacetate	5925746	1369
3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	74367343	1375
2-Hexyl-1-octanol	19780791	1485
2-Tridecanone	593088	1488
Phenol, 2,4-bis(1,1-dimethylethyl)-	96764	1502
2-Pentadecanone	2345280	1693

Table 2.5. Volatiles detected with HS-SPME GCMS in MS2, showing the relative identification chemical abstracts service (CAS) number and the linear retention index (LRI).

MS2		
Compounds	CAS	LRI
Dimethyl sulphide	75183	506
2-Propanol	67630	522
Carbon disulphide	75150	525
2,3-Butanedione	431038	577
Ethyl acetate	141786	626
Acetic acid	64197	636
1-Butanol	71363	654
2-Methylbutanal	96173	660
Pentanal	110623	697
2-Ethylfuran	3208160	700
Acetoin	513860	713
3-Methyl-1-butanol	123513	732
2-Methyl-1-butanol	137326	735
Methyl isobutyl ketone	108101	736
Dimethyl disulphide	624920	744
Isobutyl acetate	110190	753
1-Pentanol	71410	761
Toluene	108883	766
2-Hexanone	591786	788
Hexanal	66251	800
1-Octene	111660	805
Butyl acetate	123864	811
3-Methyl-butanoic acid	503742	832
Furfural	98011	835
2-Methyl-butanoic acid	116530	842
2-Furanmethanol	98000	857
Hexanol	111273	865
Heptanal	111717	869
p-Xylene	106423	870
Isopentyl acetate	123922	873
2-Heptanone	110430	888
Styrene	100425	896
2-Heptanol	543497	900
Methional	3268493	910
2,5-Dimethylpyrazine	123320	917
α -Pinene	80568	938
(E)-2-Heptenal	18829555	964
Benzaldehyde	100527	969
Hexanoic acid	142621	973
1-Octen-3-one	4312996	974
Dimethyl trisulfide	3658808	978
1-Octen-3-ol	3391864	983
3-Octanone	106683	984
6-Methyl-5-heptene-2-one	110930	985
6-Methyl-2-heptanone	928687	987

2-Pentylfuran	3777693	988
Octanal	124130	1005
D-Limonene	5989275	1022
Benzyl alcohol	100516	1037
2-Ethylhexanol	104767	1043
Benzeneacetaldehyde	122781	1051
2-Octenal	2548870	1061
Octanol	111875	1070
3,5-Octadien-2-one	38284274	1072
p-Cresol	106445	1073
Acetophenone	98862	1075
2-Nonanone	821556	1090
2-Nonanol	628999	1099
(2-Ethylcyclopentyl)methanol	36258089	1101
Nonanal	124196	1105
Phenylethyl alcohol	60128	1124
Octanoic acid	124072	1160
(E)-2-Nonenal	18829566	1163
2-Undecanone	112129	1292
Indole	120729	1306
Mesitaldehyde	487683	1362
1-[4-(1-Hydroxy-1-methylethyl)phenyl]ethanone	54549723	1477
δ -Decalactone	705862	1496

These compounds are considered FFAs metabolites, and their presence within a cheese model system without fat sources, such as MS1, was previously reported in other studies (Deetae et al., 2007). Stefanovic *et al.* (2017a) suggested that these compounds could likely be derived from the release of FFAs from cell membranes. In addition, higher percentages of sulphur compounds, esters, and S-thioesters were detected in MS1 compared to MS2, while higher percentages of aldehydes were detected in MS2, probably derived from non-enzymatic autoxidation reaction of fatty acids (Fig.2.4).

It is possible that higher concentrations of substrates derived from FAAs degradation, such as alcohols, carboxylic acids, and acyl-CoA (for esterification, or alcoholysis) were likely present in MS1, supporting the consequent ester biosynthesis (Liu et al., 2004). The formation of S-thioesters follows the same esters pathways (Liu et

al., 2004), but it is limited by the methanethiol availability, originating from the metabolism of sulphur amino acids. Cysteine and methionine are precursors of sulphur compounds, which are particularly important contributors to the flavour of surface-ripened cheese, due to their very low odour perception, and their strong garlic, cabbage, and very ripe cheese odours (Curioni and Bosset, 2002; Sablé and Cottenceau, 1999). In MS1 more sulphur compounds and S-thioesters were detected (Fig. 2.4), and some of them (dimethyl sulphide, and/or methyl thioacetate) were significantly associated ($P<0.05$) with several strains (*S. saprophyticus* DPC5671, *B. linens* B11 and DPC5699, *B. aurantiacum* DPC5683 *C. variable* DPC5313, *M. luteus* DPC6921) (Table 2.3), while in MS2 only *M. gubbeenense* DPC5286 was significantly associated ($P<0.05$) with dimethyl disulphide, suggesting that the abundant FAAs within MS1 have supported the synthesis of higher number of sulphur compounds and S-thioesters. The pathways that drive to the production of sulphur compounds, were specifically described in *B. linens* (Ratray and Fox, 1999); however, in this study, dimethyl sulphide and dimethyl disulphide were also significantly associated ($P<0.05$) with different strains, confirming the study of Bonnarme, Psoni, and Spinnler (2000) who investigated the pathways which lead to catabolism of methionine with the synthesis of sulphur compounds, which showed that other species such as *M. luteus*, *Corynebacterium glutamicum*, and *S. equorum* could be also be potentially responsible.

The volatile profiles of the bacterial strains presented some associations with the enzymatic activities analysed. Specifically, *S. saprophyticus* DPC5671 and *S. equorum* DPC4416 showed the ability to hydrolyse tributyrin, as already observed in other

metabolic studies for various species of staphylococci (Talon and Montel, 1997), and were responsible for the production of FFAs derivatives, such as methyl ketones (in MS1: 6-methyl-5-heptene-2-one, 6-methyl-2-heptanone, 2-nonaone, 2-tridecanone, and 2-pentadecanone) and secondary alcohols (in MS1: 2-ethylhexanol; in MS2: 2-heptanol) (Table 2.3), in agreement with the volatiles detected by Deetae *et al.* (2007) for *Staphylococcus* species in a synthetic model system.

Growth of *Brevibacterium* species on the surface-ripened cheese is often associated with development and synthesis of sulphur volatile compounds (through the catabolism of methionine) (Jollivet *et al.*, 1992; Rattray and Fox, 1999). Using the model systems (MS1 and MS2), dimethyl sulphide and/or methyl thiolacetate were significantly associated ($P < 0.05$) with all three *Brevibacterium* strains, but only in MS1 (Table 2.3). All *Brevibacteria* strains hydrolysed tributyrin and demonstrated esterase activity (on p-nitrophenol butyrate) using CFEs (Fig. 2.1), and correspondingly were significantly associated ($P < 0.05$) with 2-ethylhexanol, 6-methyl-2-heptanone, 2-tridecanone (in MS1), 2-heptanol, 2-nonanol, 2-heptanone, δ -decalactone (in MS2) (Table 2.3), which likely originated from lipid oxidation (Collins *et al.*, 2003; Thierry *et al.*, 2017).

Three species of *Corynebacteria* were tested in the model systems and showed a heterogeneous metabolism. *C. variabile* DPC5313 and *C. casei* DPC5298 were significantly associated ($P < 0.05$) mostly with potential FFAs metabolites, while *C. flavescentis* NCIMB 8707 was the most metabolically active strain in MS1, as observed with the PCA plot (Fig. 2.2), where it separated from all other strains. It was

significantly associated ($P<0.05$) with numerous potential metabolites from aromatic amino acids (in MS1: benzyl alcohol, phenylethyl alcohol; in MS2: phenylethyl alcohol), branched-chain amino acids (in MS1: 3-methyl-1-butanol, 3-methyl butanoic acid, isobutyl acetate, isopentyl acetate), and sulphur amino acids (in MS1: dimethyl sulfide, methyl thiolacetate) (Table 2.3). These data were in agreement with enzymatic assays using CFE, where *C. flavescentis* NCIMB 8707 displayed a significantly greater ($P<0.05$) GDH activity compared to the control (Fig. 2.1), which likely supported the FFAs degradation.

M. gubbeenense DPC5286 presented medium levels for GDH, esterase, and aromatic AT assays (Fig. 2.1), and was correspondingly significantly associated ($P<0.05$) with both potential derivatives from FFAs (in MS1: 2-hexanone, 6-methyl-2-heptanone, 2-tridecanone, 2-ethylhexanol, nonanol; in MS2: 2-heptanone, δ -decalactone, 2-heptanol) and amino acids (in MS1: 3-methyl-1-butanol, isobutyl acetate, isopentyl isovalerate) (Table 2.3). Considering the enzymatic assays, *A. arilaitensis* DPC6297 was the most active strain, presenting positive activity on tributyrin, and significantly greater ($P<0.05$) activity for the GDH and aromatic AT activity (Fig. 2.1), and was significantly associated ($P<0.05$) with metabolites of FFAs (in MS1: 6-methyl-2-heptanone, 2-tridecanone, 2-ethylhexanol; in MS2: 2-heptanone, δ -decalactone) and aromatic amino acids (in MS1: benzyl alcohol), and was the only strain associated with indole (Table 2.3), which is known to be a tryptophan derivative. *M. luteus* DPC6921 did not display hydrolytic activity on tributyrin, but did show significant higher ($P<0.05$) esterase activity (on p-nitrophenol butyrate), compared to the control (Fig.

2.1), and was significantly associated ($P<0.05$) with FFAs metabolites in MS1 (2-tridecanone, 2-pentadecanone, 2-ethylhexanol, octanol, 2-undecanol, 2-hexyl-1-octanol) (Table 2.3).

2.6. Conclusion

Model system 1 was better screening tool for surface ripening bacteria, as there were more compounds detected that one can significantly associate with the genera tested. Gram-positive bacteria, normally associated with surface-ripened cheese, are more active on a substrate including more readily available FFAs and peptides, similarly to what occurs on the cheese surface. Overall, this study suggests a novel approach for the investigation of the microbial flavour development, giving better knowledge regarding the flavour potentialities and enzymatic activities of those surface-ripened bacteria with technological importance for cheese ripening.

2.7. Acknowledgements

Thanks to Ewelina Stefanovic for her assistance in the enzymatic assays.

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Chapter 3

Use of smear bacteria and yeasts to modify flavour and appearance of
Cheddar cheese

This chapter has been published in *International Dairy Journal* (2017), 72, 44-54. DOI: 10.1016/j.idairyj.2017.04.001

In this chapter Dr Deirdre Kennedy performed cryo-SEM analysis on the cheese samples.

3.1. Abstract

Strains of *Staphylococcus saprophyticus* DPC5671 and *Corynebacterium casei* DPC5298 were applied in combination with *Debaryomyces hansenii* DPC6258 to the surface of young Cheddar cheese curd to obtain two different surface-ripened cheeses. A surface microbiota developed over the incubation period, comprising of both yeast and bacteria; pulsed field gel electrophoresis and cryo-SEM confirmed that the inoculated strains of *S. saprophyticus* DPC5671 or *C. casei* DPC5298 were the dominant strains on the surface of the cheese at the end of the ripening period. The smear cultures changed the appearance and aroma, which were significantly different from the control cheese. The approach presented in this study represents a method for the development of new cheese varieties with short ripening time and novel aromas.

3.2. Introduction

Surface-ripened cheese is a traditional dairy product, which has plays an important role in both small and industrial scale dairy production. Surface-ripened cheese is characterized by a short ripening time and strong aroma produced by the growth of smear microbiota on the cheese surface. Surface-ripened cheeses are manufactured by inoculating the surface of the cheese curd, by dipping, spraying or brushing with a mixture of bacteria and yeasts. The traditional method of production is called “old-young smearing” and consists of washing young curds with the brine from old cheese, to encourage the transfer of the microbiota from the old to the young cheeses (Desmaures et al., 2015; Fox et al., 2017a).

The microbiota on the surface of surface-ripened cheese is composed of a variety of microorganisms which coexist in symbiotic relationships. Yeasts are normally the first resident microorganisms to establish themselves on the surface of the cheese due to their tolerance to low pH and salt. Yeasts metabolize lactate, producing H₂O and CO₂ and increase the pH (Cholet et al., 2007; Corsetti et al., 2001). Moreover, they produce metabolites and growth factors (vitamins and amino acids) which encourage the growth of Gram-positive bacteria, such as *Corynebacterium*, *Staphylococcus* and *Brevibacterium* species (Cogan et al., 2014; Larpin et al., 2011).

The growth of smear microorganisms on the surface of cheese curd modifies the appearance, aroma, proteolysis and lipolysis of the cheese within a relatively short ripening time (McSweeney, 2004). The combined growth of the bacteria and yeasts on

the surface of the cheese results in the production of proteolytic and lipolytic enzymes, increasing the amount of free amino acids (FAAs) and free fatty acids (FFAs) (McSweeney and Sousa, 2000; Sousa et al., 2001). Yeasts and Gram-positive bacteria isolated from surface-ripened cheeses have a wide range of proteolytic enzymes that display various peptidase activities, with FAAs increasing within the cheese as a consequence. Additionally, yeasts and Gram-positive bacteria possess esterolytic/lipolytic enzymes capable of catabolizing triacylglycerols in cheese, producing FFAs (Curtin et al., 2002; Fox et al., 2017b).

The further metabolism of FAAs and FFAs during the ripening produces flavour compounds important for cheese aroma. The catabolism of FAAs, especially of branched chain amino acids, aromatic amino acids and sulphur amino acids, produces mainly aldehydes, alcohols, carboxylic acids and sulphur compounds. Moreover, FFAs are involved in reactions leading to the production of flavour compounds such as secondary alcohols, carboxylic acids, esters, lactones and ketones (McSweeney and Sousa, 2000; Singh et al., 2003; Smit et al., 2005; Yvon and Rijnen, 2001).

The characteristics of surface-ripened cheese are not strictly controlled inside artisanal cheese plants. The resulting product is also affected by the final microbial consortia of the cheese, which is influenced by the individual in-house microbiota (contaminant microbial communities) of the cheese-making facilities. Microorganisms detected in the environment of artisanal cheese-making plants have also been found on the surface of surface-ripened cheeses, indicating a strong relationship between product

and the environment in which the cheese is manufactured and ripened (Bokulich and Mills, 2013; Goerges et al., 2008; Mounier et al., 2006).

In previous studies, smear strains were added on the surface during the ripening, or as adjunct cultures to the milk during manufacture of surface-ripened cheese; however, some of the added strains were not detected at the end of ripening (Feurer et al., 2004; Goerges et al., 2008). These commercial smear strains have to compete with the in-house microbiota and do not always successfully establish themselves on the cheese surface (Bokulich and Mills, 2013; Feuerer et al., 2004; Goerges et al., 2008). It is likely that the relationship within the smear microbiota promotes the survival of a particular group of microorganisms to the detriment of others.

With the abolition of the milk quotas within the EU in 2015 there is a renewed interest in developing novel cheeses with a range of flavours. There is a progressive increase in global cheese consumption, with an annual production in Ireland of 207.100 tonnes in 2015 (data from Eurostat). Therefore, the aim of this work was to develop a novel cheese with diverse aromas and short ripening time using cheese curd made in a traditional Cheddar cheese plant. Ripening time for Cheddar cheese can be from a little as 3 months for mild cheese up to > 9 months for mature/extra mature varieties. In this study, the ability of smear bacteria and yeast to grow on the surface of young Cheddar cheese curd was investigated in order to produce a cheese variety with different flavour and appearance compared to Cheddar cheese within a short time frame, of 35 days.

3.3. Materials and methods

3.3.1. Preparation of smear suspensions

For the preparation of *D. hansenii* DPC6258 suspension, the strain was streaked onto yeast extract glucose chloramphenicol agar (YGC agar; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated aerobically at 25°C for 96 h. Using a 5 µL loop, the strain was inoculated into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated, shaking at 100 rpm, at 25°C. When the OD₆₀₀ reached ~1, the cells were centrifuged at $6,000 \times g$ at 4°C for 15 min, washed twice with sterile 0.75% NaCl and the pellet was resuspended in sterile 0.75% NaCl to obtain a suspension of $\sim 10^6$ cfu mL⁻¹.

For the preparation of the *C. casei* DPC5298 and *S. saprophyticus* DPC5671 suspensions, the strains were streaked onto trypticase soy agar (TSA; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated aerobically at 30°C for 48 h. Using a 5 µL loop, the strains were inoculated into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated, shaking at 100 rpm, at 30°C. When the OD₆₀₀ reached ~1, the cells were centrifuged at $6,000 \times g$ at 4°C for 15 min, washed twice with sterile 0.75% NaCl and the pellets resuspended in sterile 0.75% NaCl to obtain a suspensions of $\sim 10^5$ cfu mL⁻¹.

3.3.2. Smearing of cheese blocks

Cheddar cheese was supplied by a commercial cheese company as 20 kg blocks, <24 h post manufacture. The large cheese block was aseptically cut into smaller blocks ($\sim 8 \times 6.5 \times 30$ cm). These blocks were then inoculated by placing them for few seconds (3-5 sec) in a saline suspension containing *D. hansenii* DPC6258 (10^6 cfu mL⁻¹), ensuring an even coating of yeast. Subsequently the blocks of cheese were placed on sterile, plastic coated racks and allowed to drain. Once the excess liquid had completely drained, the cheese pieces were placed inside a sterile plastic bag (Südpack Verpackungen, Ochsenhausen, Germany), onto a sterile rack (approx. 2cm in height) within a frame (approx. 10cm inches in height), ensuring that the surfaces of the cheese did not make contact with the plastic bag. Relative humidity % (RH%) was maintained by pouring 100 mL of sterile 0.75% NaCl into the base of the bag and the bag was sealed. The cheese was ripened at 15°C with a RH% of $\sim 97\%$. After 5 days of ripening, the blocks of cheese were removed from the bag and dipped for few seconds (3-5 sec) in saline suspensions containing *C. casei* DPC5298, or *S. saprophyticus* DPC5671 (10^5 cfu mL⁻¹). The blocks of cheese were placed on the sterile rack and incubated for a further 30 days (for a total ripening period of 35 days), as described above, to produce a surface-ripened cheese with *D. hansenii* DPC6258 in combination with *S. saprophyticus* DPC5671 (cheese A) or *C. casei* DPC5298 (cheese B). During the ripening period the surface of the cheese blocks was washed with a sterile sponge soaked in a sterile brine solution (5% NaCl) at day 7, 10, 15 of ripening to ensure an even growth of the smear microbiota. As a control, blocks of Cheddar cheese were vacuum packed in sterile bags

and incubated at 15°C. These blocks were not smeared with either bacteria or yeasts and were not washed with NaCl solution during the ripening period. However, the control cheese differs from normal Cheddar cheese in that the temperature of ripening was higher (15°C) than the ripening temperature normally associated with Cheddar cheese (~8°C). Three replicate cheese trials were performed.

3.3.3. Sampling cheese

The surface of the cheese A and B was aseptically sampled for enumeration and isolation of bacteria and yeast at 3, 5, 7, 10, 15, 21, 25, 30 and 35 days of ripening.

When analysing the control cheese samples, a composite sample of core and surface was analysed, while for the test cheeses both the surface and core were analysed separately. At day 0 and day 35, samples were taken from the control cheese, cheese A and B for composition, urea-polyacrylamide gel electrophoresis (urea-PAGE), free fatty acids (FFAs) and free amino acids (FAAs) analysis. At day 35, samples were taken from the control cheese, cheese A and B for sensory evaluation and volatile analysis. At days 0, 10, 15, 21, 25, 30, 35 samples were taken from the control cheese, cheese A and B for analysis of proteolysis and colorimetric analysis.

3.3.4. Enumeration of bacteria and yeast from cheese surface

During ripening, a sample of $\sim 5 \text{ cm}^2$ was aseptically removed from the cheese surface and resuspended in 2% trisodium citrate, serially diluted and plated on TSA 5%

NaCl with 50 U mL⁻¹ of nystatin (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) and YGC agar, for the enumeration of smear bacteria and yeasts, respectively. Nystatin was added to TSA to prevent the growth of yeast and moulds. The TSA plates were incubated at 30°C for 48 h, while YGC plates were incubated at 25°C for 96 h. Colonies were counted and the results expressed as log cfu g⁻¹ of cheese. Five colonies from the highest countable dilution were restreaked onto TSA and incubated at 30°C for 48 h. Isolates were stocked at -80°C in glycerol for further analysis.

3.3.5. Pulsed Field Gel Electrophoresis (PFGE)

The cultures isolated from cheese A and B were grown on TSA, incubated aerobically at 30°C for 24 h and then inoculated in 8 mL of TSB, with shaking at 100 rpm at 30°C for 24 h. The PFGE was carried out as described by Bannerman *et al.* (1995) for *S. saprophyticus*, while the method outlined by Brennan *et al.* (2001) was used for *C. casei*. Before digestion the agarose plugs were cut into small slices (1 by 2 mm), transferred into 100 µL restriction buffer (New England Biolabs, Hitchin, UK) containing 20 U of *Sma*I (New England Biolabs, Hitchin, UK) for *S. saprophyticus*, and 20 U of *Spe*I (New England Biolabs, Hitchin, UK) for *C. casei* and incubated over night at 25°C or 37°C, respectively. The gel was run in a CHEF-DR III PFGE apparatus at 1 V (6 V cm⁻¹) at 14°C for 20 h, with the pulse ramped from 5 to 40 s for *S. saprophyticus*, while 1 V (6 V cm⁻¹) at 14°C for 16 h, from 1 to 20 s for *C. casei*. Gels were stained with ethidium bromide (0.5 µg mL⁻¹) in water, destained in water and then

photographed using a gel imaging system (AlphaImager 2000, Alpha Immotech, San Leonardo, CA, USA).

3.3.6. Compositional analysis

Cheese samples were analysed for moisture content by oven-drying 3 g of cheese sample at 102°C for 5 h and for salt content by a potentiometric method on a mixture of 2 g of grated cheese and 60 g of water (International Dairy Federation, 1988). Total protein was determined on 0.2 g of cheese sample and total fat was determined on 3 g of cheese sample by standard methods (International Dairy Federation, 1993, 1996). The pH was measured with a standard pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland) on a mixture of 20 g of grated cheese and 12 g of water, as described by BSI standard (British Standards Institution, 1976).

3.3.7. Cryo-SEM

Cryo-SEM was performed using a modification of the method of Rößle *et al.* (2010). Thin sections of samples (1 mm × 1 mm × 4 mm) were cut perpendicularly at the surface of the cheese and were immediately frozen in liquid nitrogen slush at −207°C. The samples were then etched at −80°C for 15 min, cooled to −125°C, and coated with sputtered platinum (10 mA for 60 s). Samples were transferred under vacuum to the cold stage in a Zeiss Supra 40VP scanning electron microscope (Carl Zeiss AG, Darmstadt,

Germany), where they were maintained at -125°C and imaged using an accelerating voltage of 1 kV.

3.3.8. Determination of colour

The development of the colour during the ripening was measured in triplicate on the surface of the cheese at room temperature, using a Minolta Colorimeter CR-300 (Minolta Camera, Osaka, Japan). A white colour tile standard was used to calibrate the instrument and the colour was analysed using L^* , a^* and b^* -values, which describe the colour space. L^* -value measures the visual lightness (as values increase from 0 to 100), a^* -value measures from the redness to greenness (positive to negative values, respectively) and b^* -value from the yellowness to blueness (positive to negative values, respectively).

3.3.9. Proteolysis

The proteolysis was determined by measuring the levels of the non-casein nitrogen content, soluble at pH 4.6 (pH4.6-SN) (Fenelon and Guinee, 2000) and total nitrogen (TN) on a water soluble extract of a mixture of 60 g of grated cheese and 120 g of water, using the macro-Kjeldahl method (International Dairy Federation, 1993). The levels of proteolysis were expressed as a percentage of the ratio between non-casein nitrogen content and total nitrogen (% pH4.6-SN/TN).

3.3.10. Urea-PAGE

Urea-PAGE was performed according to the method described by Rynne *et al.* (2004). The gel system was composed of a separating and stacking gel, using a PROTEANs II xi cell vertical slab gel unit (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK). The samples were prepared, maintaining the same concentration of protein (4.25 g of protein content L⁻¹ sample buffer) to have a final volume of 1 mL. Ten microliters of sample solution were loaded into individual wells. The sample buffer (pH 8.7), the sample preparation and the running conditions were as described by Henneberry *et al.* (2015). After the run, the gel was removed from the plates and stained overnight in an aqueous solution of Coomassie Blue G250 (0.25 % w/v), destained in a destaining solution (acetic acid 10%, methanol 25%), and washed in distilled water. The images were acquired by a gel imaging system (AlphaImager 2000, Alpha Immotech, San Leonardo, CA, USA).

3.3.11. Free amino acids analysis

Individual FAAs were determined on the soluble N extracts as described by McDermott *et al.* (2016) using a Jeol JLC-500V AA analyser fitted with a Jeol Na⁺ high performance cation exchange column (Jeol Ltd., Garden city, Herts, UK). The chromatographic analyses were conducted at pH 2.2. Results are expressed as µg mg⁻¹ of cheese.

3.3.12. Free fatty acids analysis

FFAs extraction was performed on 10 g of grated cheese, according to the method described by De Jong and Badings (1990). The FFAs extracts were aliquoted into amber glass vials and capped with PTFE/white silicone septa (Agilent Technologies, Little Island, Cork, Ireland). The FFAs extracts were derivitised as methyl esters as outlined by Mannion *et al.* (2016) using a Sample Prep Workbench (Agilent Technologies, Little Island, Cork, Ireland). Fatty acid methyl esters extracts were analysed using Varian CP3800 gas chromatograph (Aquilant, Dublin 22, Ireland) with a CP84000 auto-sampler and flame ionisation detector (GC-FID) and a Varian 1079 injector (Aquilant, Dublin 22, Ireland). For the GC-FID analysis, 0.7 μL were injected into a CP FFAP CB capillary column (30 m $\times$ 0.25 mm $\times$ 0.32 μm) (Agilent Technologies, Little Island, Cork, Ireland). Results are expressed as $\mu\text{g mg}^{-1}$ of cheese

3.3.13. Volatile analysis

After sampling the cheese samples were wrapped in foil, vacuum packed and stored at -20°C . Before analysis the samples were defrosted at room temperature and blended with a cheese grater. For the analysis, 4 g of cheese sample were placed in a screw capped SPME vial with a silicone/PTFE septum (Apex Scientific, Maynooth, Ireland). The SPME vials were equilibrated to 40°C for 10 min with pulsed agitation (5 s on/2 s off) at 500 rpm. Sample introduction was performed using AOC-5000 injection system (Shimadzu, Albert-Hahn-Str., Duisburg, Germany) and a single 50/30 μm Carboxen TM137 / divinylbenzene / polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre was

used for the volatile extraction (Agilent Technologies, Little Island, Cork, Ireland). The SPME fibre was exposed to the headspace above the samples for 20 min at depth of 54 mm, then was retracted and injected into the GC inlet and desorbed for 2 min at 250°C. Injections were made on a Shimadzu 2010plus with an Agilent DB-5ms column (60 m $\times$ 0.25 mm $\times$ 0.25 μ m) (Agilent Technologies, Little Island, Cork, Ireland), using a multipurpose injector with a Merlin microseal. The temperature of the column oven was initially held for 30 s at 35°C, increased to 230°C at a rate of 6.5°C min⁻¹, and to 320°C at a rate of 15°C min⁻¹, yielding at total GC run time of 41.5 min. The carrier gas, helium, was at a constant pressure of 1.58 bar, performing a splitless injection. The detector, a Shimadzu TQ8030 MSD triple quadrupole mass spectrometer, was used in single quadrupole mode. The ion source temperature and interface temperature were set at 220°C and 280°C, respectively, and the MS mode was electronic ionisation (70 v) with the mass range between 35 and 250 amu. The chromatograms were deconvoluted and the peaks quantified with TargetView (Markes International Ltd, Llantrisant, UK). The compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, and using an internal database with known target and qualifier ions for each compound. Ten microliters of the standard solution [1-butanol, dimethyldisulphide, butyl acetate, cyclohexane, benzaldehyde at 10 ppm, and 2-phenylethanol at 50 ppm, in methanol/:water (1:99)] in an empty SPME vial were run before and after every series of samples to ensure that both the SPME extraction and MS detection were performing within specification. An autotune of the GCMS was carried out prior to the analysis to ensure optimal GCMS performance. All analyses were performed in triplicate.

3.3.14. Sensory affective evaluation and ranking descriptive analysis

The blocks of cheese sample (~500 g), vacuum packed and stored at -20°C, were defrosted at 4°C the day before sensory evaluation. The surface of cheese A and B was removed and the blocks were cut into portions for the sensory test. Twenty naive assessors were recruited in University College Cork, Ireland. Sensory acceptance testing was conducted using these untrained assessors, both Cheddar and surface-ripened cheese consumers, (age 21 - 48 years). Assessors used the sensory hedonic descriptors for the control, cheese A and B. Samples underwent monadic presentation to the panel at ambient temperatures (~21°C) and coded with a randomly selected 3 digit code. Each assessor was asked to indicate their degree of liking on a 10-cm line scale ranging from 0 (extremely dislike) to 10 (extremely like). Ranking descriptive analysis (RDA) was then undertaken using the consensus list of sensory descriptors, which was also measured on a 10 cm line scale. All samples were presented in duplicate.

3.3.15. Aminopeptidase activity

After the growth of *D. hansenii* DPC6258, *S. saprophyticus* DPC5671, and *C. casei* DPC5298 in 10 mL of TSB (as previously described in the paragraph of this chapter “Preparation of smear suspensions”, p 131), the cells were manipulated to obtain cell free extract (CFE), as described previously in the chapter 2 (“Preparation of cell free extract”, p 94). The negative control for the assays was CFE heated at 98°C for 15 min. The concentration of the protein content of the CFE was determined using Qubit®

Protein Assay Kit (Cambio Ltd, Cambridge, UK), following the procedure of the supplier.

To measure the aminopeptidase activity on 96 well plate. Chromogenic substrates, namely L-Lys-paranitroanilide (Sigma Aldrich) (for PepN), and H-Gly-Pro-pNA (Bachem, Bubendorf, Switzerland) (for PepX) were used, modifying the method outlined by Jensen and Ardo (2010). These substrates were separately diluted in 2 solutions of 50 mmol L⁻¹ sodium-phosphate buffer pH 7.5 at concentration of 1 mmol L⁻¹. Subsequently 50 µl of PepX, or PepN reaction mixture, were added to 50 µl of CFE and the plate was incubated for 30 min, at 30°C in plate reader (Synergy HT, Bio-Tek Multi Detection Plate Reader). Absorbance was measured at 405 nm (Synergy HT, Bio-Tek Multi Detection Plate Reader), Aminopeptidase activity was expressed as nmol of p-nitroaniline (resultant product of the reactions) released per min for mg of protein, calculated with the use of a standard curve previously obtained for standard samples of p-nitroaniline ranging between 0 to 50 nmol. PepX and PepN analysis were performed in triplicate.

3.3.16. Tributyrin agar assay

The assay was prepared using tributyrin agar (Sigma Aldrich) as previously described in chapter 2 (“Tributyrin agar assay”, p 93-94).

3.3.17. Statistical analysis

The statistical analysis for cheese composition, sensorial analysis, FAAs and FFAs were tested with one-way analysis of variance (ANOVA) using Minitab (Minitab 17, Minitab Inc, Coventry, UK). A split plot designed with SAS (SAS 9.3; Dublin 2; Ireland) was used to determine the individual effect of smear treatments, ripening time and their interaction on the microbiology, pH, % pH 4.6-SN/TN, L*, a* and b* -values, measured at several time points during the ripening. The statistical analysis of the volatile compounds was tested with principal component analysis (PCA) and ANOVA-Partial Least Squares Regression (APLSR) using Unscrambler (The Unscrambler X 10.3, Camo Software, Oslo, Norway). From the results of the APLSR, the individual volatile compounds positively correlated with the samples were tested with one-way analysis of variance (ANOVA) using Minitab 17 (Minitab 17, Minitab Inc, Coventry, UK), to evaluate the significant differences among the samples for each volatile compound. The level of significance for all analyses was determined at P value < 0.05.

3.4. Results

3.4.1. Growth of the strains and pH development

PFGE analysis established that the inoculated cultures of *S. saprophyticus* DPC5671 and *C. casei* DPC5298 were the dominant strains isolated at the end of the ripening as all the colonies taken from the highest dilution had similar PFGE restriction patterns to the inoculated strains (Fig. 3.1).

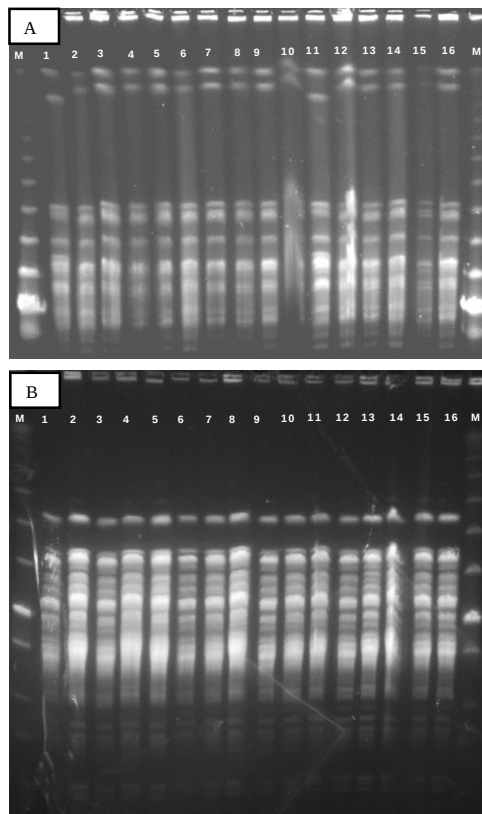


Figure 3.1. PFGE performed on the strains isolated at day 35 of ripening. Gel A: markers (lanes M), strains isolated strains from cheese A (lane 1-15), *S. saprophyticus* DPC5671 (lane 16). Gel B: markers (lanes M), strains isolated strains from cheese B (lane 1-15), *C. casei* DPC5298 (lane 16).

This was confirmed by scanning electron micrographs (Fig. 3.2) which shows the microbiota established on the surface of cheese A and cheese B at the end of the ripening. The morphologies and dimensions of the microorganisms detected were compatible with *D. hansenii* DPC6258, *S. saprophyticus* DPC5671 and *C. casei* DPC5298.

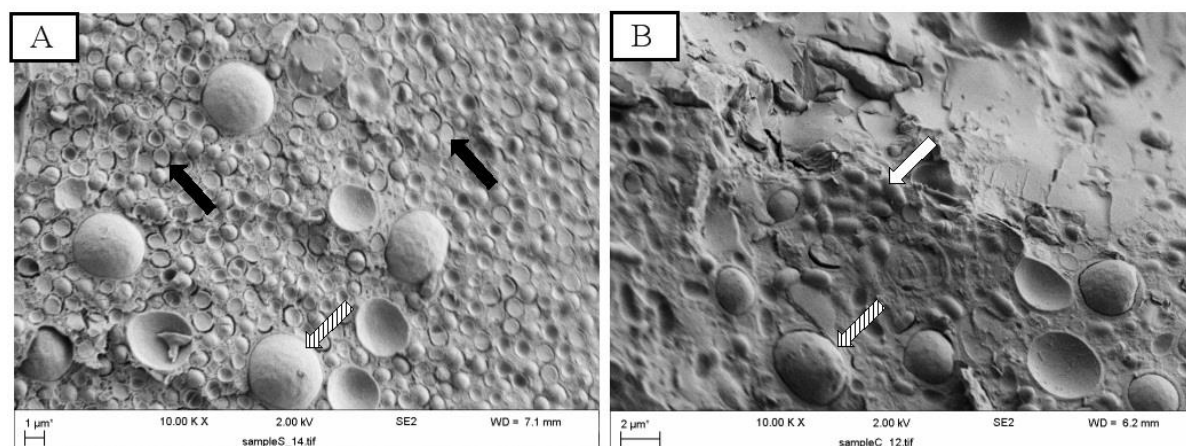


Figure 3.2. Cryogenic scanning electron micrographs, acquired with a Zeiss Supra 40VP scanning electron microscope (Carl Zeiss AG, Darmstadt, Germany), of the surface of cheese A and cheese B at the end of the ripening time. Scanning electron micrographs showed *D. hansenii* DPC6258 (striped arrows), *S. saprophyticus* DPC5671 (black arrows) and *C. casei* DPC5298 (white arrow) established on the surface of the test cheeses.

The total count of yeasts and smear bacteria during ripening is shown in the Fig. 3.3. A significant interactive effect ($P < 0.05$) between ripening time and smear treatments was observed for the growth of the surface microbiota. No significant differences were observed on the growth of yeast and bacteria between cheese A and cheese B.

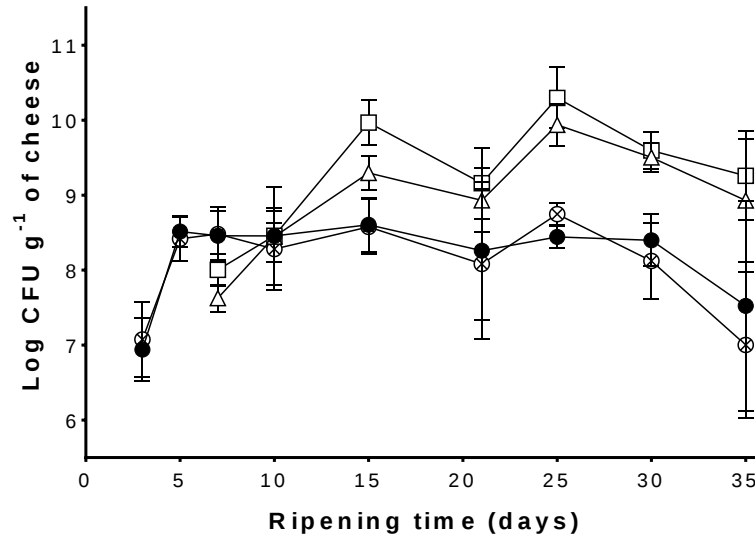


Figure 3.3. Enumeration in CFU g⁻¹ of the cheese surface of cheese A and cheese B during the ripening time; total yeasts count in cheese A (●) and in cheese B (⊗), total smear bacteria count in cheese A (△) and in cheese B (□). Values presented are the means and standard deviations from three replicate trials.

The variation in pH of the control, and the core and the surface of cheese A and B is shown in Fig. 3.4. A significant interactive effect ($P < 0.05$) between smear treatments and ripening time was observed for pH. From day 21 to 35, the pH was significantly higher ($P < 0.05$) at the surface of cheese A compared to the respective core and control. From day 15 to 35, the pH was significantly higher ($P < 0.05$) at the surface of cheese B compared to the respective core and control.

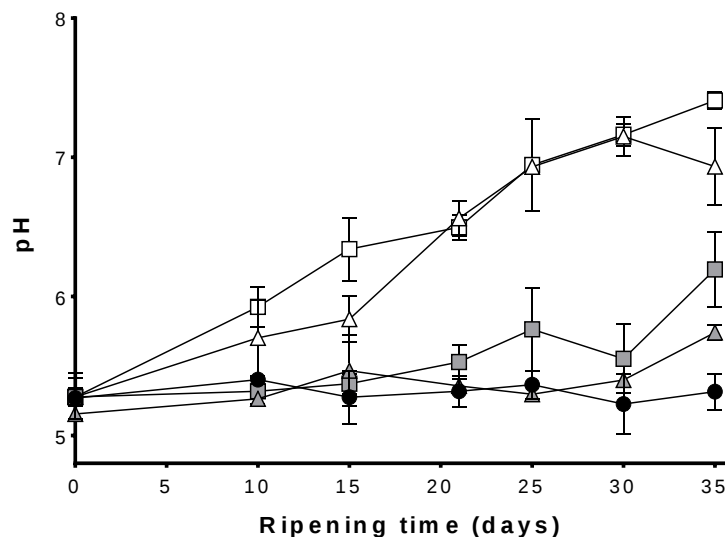


Figure 3.4. Change in pH over time; control cheese (●), surface of cheese A (△), core of cheese A (▲), surface of cheese B (□) and core of cheese B (■). Values presented are the means and standard deviations from three replicate trials.

3.4.2. Cheese composition

The mean composition of the Cheddar cheese before the smearing process was typical of a commercial Cheddar cheese after manufacture, but the smearing treatments influenced the compositional parameters at the end of the ripening (35 days). Compositional data and significant differences ($P < 0.05$) are presented in Table 3.1.

Table 3.1. Composition of the control at day 0 and 35 and composition of the surface and core of cheese A and cheese B at day 35.

Composition	Control d0	Control d35	Surf A d35	Core A d35	Surf B d35	Core B d35
Moisture (% w/w)	38.80±0.84 ^{bc}	38.32±0.62 ^c	39.71±1.20 ^{bc}	40.49±0.86 ^{ab}	39.61±1.43 ^{ab}	41.27±0.39 ^a
Fat (% w/w)	30.45±0.33 ^a	30.41±0.27 ^a	28.30±0.14 ^b	30.09±0.69 ^a	28.01±0.25 ^b	29.80±0.30 ^a
Protein (% w/w)	24.87±0.21 ^a	25.17±0.11 ^a	24.28±0.57 ^a	24.5±0.83 ^a	24.94±0.32 ^a	24.72±0.44 ^a
MNFS (% w/w)	55.79±1.44 ^{bc}	55.07±1.02 ^c	55.39±1.77 ^{bc}	57.89±1.33 ^{ab}	55.02±2.13 ^b	58.79±0.39 ^a
FDM (% w/w)	49.77±1.16 ^a	49.32±0.80 ^a	46.97±1.13 ^b	50.51±1.33 ^a	46.41±1.44 ^b	50.74±0.24 ^a
S/M (% w/w)	4.02±0.10 ^{ab}	4.17±0.26 ^a	3.74±0.20 ^{ab}	3.81±0.33 ^{ab}	3.58±0.10 ^b	3.88±0.51 ^{ab}
Salt (% w/w)	1.56±0.02 ^a	1.60±0.09 ^a	1.48±0.11 ^a	1.55±0.12 ^a	1.42±0.08 ^a	1.60±0.22 ^a

The compositional values for moisture, fat, protein, MNFS (moisture in non fat substances), FDM (fat in dry matter), S/M (salt in moisture) and salt of control at day 0 (Control d0), for control at day 35 (Control d35), for the surface of cheese A at day 35 (Surf A d35), for the core of cheese A at day 35 (Core A d35), for the surface of cheese B at day 35 (Surf B d35) and for the core of cheese B at day 35 (Core B d35). Values presented are the means±standard deviations of three replicate trials. The significant differences ($P<0.05$) are indicated with ^{a, b, c}.

3.4.3. Proteolysis

The proteolysis was particularly high at the surface of cheese A and B, where the smear treatments significantly ($P<0.05$) influenced the level of % pH 4.6-SN/TN (Fig. 3.5).

Urea-PAGE (Fig. 3.6) confirmed the high level of proteolysis on the surface of cheese A and B. α_{s1} -Casein was partially degraded to α_{s1} -CN (f102-199) and α_{s1} -CN (f24-199) in the control cheese and in the core of cheese A and B. However, there was evidence of further breakdown products on the surface samples of the test cheeses. Similar β -casein degradation patterns were observed for the control and core of test cheeses, while samples from the surface of the test cheeses showed that the β -casein was almost totally degraded producing β -CN (106-209) and β -CN (108-209) with higher intensity (Fig 3.6).

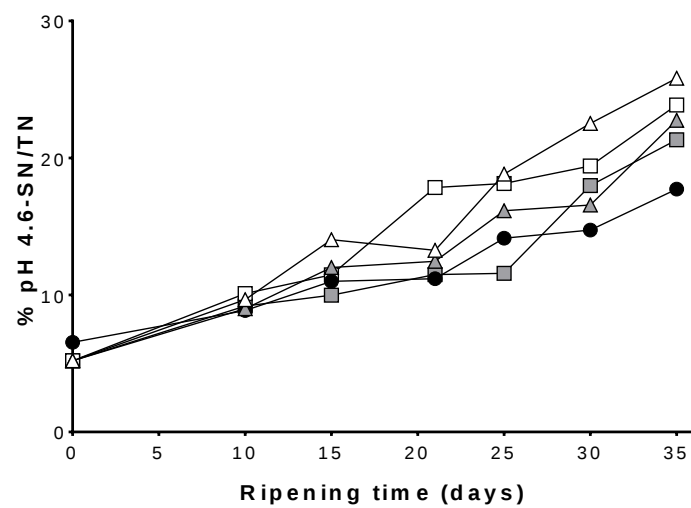


Figure 3.5. Proteolysis, expressed as % pH4.6-SN/TN during ripening: control cheese (●), surface of cheese A (△), core of cheese A (▲), surface of cheese B (□) and core of cheese B (■). Values presented are the means from three replicate trials.

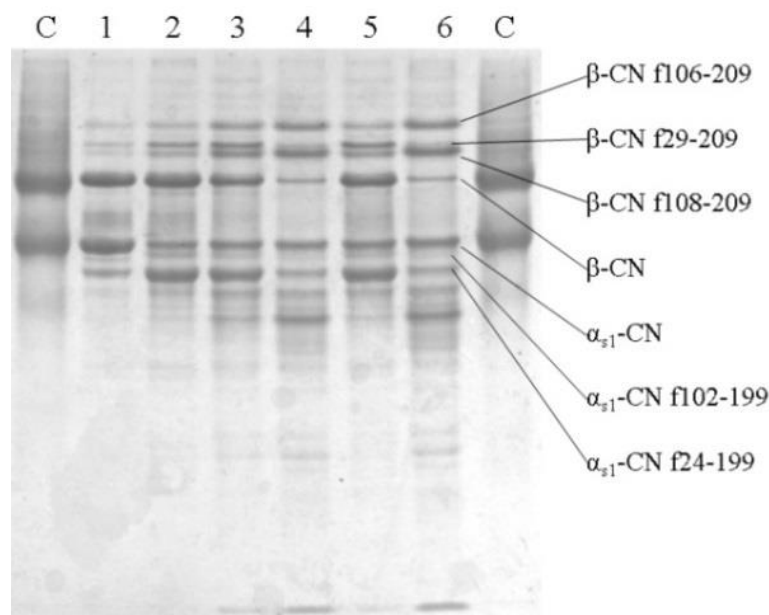


Figure 3.6. Urea-PAGE electrophoretogram of sodium caseinate (*lane C*), control cheese at day 0 (*lane 1*), control cheese at day 35 (*lane 2*), core of cheese B at day 35 (*lane 3*), surface of cheese B at day 35 (*lane 4*), core of cheese A at day 35 (*lane 5*) and surface of cheese A at day 35 (*lane 6*). The urea-PAGE was performed on the basis of fixed weight protein for all three replicate trials.

3.4.4. Free amino acids and free fatty acids

Significant differences ($P < 0.05$) on the total amount of FAAs were observed between the control and the surface of cheese A and B at the end of ripening on day 35 (Fig. 3.7). In the cheese A and B, some individual FAAs were significantly higher ($P < 0.05$) on the surface compared to their respective cores or the control. No significant difference was determined between the surface samples of cheese A and B with respect to the total FAAs content. Significant differences ($P < 0.05$) in the total amount of FFAs were observed between the control and the surface of cheese A and B at day 35 (Fig.

3.8). In the cheese A and B, all individual FFAs detected on the surface were significantly higher ($P<0.05$) compared to the levels of the respective cores or the control. Significantly higher levels ($P<0.05$) of total FFAs were detected on the surface of cheese A ($20169\pm2120 \mu\text{g mg}^{-1}$) compared to the surface of cheese B ($12338\pm3382 \mu\text{g mg}^{-1}$).

3.4.5. Cheese colour

The measurements of the colour on the surface of cheese A and B during ripening are shown in Fig. 3.9. A significant interactive effect ($P<0.05$) between time and smear treatments was observed on a^* and b^* values. From day 15, a^* value was significantly higher ($P<0.05$) compared to the control for the cheese A and B. At the end of the ripening (day 35), a^* value was significantly higher ($P<0.05$) for cheese B compared to cheese A, indicating a redder colour on the surface of cheese B.

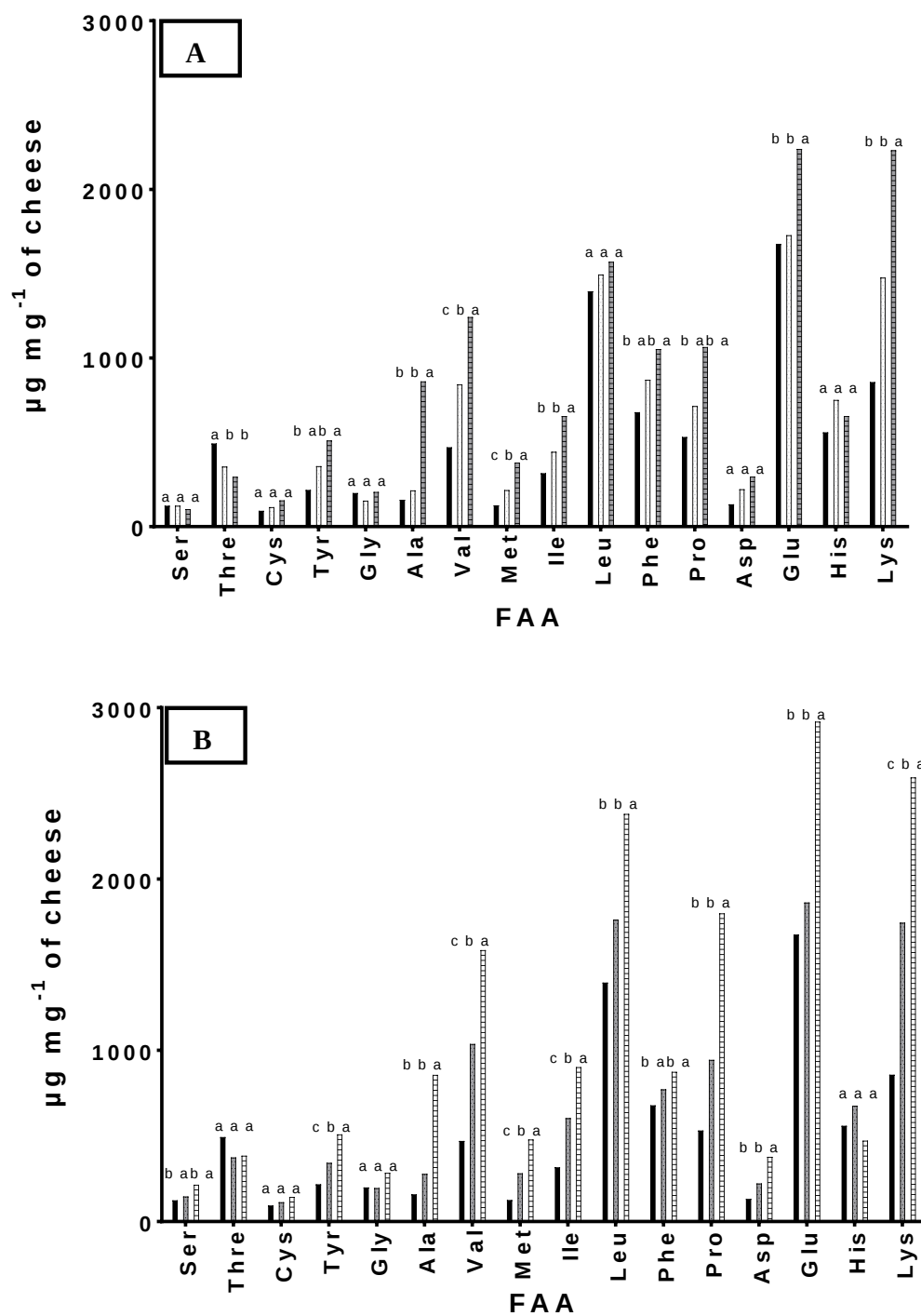


Figure 3.7. Individual free amino acids content of the control (■), core cheese A (□), surface cheese A (▨), core cheese B (▤) and surface cheese B (▧) at day 35. The values presented are the means of the three replicate trials. The significant differences ($P < 0.05$) are indicated with a, b, c.

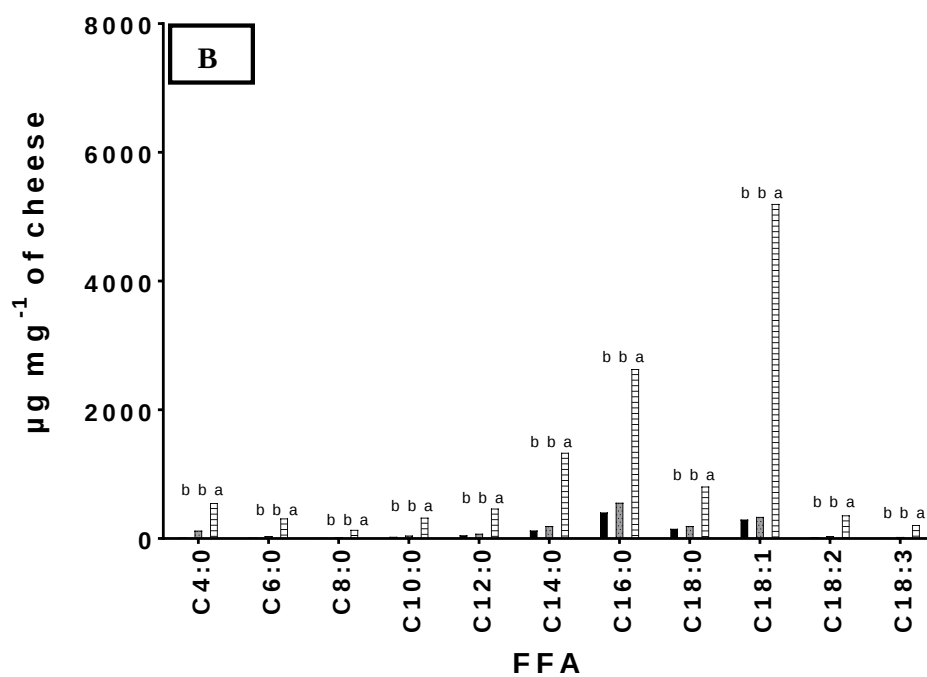
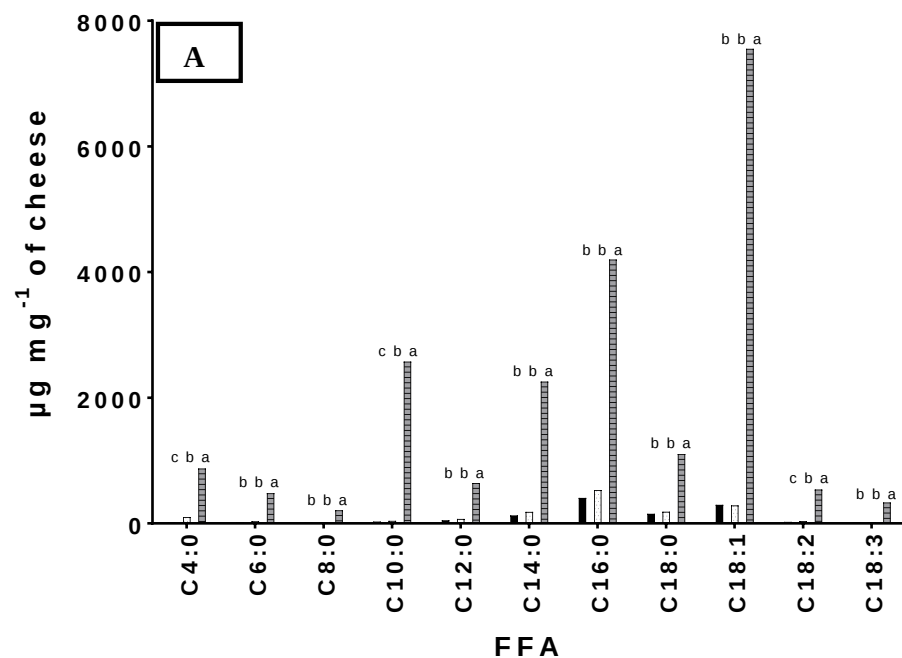


Figure 3.8. Individual free fatty acids content of the control (■), core cheese A (▣) and surface cheese A (▤), core cheese B (▣) and surface cheese B (▤) at day 35. The values presented are the means of the three replicate trials. The significant differences ($P < 0.05$) are indicated with a, b, c.

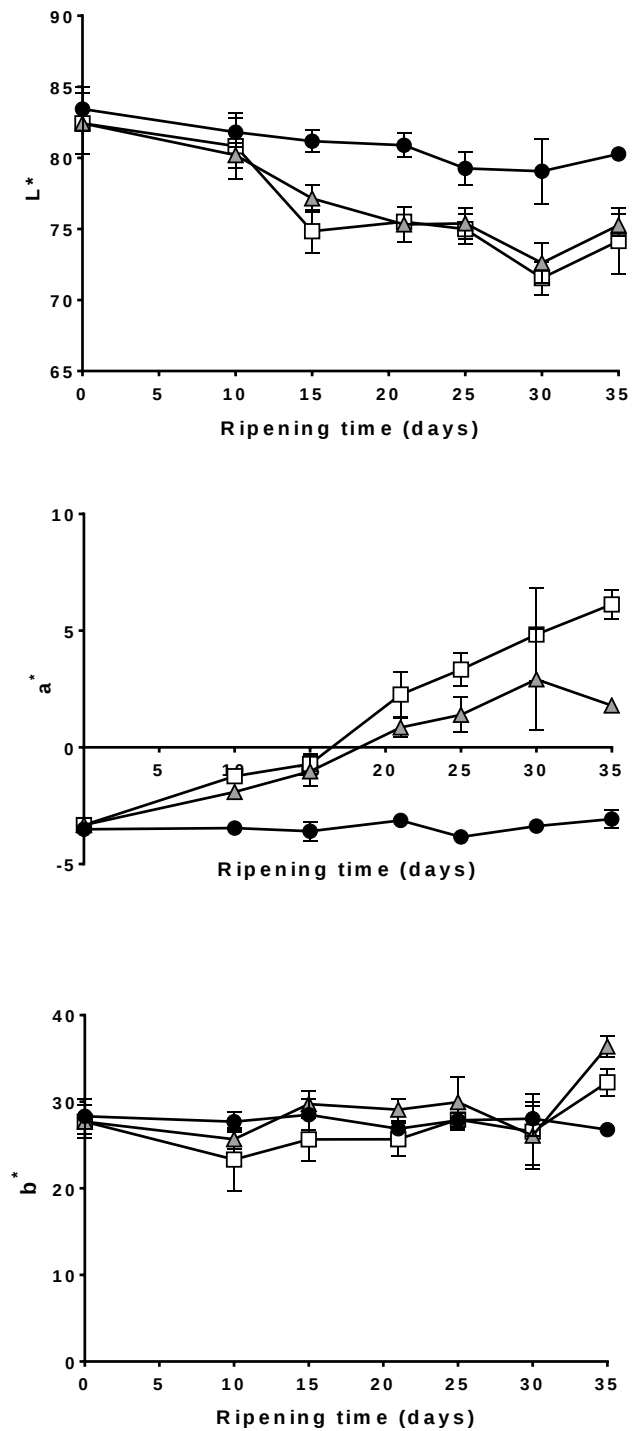


Figure 3.9. Colour development on the surface of the cheeses during the ripening. The colour values (L^* , a^* , b^*) of the control cheese (●), of cheese A (▲) and of cheese B (□). Values presented are the means and standard deviations from three replicate trials.

3.4.6. Volatile compounds and sensory analysis

The analysis of variance enabled the selection of 40 volatile compounds which were significantly different ($P < 0.05$) and positively correlated with the samples (Table 3.2). In total, 22 volatile compounds (7 acids, 4 alcohols, 5 esters, 4 sulphur compounds, 1 ketone and 1 aromatic hydrocarbon) were significantly associated ($P < 0.05$) with the surface of cheese A. Eight compounds (2 acids, 2 aldehydes, 1 alcohol 1 ester, 1 pyrazine and 1 sulphur compound) were significantly associated with the surface of cheese B. Two compounds (1 ketone and 1 hydrocarbon) were significantly associated ($P < 0.05$) with the core of cheese A. Two compounds (1 alcohol and 1 ketone) were significantly associated ($P < 0.05$) with the core of cheese B. Nine compounds (4 alcohols, 4 ketones and 1 ester) were significantly associated ($P < 0.05$) with the control cheese.

Table 3.2. Volatile compounds detected with SPME-GCMS in cheese A, cheese B and control, and relative aroma notes.

Volatile compound	CAS number	Aroma note
ALDEHYDES		
3-Methyl-butanal ^b	590-86-3	Malty, powerful, cheese, green, dark chocolate (Kilcawley, 2017)
2-Methyl butanal ^b	96-17-3	Malty, dark chocolate, almond, cocoa (Qian et al., 2006; Singh et al., 2003; Urbach, 1993)
ALCOHOLS		
Ethanol ^e	64-17-5	Dry, dust, alcohol (Kilcawley, 2017)
2-Butanol ^e	78-92-2	Sweet, fruity, fusel oil, wine-like (Kilcawley, 2017)
3-Methyl-1-butanol ^a	123-51-3	Fresh cheese, breath-taking, alcoholic, fruity, grainy (Kilcawley, 2017)
2-Methyl-1-butanol ^a	137-32-6	Malty, wine, onion (Kilcawley, 2017)
2,3-Butanediol ^e	513-85-9	Fruity (Singh et al., 2003)
2-Heptanol ^{a, b}	543-49-7	Fruity, earthy, green, sweetish, dry (Kilcawley, 2017)
2-Ethyl-1-hexanol ^{d, e}	104-76-7	Animal, cardboard (Thomsen et al., 2012)
Phenylethyl-alcohol ^a	60-12-8	Unclean, rose, violet-like, honey, floral (Kilcawley, 2017)
KETONES		
2,3-Butanedione ^c	431-03-8	Buttery, strong (Kubičková and Grosch, 1997; Singh et al., 2003)
2-Pentanone ^e	107-87-9	Orange peel, sweet, fruity (Kilcawley, 2017)
3-Methyl-2-pentanone ^a	565-61-7	Minty-camphoraceous, sharp (Barron et al., 2005)
2-Hexanone ^e	591-78-6	Floral, fruity (Qian et al., 2006)
8-Nonen-2-one ^d	5009-32-5	Animal, stinky (Poveda et al., 2008; Varming et al., 2013)
2-Nonanone ^e	821-55-6	Malty, rotten fruit, hot milk, green, earthy (Kilcawley, 2017)
2-Decanone ^e	693-54-9	Fruity, musty (Qian et al., 2006; Varming et al., 2013)
ACIDS		
Acetic acid ^a	64-19-7	Vinegar, peppers, green, fruity, floral (Kilcawley, 2017)
2-Methyl-propanoic acid ^a	79-31-2	Rancid butter, sweaty, sweet, apple-like (Curioni and Bosset, 2002)
Butanoic acid ^a	107-92-6	Sweaty, butter, cheese, strong, acid (Kilcawley, 2017)
3-Methyl- butanoic acid ^b	503-74-2	Cheesy, sweaty, socks, rancid, rotten fruit (Kilcawley, 2017)
2-Methyl-butanoic acid ^a	116-53-0	Fruity, waxy, sweaty (Singh et al., 2003)
Pentanoic acid ^a	109-52-4	Rain, wood, vegetable, spicy, nutty, grain, swiss cheese, stable, sweaty, sheep (Curioni and Bosset, 2002)
Heptanoic acid ^a	111-14-8	Soapy, fatty, goaty, rancid (Curioni and Bosset, 2002)
Octanoic acid ^b	124-07-2	Cheesy, rancid, pungent, sweat (Kilcawley, 2017)
n-Decanoic acid ^a	334-48-5	Stale, butter, sour, fruity, pungent (Kilcawley, 2017)
ESTERS		
Ethyl acetate ^a	141-78-6	Solvent, pineapple, fruity (Kilcawley, 2017)

Ethyl propionate ^e	105-37-3	Pineapple, solvent, fruity (Barron et al., 2005; Qian et al., 2006)
3-methylbutyl acetate ^a	123-92-2	Fruity, banana, candy, sweet (Barron et al., 2005; Curioni and Bosset, 2002; Qian et al., 2006)
Methyl hexanoate ^a	106-70-7	Pineapple, fruity (Qian et al., 2006; Varming et al., 2013)
Ethyl hexanoate ^b	123-66-0	Pineapple, sweet, fruity, banana (Kilcawley, 2017)
Ethyl octanoate ^a	106-32-1	Pear, apricot, sweet, fruity, banana, pineapple (Kilcawley, 2017)
Isopentyl hexanoate ^a	2198-61-0	Sweet, fruity (Gürbüz et al., 2006)
SULPHUR COMPOUNDS		
Methionol ^a	505-10-2	Orange (Carpino et al., 2004)
Methanethiol ^{a, b}	74-93-1	Rotten cabbage, cheese, vegetative, sulphur (Kilcawley, 2017)
Dimethyldisulphide ^a	624-92-0	Green, sour, onion (Kilcawley, 2017)
Dimethyltrisulphide ^a	3658-80-8	Vegetable-like, sulphurous, garlic, putrid, cabbage-like (Kilcawley, 2017)
AROMATIC HYDROCARBONS		
Benzaldehyde ^c	100-52-7	Bitter almond, sweet cherry (Singh et al., 2003; Smit et al., 2005)
Benzeneacetaldehyde ^a	122-78-1	Honey-like, rose, violet-like, hyacinth, green (Kubíčková and Grosch, 1997; Qian et al., 2006; Singh et al., 2003; Smit et al., 2005; Varming et al., 2013)
PYRAZINES		
3-Ethyl-2,5-dimethyl-pyrazine ^b	13360-65-1	Roasted, baked (Qian and Reineccius, 2002)

^a volatile compounds significantly different ($P<0.05$) and positively correlated to the surface of cheese A.

^b volatile compounds significantly different ($P<0.05$) and positively correlated to the surface of cheese B.

^c volatile compounds significantly different ($P<0.05$) and positively correlated to the core of cheese A.

^d volatile compounds significantly different ($P<0.05$) and positively correlated to the core of cheese B.

^e volatile compounds significantly different ($P<0.05$) and positively correlated to the control cheese.

Using hedonic sensory analysis (Fig. 3.10) the control cheese scored significantly higher ($P<0.05$) for “Liking of Aroma” compared to the cheese A and B. Cheese A scored significantly higher ($P<0.05$) for “Liking of Aroma” compared to cheese B. The control and cheese A scored significantly higher ($P<0.05$) for “Liking of Flavour” compared to cheese B. As seen in Figure 3.11, the control cheese as expected scored significantly higher ($P<0.05$) for “Cheddar flavour” compared to cheese A, which scored significantly higher ($P<0.05$) for “Cheddar flavour” compared to cheese B. Cheese A and B scored significantly higher ($P<0.05$) for “Pungent flavour” and “Mould

cheese flavour” compared to the control cheese. Cheese B scored significantly higher ($P<0.05$) for descriptors “Sweaty/Sour Aroma”, “Pungent Aroma” and “Off-Aroma” compared to the control cheese. The control scored significantly higher ($P<0.05$) for “Crumbly texture” compared to cheese A and B.

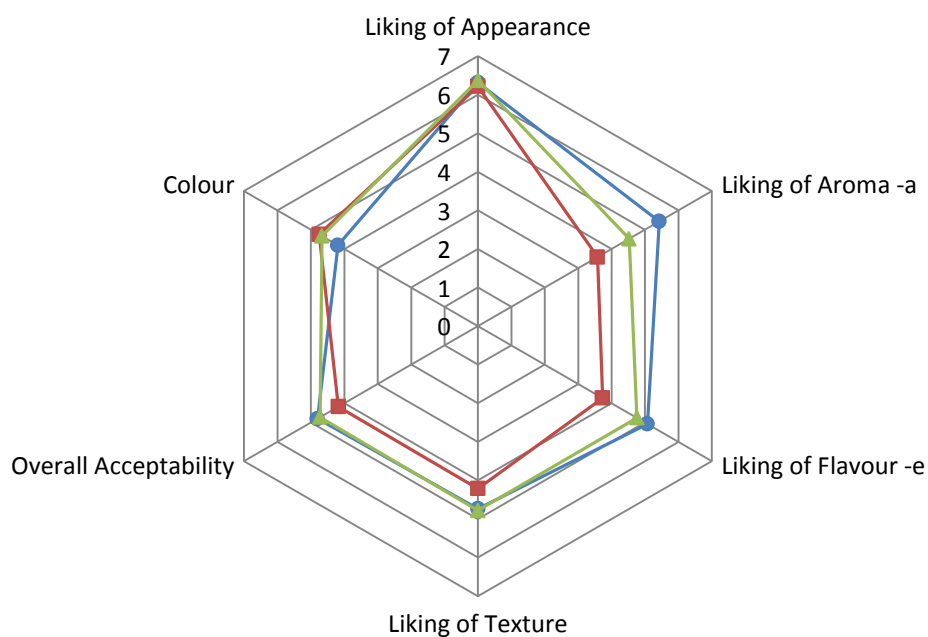


Figure 3.10. Sensory affective (hedonic) analysis performed in duplicate on cheese A (▲), cheese B (■) and control (●) at 35 days of ripening.

-a: control significantly higher ($P<0.05$) compared to cheese A and B.

-e: cheese A and control significantly higher ($P<0.05$) compared to cheese B.

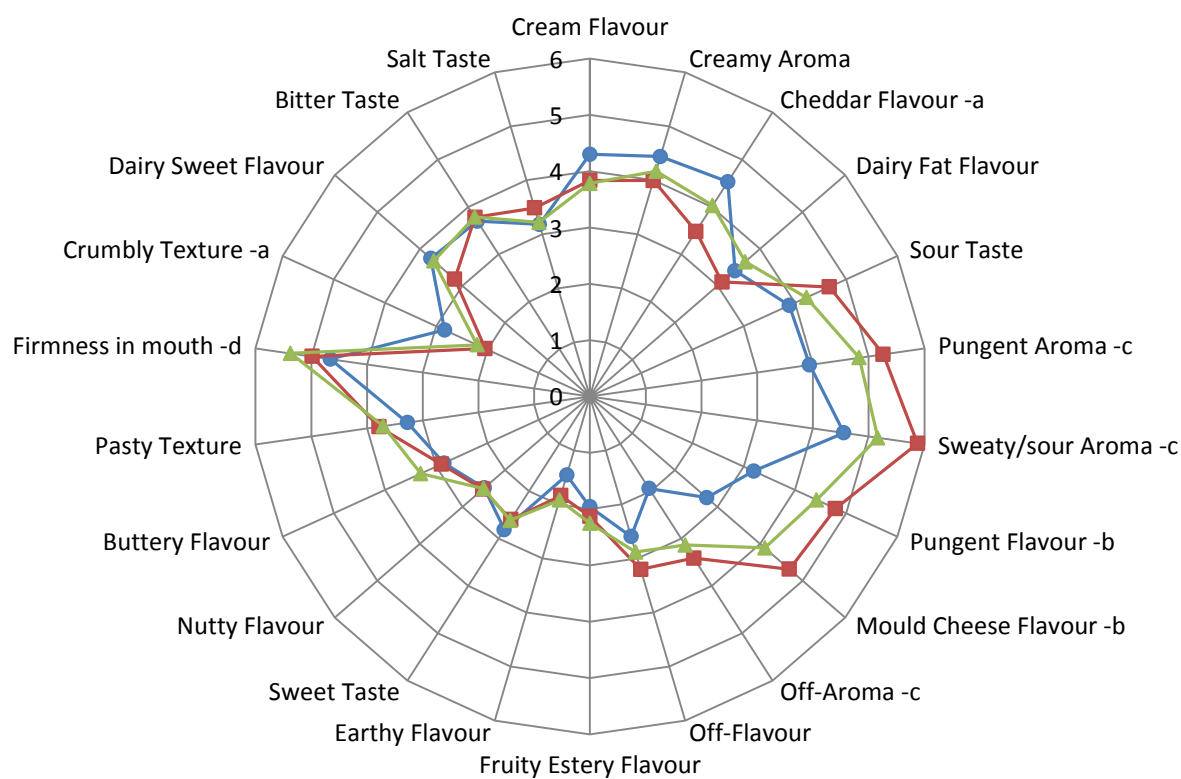


Figure 3.11. Ranking descriptive analysis performed in duplicate on cheese A (Δ), cheese B ($\blacksquare$) and control ($\bullet$) at 35 days of ripening.

-a: control significantly higher ($P < 0.05$) compared to cheese A and B.

-b: cheese A and B significantly higher ($P < 0.05$) compared to control.

-c: cheese B significantly higher ($P < 0.05$) compared to cheese A and control.

-d: cheese A significantly higher ($P < 0.05$) compared to cheese B and control.

3.4.7. Enzymatic activities

The aminopeptidase analysis performed on the pure cultures showed high levels of activity for *D. hansenii* DPC6258 (Fig. 3.12). PepX was significantly higher ($P<0.05$) for *D. hansenii* DPC6258, compared to *S. saprophyticus* DPC5671, which presented significant higher activity ($P<0.05$), compared to *C. casei* DPC5298. PepN was significantly higher ($P<0.05$) for *D. hansenii* DPC6258, compared to *C. casei* DPC5298, which presented significant higher activity ($P<0.05$), compared to *S. saprophyticus* DPC5671. On the tributyrin agar, *S. saprophyticus* DPC5671 was the only strain with hydrolytic activity (Fig. 3.13).

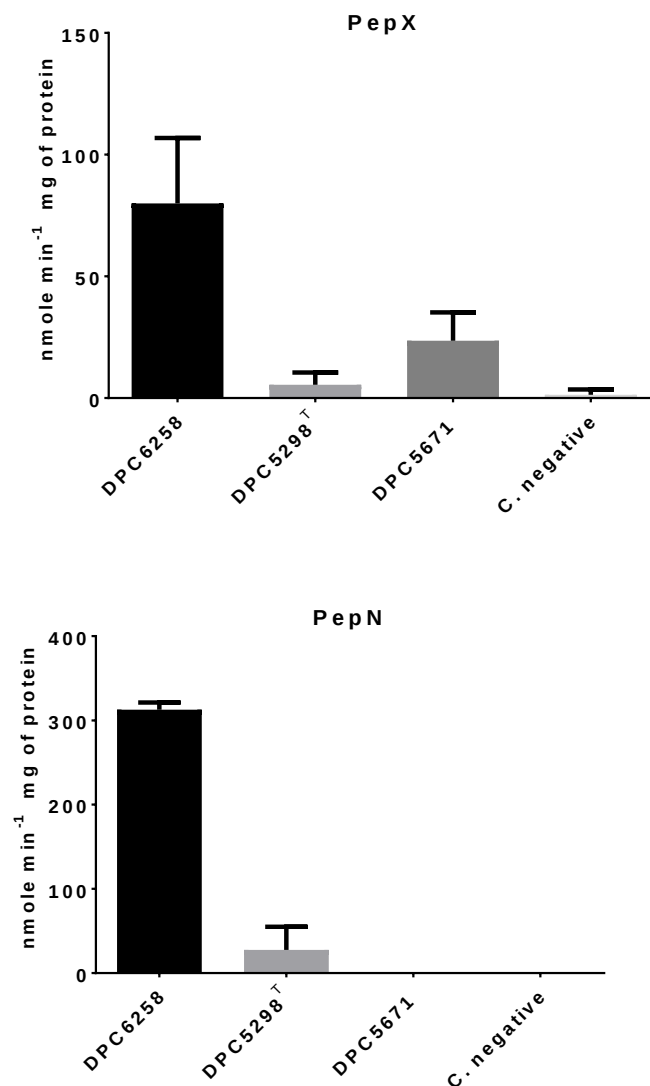


Figure 3.12. Aminopeptidase activities (PepX and PepN) of cell free extract of *S. saprophyticus* DPC5671 (DPC5671), *C. casei* DPC5298 (DPC5298), *D. hansenii* DPC6258 (DPC6258) and control negative (cell free extract heated at 98°C for 10 min to denature the enzymes). PepX and PepN are expressed as the nmole min⁻¹ of product per mg of protein in cell free extract. Values presented are the means from three replicate trials.

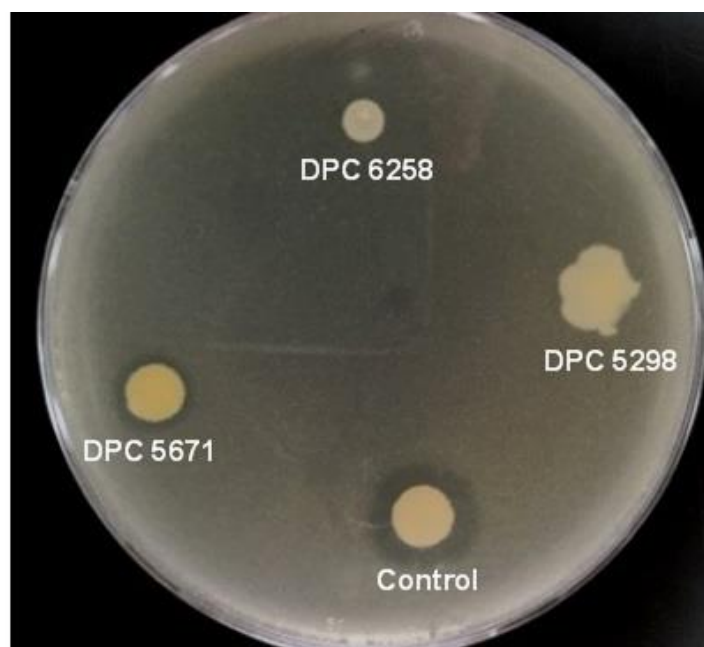


Figure 3.13. Tributyrin agar test on *S. saprophyticus* DPC5671 (DPC5671), *C. casei* DPC5298 (DPC5298), *D. hansenii* DPC6258 (DPC6258) and *Yarrowia lipolytica* (Control). The test was performed in triplicate.

3.5. Discussion

C. casei and *S. saprophyticus*, bacteria commonly isolated from surface-ripened cheeses (e.g. Limburger, Reblochon, Livarot, Tilsit, Gubbeen) (Cogan et al., 2014; Larpin et al., 2011), do not belong to the traditional microbiota of Cheddar cheese, although in this study both strains established themselves on the surface of young Cheddar cheese curd and they were the dominant population on the cheese surface throughout the ripening. *C. casei* DPC5298 or *S. saprophyticus* DPC5671 in combination with *D. hansenii* DPC6258 developed a coloured layer on the cheese surface after 15 days of ripening.

Unlike studies with *Brevibacterium linens* which showed that colour development was influenced by the yeast strain used (Leclercq-Perlat et al., 2004b), in this study the type of colour developed was dependant on the bacteria used. The combination of *D. hansenii* DPC6258 with *C. casei* DPC5298 on cheese B developed a different colour, which was redder compared to the combination of *D. hansenii* DPC6258 with *S. saprophyticus* DPC5671 on cheese A. Similar results were shown by Mounier *et al.* (2006) who reported in a cheese model an increase of the colour after the 15th day of ripening at 14°C and higher a* value for the combination *D. hansenii* with *C. casei*, compared to *D. hansenii* with *S. saprophyticus*, resulting in higher development of red colour.

The development of the typical aroma and flavour in cheese A and B is associated with the lipolytic and proteolytic processes of the yeast and bacterial component of the

smear consortium. These processes were slower in the control cheese, which did not develop the same levels of proteolysis, FAAs and FFAs (Fig. 3.5, 3.6, 3.7, 3.8) in a short ripening time (35 days).

During cheese ripening, a gradual decomposition of caseins into small peptides and FAAs occurs. FAAs are considered precursors of flavour compounds during the development of cheese flavour. It is likely that the smear treatments increased the proteolysis of β -casein by plasmin (more active at alkaline pH) on the surface of the test cheeses (Fig. 3.6), increasing the pH levels. At the end of ripening the dominant FAAs detected in high amount on the surface of the test cheeses were valine, leucine, proline, glutamate and lysine (Fig. 3.7), due to their relative concentration in casein and the peptidase activity of the smear consortium, especially *D. hansenii* DPC6258. Analysis of cell-free supernatants of the cultures used in this study confirmed that *D. hansenii* DPC6258 had high peptidase activities (PepX and PepN), while activities were low for *S. saprophyticus* DPC5671 and *C. casei* DPC5298 (Fig. 3.13). Similar results have also been reported for other *D. hansenii*, *S. saprophyticus* and *C. casei* strains (Bintsis et al., 2013; Casaburi et al., 2006; Curtin et al., 2002).

The hydrolysis of triglycerides is the main biochemical transformation of fat during cheese ripening, which leads to the production of FFAs. Individual FFAs contribute to the cheese aroma with their specific flavours and especially with their metabolites. At the end of ripening high amounts of FFAs were detected on the surface of cheese A and B. The levels of all individual FFAs detected were higher on the surface of cheese A compared with cheese B, especially for C10:0 (Fig. 3.8). It has been

previously reported that the lipolysis by *D. hansenii* is weak, while studies on Gram-positive bacteria showed good activity on substrates with different glyceride chains length (Bintsis et al., 2003; Cardoso et al., 2015; van den Tempel and Jakobsen, 2000). Experimental work showed that *S. saprophyticus* DPC5671 had greater lipolytic activity on tributyrin than either *C. casei* DPC5298 or *D. hansenii* DPC6258 (Fig. 3.14). These results are in agreement with what was previously reported by Talon and Montel (1997), who detected lipolytic activity on tributyrin in a range of staphylococcus strains, including *S. saprophyticus*. The higher lipolytic activity of *S. saprophyticus* DPC5671 compared to *C. casei* DPC5298 may explain the higher amount of FFAs in cheese A.

Numerous volatile compounds were significantly ($P<0.05$) associated to cheese A and B, especially those particularly characterized by strong aroma notes, such as some specific carboxylic acids, alcohols, esters, ketones and sulphur compounds. However, in the control cheese, the majority of volatile compounds detected were characterized by mild aroma notes, suggesting that the smear treatments have modified the aroma profile of Cheddar cheese curd in only 35 days of ripening (Table 3.2).

The metabolism of FAAs in the cheese A and B in this study was responsible for the development of specific branched alcohols and branched chain acids detected (3-methyl-1-butanol, phenylethyl-alcohol, 3-methyl-butanoic acid). *D. hansenii* has been identified as a possible producer of alcohols in previous studies (Arfi et al., 2002; Gori et al., 2012; Leclercq-Perlat et al., 2004a), while the production of carboxylic acids has been previously attributed to both yeasts and smear bacteria (*Geotrichum candidum* and *B. linens*) (Jollivet et al., 1992; Jollivet et al., 1994).

The high amount of esters detected in cheese A (ethyl acetate, ethyl octanoate, methyl hexanoate, 3-methylbutyl acetate and isopentyl hexanoate) is likely related to the high FFAs content and the presence of alcohols in cheese A, considering they originate from the esterification or alcoholysis of alcohols with carboxylic acids. While information on the biosynthesis of esters by corynebacteria is sparse, numerous studies reported ester production by staphylococci isolated from fermented foods, including *S. saprophyticus* strains (Talon et al., 1998). The formation of esters in cheese A, not detected in high amount in cheese B, was likely due to the metabolic activity of *S. saprophyticus* DPC5671 rather than by *D. hansenii* DPC6258.

Other products of FFAs metabolism such as ketones and alcohols were detected in all cheeses. However 2-pentanone, 2-hexanone, 2-nonanone and 2-decanone were particularly associated with the control cheese and not with cheese A and B, suggesting an involvement of LAB rather than the smear cultures. It is known that methyl ketones result from the β -oxidation of FFAs by lipolytic enzymes due to autolysis of the LAB during ripening (Collins et al., 2003), although it is also postulated that production is enhanced by heating milk or directly from esterified β -keto acids (Alewijn, 2006; Forss, 1979).

Sensory analysis showed different results between the cheese A, cheese B and control cheese. The sensory panel was not influenced by the colour of the cheese, because the surface was removed before the sensory test. “Mould Cheese Flavour” and “Pungent Flavour” are descriptors associated with the surface-ripened cheeses and they can be correlated with a wide range of volatiles with strong aroma notes (Table 3.2)

detected on the surface and core of both cheese A and B, namely butanoic, octanoic acid (originated from lipolysis of lipids), 2-heptanol (from reduction of ketones), 2-methyl-propanoic, 3-methyl-butanoic, 2-methyl-butanoic, pentanoic acid, 3-methyl-butanol, 2-methyl-butanol, phenylethyl-alcohol, 3-methyl-butanal (from metabolism of branched chain amino acids or possibly phenylalanine for phenylethyl-alcohol), methanethiol, dimethyldisulphide and dimethyldisulphide (from metabolism of sulphur amino acids). As expected, these descriptors were significantly associated ($P < 0.05$) to the cheese A and/or B, suggesting that the activities of yeast and Gram-positive bacteria on the cheese conferred a typical surface-ripened cheese flavour, not perceived in the control. The descriptors significantly associated ($P < 0.05$) to cheese B, as “Sweaty/Sour Aroma”, “Pungent Aroma”, “Off-Aroma”, are considered as “unclean” and off-odorants and were associated with some compounds detected in abundance on the surface and core of cheese B, such as 3-methyl-butanoic acid, octanoic acid, methanethiol and particularly 8-nonen-2-one (from β -oxidation of fatty acids) which was identified only in cheese B.

Sensory analysis showed the smear cultures on the cheese surface have affected the cheese ripening giving strong and intense aroma and flavour to cheese A and B, while the control cheese was characterized by mild aroma and flavour.

The smear treatments have induced the production of new variants of cheese with novel aroma notes in only 35 days. Overall, the results of the volatile compounds and sensory analysis suggested that the bacterial strains in conjunction with *D. hansenii* may have potential to produce novel cheeses with different aroma profiles using a Cheddar cheese curd.

3.6. Conclusion

The cheese-making method described in this paper gives a new approach for the production of novel surface-ripened cheeses starting from a Cheddar cheese curd. Both the yeast and bacterial cultures were able to establish themselves on the surface of the cheese and become the dominant microbiota on the cheese surface, producing a cheese variety with acceptable appearance and novel flavour aroma profiles. This study represents a model to produce novel cheese types with a range of flavours and aromas through the growth of combinations of yeast and bacterial cultures on the using cheese curd produced on a traditional Cheddar cheese plant. The method proposed could potentially represent a low-cost solution for medium-small cheese industries to diversify cheese production.

3.7. Acknowledgements

Thanks to Sarah Henneberry for the assistance in urea-PAGE analysis. Thanks to Dr Deirdre Kennedy for cryo-SEM micrographs.

3.8. References

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Chapter 4

Staphylococcus saprophyticus DPC5671, a strain isolated from the surface of Cheddar cheese with the potential to diversify flavour

This chapter has been published in *Genome Announcements* (2017), 16, e00193-17. DOI: 10.1128/genomeA.00193-17

In this chapter Dr Fiona Crispie performed 454 DNA sequencing of *S. saprophyticus* DPC5671 and Dr Caitriona Guinane performed DNA quality analysis and contig assembly.

4.1. Abstract

Staphylococcus saprophyticus is a coagulase negative staphylococcus (CNS), which is involved in the flavour development of fermented food, and thus has been previously used as an adjunct culture during ripening for the production of cheese and sausages. However, it is not clear if some CNSs might present health implications, which could prevent their use in food preparation. In this study, a strain previously isolated from the surface of Cheddar cheese, *S. saprophyticus* DPC5671 was extensively analysed using both genotypic and phenotypic methods. Initial confirmation of the species was established by sequence analysis of the *16S rRNA*, *dnaJ* and *rpoB* genes. Using phenotypic-based methods, it was determined that the strain did not produce any detectable enterotoxins, presented as coagulase negative, non-haemolytic and did not show resistance to a panel of antibiotics as set by European Food Safety Authority (EFSA). The draft genome sequence was obtained for *S. saprophyticus* DPC5671 and analysed for potential virulence factors, and potential gene loci encoding for enzymes involved in cheese flavour development. Although the genome analysis of *S. saprophyticus* DPC5671 detected a protein-coding sequence potentially involved in some cases of urinary tract infection, this strain presented a history of safe use within fermented food, and its infection mechanism is not related to the food consumption. Genomic analysis and annotation did not reveal any other potential virulence factors (genes involved in antibiotic resistance, biogenic amines, and enterotoxins), while

several CDSs encoding for enzymes implicated in proteolysis (proteinases and peptidases), free amino acids degradation (aminotransferases and lyases) and lipolysis (lipases/esterases) were observed and are outlined in this chapter.

4.2. Introduction

Surface-ripened cheese is characterized by the growth of complex microbiota on the cheese surface, composed of both yeasts and bacteria, which are responsible, through their metabolic activity, for the development of the typical flavour, and the external “glistening” appearance of this cheese variety (Mounier et al., 2017). Due to their low pH tolerance, yeasts (e.g. *Debaryomyces sp.*, *Kluyveromyces sp.*, *Geotrichum sp.*) are the first microorganisms to establish themselves on the cheese surface, and they are able to increase the pH through the metabolism of lactate, and the secretion of alkaline compounds, such as ammonia (from free amino acids degradation) (Ferreira et al., 2002). The favourable higher pH determines the growth of Gram-positive bacteria, such as *Brevibacterium*, *Corynebacterium* and *Microbacterium*, species which are considered, with *Staphylococci*, the most important genera which make up the microbiota of surface-ripened cheese (Bertuzzi et al., 2017; Mounier et al., 2006; Mounier, 2015).

The *Staphylococcus* genus includes both highly pathogenic coagulase positive species (e.g. *Staphylococcus aureus*, *Staphylococcus haemolyticus*) and coagulase negative species (CNSs). CNSs (e.g. *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus equorum*) are favoured for meat and dairy fermentation, due to their tolerance to salt and production of flavour compounds (Blaiotta et al., 2004; Ruaro et al., 2013; Vergnais et al., 1998). CNSs, together with the other Gram-positive bacteria,

moulds and yeasts, are considered the microorganisms responsible for the production of the intense flavour of surface-ripened cheese (Bertuzzi et al., 2017; Desmaures et al., 2015; Mounier et al., 2017). In some food preparations, it is common practice to add starter cultures, containing CNSs, to meat and dairy fermentations to increase the flavour development of the food products during ripening (Di Maria et al., 2004).

The flavour development in cheese is a particularly complex process which involves both proteolytic, and lipolytic pathways (McSweeney, 2017). The casein in cheese can be used as substrate for microbial or indigenous milk proteinases, resulting in an increase of peptides, which are metabolized by the microbial aminopeptidases with the subsequent release of free amino acids (FAAs). Flavour volatile compounds can originate from FAAs catabolism, by the activity of decarboxylases, deaminases, transaminases, lyases, and dehydratases. In addition, triacylglycerols can be metabolized by the microbial, or indigenous milk lipases or esterases and the free fatty acids (FFAs) can be degraded through an oxidation process, with a consequent biosynthesis of flavour volatile compounds.

Despite a history of safe use in fermented foods, some concern has been expressed around the use of CNSs in food, as research studies on their safety assessment showed their ability to produce enterotoxins, or to be antibiotic resistant (Irlinger, 2008; Podkowik, Park, Seo, Bystron, and Bania, 2013). The pathogenic potential of CNSs is a strain-dependent characteristic, with specific strains of *S. simulans*, *S. xylosum*, *S. equorum*, *S. lentus* and *S. capitis*, isolated from goat's milk and cheese, capable of producing enterotoxins (Vernozy-Rozand et al., 1996), and other strains of *S.*

epidermidis, *S. chromogenes*, *S. warneri*, *S. hyicus* and *S. simulans*, isolated from milk of cows with mastitis, displaying methicillin-resistance (Silva et al., 2014).

In the present study, *S. saprophyticus* DPC5671, which was used to produce surface-ripened cheese, was firstly analysed phenotypically to determine its safe utilization in food system, and subsequently its full genome was characterized and investigated for the presence of virulence factors and enzymatic pathways related to cheese flavour development.

4.3. Materials and methods

4.3.1. Bacterial strains and growth conditions

The bacterial strain of *S. saprophyticus* DPC5671 was isolated from the surface of Cheddar cheese and maintained at -80°C for long term storage. For routine use, the strain was streaked from frozen stocks onto Trypticase Soya Agar (TSA; Becton, Dickinson and Company) and subsequently sub-cultured into 10 mL of Trypticase Soy Broth (TSB; Becton, Dickinson and Company), shaking at 30°C, for 24 h.

4.3.2. DNA extraction for PCR

Microbial DNA was extracted from a culture, grown in TSB, for 24 h. The culture (1 mL) was centrifuged at $15,000 \times g$, for 5 min, and the DNA was extracted from the cell pellet with the QIAamp DNA Mini Kit QIAGEN (Hilden, Germany), according to the manufacturer's protocol.

4.3.3. 16S rRNA sequencing

The 16S rRNA gene (~1500 bp) was PCR amplified using the UniF (5' - AGAGTTTGATCCTGGCTCAGG- 3') and UniR (5' - ACGGCAACCTTGTACGAGT- 3') primers. The PCR reaction conditions were as follows, initial denaturation at 94°C for 5 min, 30 cycles of annealing of 94°C for 40 sec, 55°C for 30 sec, 72°C for 1 minute, and a final extension of 72°C for 10 min. The PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification

Kit (GE Healthcare, Little Chalfont, UK), and analysed through agarose gel electrophoresis (1% of agarose) to check the size of the amplicons. Amplicons were sequenced through conventional Sanger sequencing by Beckman Coulter (Brea, CA, USA) and aligned with BLASTN server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the NCBI database.

4.3.4. *rpoB* and *dnaJ* gene sequencing

To confirm the species, genomic DNA from *S. saprophyticus* DPC5671 was extracted as described above and used to amplify the *rpoB* and *dnaJ* genes. The PCR amplification for *rpoB* was performed using primers 2491F (5' – AACCAATTCCGTATIGGTTT- 3') and 3554R (5' -CCGTCCCAAGTCATGAAAC- 3'). The PCR reaction conditions were as follows: initial denaturation of 95⁰C for 2 min, 35 cycles of 94⁰C for 30 sec, 52⁰C for 40 sec, 72⁰C for 1 min, and a final extension of 72⁰C for 5 min. The sequencing of *rpoB* PCR product was carried out with internal degenerate primers 2643F (5' -CAATTCATGGACCAAGC- 3') and 3241R (5' -GCIACITGITCCATACCTGT- 3'), on a fragment of *rpoB* gene of approximately 600 bp (Drancourt and Raoult, 2002).

The PCR amplification for *dnaJ* was performed using primers SA-(F) (5'-GCCAAAAGAGACTATTATGA- 3') and SA-(R) (5' -ATTGYTTACCYGTTTGTGTACC- 3'). The PCR reaction conditions were as follows: an initial denaturation of 94⁰C for 3 min, 5 cycles of 94⁰C for 30 sec, 52⁰C for 40 sec, 72⁰C for 1 min, and 30 cycles of 94⁰C for 30 sec, 50⁰C for 30 sec, 72⁰C for 1 min and

a final extension of 72°C for 3 min. The amplicon size of *dnaJ* PCR reaction was 920 bp (Shah et al., 2007). The PCR products were purified with Illustra GFX PCR DNA Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), following the protocol of the supplier, and analysed with agarose gel electrophoresis (1% of agarose) to check the amplicon size. Amplicons were sequenced with conventional Sanger sequencing at Beckman coulter (Brea, CA, USA) and aligned with BLASTN server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the NCBI database.

4.3.5. Coagulase activity

The coagulase activity of *S. saprophyticus* DPC5671 was analysed with a commercial coagulase test (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) as follows: 100 µL of an overnight culture of *S. saprophyticus* DPC5671, grown in TSB, (shaking at 30°C, for 24 h) was mixed in a tube with 300 µL of lyophilized rabbit plasma with EDTA solubilised in sterile distilled water. The tube was incubated at 37°C for 24 h. The coagulase test was positive if more than 75% of the tube contents had formed a coherent clot. The test was performed in triplicate and the strains of *S. aureus* R963 and *S. aureus* R4071 were used as positive controls, while a water solution of uninoculated lyophilized rabbit plasma with EDTA was used as negative control. Coagulase activity was examined in triplicate.

4.3.6. Detection of Staphylococcal enterotoxins

S. saprophyticus DPC5671 was tested with 72-wells plate 3M™ Tecra™ Staph Enterotoxins ID Test (3M™ St. Paul, MN, USA) to screen for the production of enterotoxins A-E. *S. aureus* SA45, *S. aureus* FRI13, *S. aureus* R963, and *S. aureus* R4071 were used as positive controls, in addition to the positive control provided with the kit. All the strains used in this test were stored at -80°C, and were grown in TSB, using the same conditions described above for *S. saprophyticus* DPC5671. Subsequently, 50 µL of sub-cultured broth were inoculated into 5 mL of sterile skim milk solution (10% w/v) and grown at 37°C, for 24 h. An uninoculated sterile skim milk solution was used as negative control for the test. Initially, the 72-wells plate was washed with the washing solution (provided with the kit), then 200 µL of inoculum were added to each well, and the plate was incubated at 37°C, for 2 h. Subsequently, the 72-wells plate was emptied and washed with washing solution for 4 times, and 200 µL of conjugate solution (provided with the kit) were added to each well and the plate was incubated at 25°C, for 1 h. Again, the plate was emptied and washed with washing solution 5 times, and 200 µL of substrate solution (provided with the kit) was added to each well and the plate was incubated at 25°C, for 30 min. At the end of the incubation, 20 µL of stop solution (provided with the kit) was added to each well, and the colour of each well was visually compared with the colour card (provided with the kit) to verify the production of enterotoxins. *S. saprophyticus* DPC5671 was tested in triplicate for the production of staphylococcal enterotoxins.

4.3.7. Haemolysis agar assay

The haemolytic activity of *S. saprophyticus* DPC5671 was evaluated by streaking the culture onto TSA containing 5 % rabbit blood v/v (Difco) and incubated at 30°C, for 24 h. The positive strains showed a clear halo around the bacterial colony, which indicated β -haemolysis. *S. aureus* SA45 and *S. aureus* FRI13 were used as positive controls. Haemolytic activity was examined in triplicate.

4.3.8. Antibiotic sensitivity

Minimum inhibitory concentrations (MICs) for antibiotics were evaluated using VetMIC system (National Veterinary Institute of Sweden, Uppsala, Sweden). Each micro-titre plate contained 10 fold serial dilutions of 9 antibiotics (ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, and chloramphenicol), and wells for negative (uninoculated broth) and positive (strains grown in the absence of antibiotic) controls. Five mL of *S. saprophyticus* DPC5671 broth culture, grown in TSB as described above, was centrifuged at $4,500 \times g$ at 4°C for 15 min, and was resuspended in maximum recovery diluent (Becton, Dickinson and Company) as per the manufacturer's instructions to obtain a cell density of $\sim 3 \times 10^5$ CFU mL⁻¹. One hundred μ L of cell suspension was added to each well on the micro-titre plate and incubated for 48 h at 30°C. The lowest antibiotic concentration at which no growth occurred was defined as the minimum inhibitory concentration for each antibiotic. Antibiotic sensitivity was examined in triplicate.

4.3.9. DNA extraction for genome sequencing

Genomic DNA was extracted from *S. saprophyticus* DPC5671 as follows: *S. saprophyticus* DPC5671 was grown in 10 mL of TSB as described above. The cells were harvested by centrifugation at 6,000× g for 10 min, and resuspended in 5 mL of sterile buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5), containing 200 µg mL⁻¹ of lysostaphin (Sigma Aldrich) and incubated at 37°C, for 1 h. Subsequently, a 1 mL of solution of SDS (20%) and proteinase K (0.5 mg mL⁻¹) was added and incubated at 55°C, for 2 h, with occasional inversion. Then, 2 mL of NaCl 5M, and 6 mL of chloroform were added to the sample and incubated at room temperature for 30 min, with frequent inversion. The sample was centrifuged at 4,500× g for 15 min, and the aqueous (upper) phase was transferred into a new sterile tube and 5.5 mL of isopropanol were added to the sample, which was gently inverted for few times. The DNA solution was transferred to an Eppendorf tube and washed with 70% (ethanol/H₂O) with centrifugation at 6,000 × g, for 1 min. The DNA, precipitated on the bottom of the tube, was dried for 4-5 hours and then resuspended in 100 µL of ultra-pure water.

4.3.10. Genome sequencing

The draft genome of *S. saprophyticus* DPC5671 was sequenced using paired end 454 pyrosequencing, to coverage of 23X. Sequencing took place at the Teagasc 454 sequencing facility on a genome sequencer FLX platform (Roche Diagnostics Ltd., West Sussex, United Kingdom) according to the manufacturer's protocols. This was followed by initial assembly into 24 contigs using the Newbler program (Roche-applied-

science.com). Prodigal software (Hyatt et al., 2010) was used to predict open reading frames within the draft genome and the RAST annotation server (Aziz et al., 2008) was used to determine complementary gene calling and automated annotation. The draft genome was manually analysed using the ARTEMIS genome browser (Carver et al., 2012), and comparative analysis with the genome of *S. saprophyticus* ATCC 15305 (Kuroda et al., 2005) was performed using the Artemis Comparison Tool (ACT) (Carver et al., 2005). PHAST (Phage Search Tool) web server (Zhou et al., 2011) was used to determine the presence of putative phage within the genome. The comprehensive antibiotic resistance database (CARD) software (McArthur et al., 2013) was used to determine the presence of genes potentially involved in antibiotic resistance and the presence coding DNA sequences (CDSs) encoding for virulence factors and potential flavour enzymes were manually analysed using Artemis and the BLASTP web server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al., 1997).

4.4. Results

4.4.1. Strain identification

Prior to whole genome sequence analysis, alignment of the sequenced PCR amplicons (*16S rRNA*, *rpoB*, *dnaJ*), with BLASTN, determined that the strain isolated from Cheddar cheese surface was *S. saprophyticus*. Alignment of *16S* showed 100% of query cover, and 100% of identity with *Staphylococcus saprophyticus* subsp. *saprophyticus* strain IF1SW-B3 *16S* ribosomal RNA gene, the alignment of *dnaJ* presented 100% of query cover, and 100% of identity with *Staphylococcus saprophyticus* subsp. *bovis* strain CIP 105260 *DnaJ* (*dnaJ*) gene, and the alignment of *rpoB* showed 100% of query cover, and 99% of identity with *Staphylococcus saprophyticus* strain FDAARGOS_355 (Table 4.1).

Table 4.1. Results of the best three alignments of the PCR products (*16S*, *dnaJ*, and *rpoB*) with BLASTN.

PCR products	BLASTN hits	Query cover	Identity
<i>16S</i>	<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i> strain IF1SW-B3 <i>16S</i> ribosomal RNA gene	100%	100%
	<i>Staphylococcus saprophyticus</i> strain H72 <i>16S</i> ribosomal RNA gene	99%	100%
	<i>Staphylococcus saprophyticus</i> strain N33 <i>16S</i> ribosomal RNA gene	100%	99%
<i>dnaJ</i>	<i>Staphylococcus saprophyticus</i> subsp. <i>bovis</i> strain CIP 105260 <i>DnaJ</i> (<i>dnaJ</i>) gene	100%	100%
	<i>Staphylococcus saprophyticus</i> subsp. <i>bovis</i> <i>dnaJ</i> gene for <i>dnaJ</i> protein	100%	99%
	<i>Staphylococcus saprophyticus</i> strain FDAARGOS_336	100%	99%
<i>rpoB</i>	<i>Staphylococcus saprophyticus</i> strain FDAARGOS_355	100%	99%
	<i>Staphylococcus saprophyticus</i> strain FDAARGOS_336	100%	99%
	<i>Staphylococcus saprophyticus</i> strain FDAARGOS_137	100%	99%

4.4.2. Coagulase activity

The growth of *S. saprophyticus* DPC5671 within the solution of lyophilized rabbit plasma with EDTA, did not form any clot, suggesting the strain was coagulase negative (Fig. 4.1). The strains used as control positive, *S. aureus* R963 and *S. aureus* R4071, formed a clot inside the test tube, due to their ability to produce a fibrin clot (Fig. 4.1).

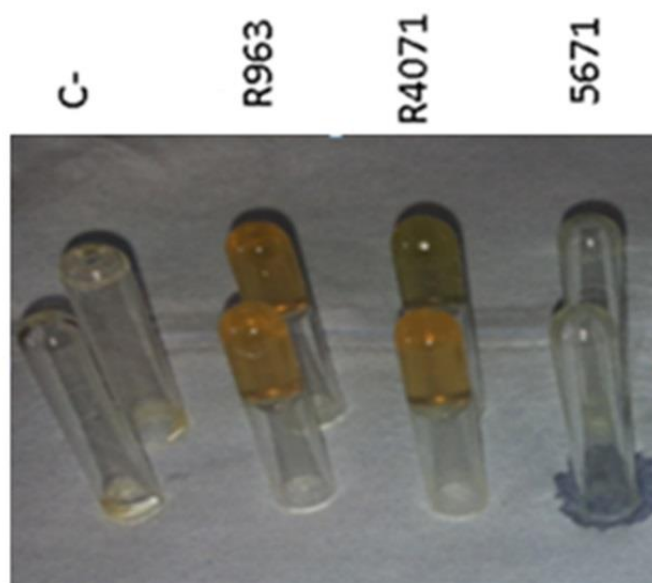


Figure 4.1. Coagulase test performed on *S. saprophyticus* DPC5671 (5671). *S. aureus* R963 (R963) and *S. aureus* R4071 (R4071) were used as positive controls, while water solution of lyophilized rabbit plasma with EDTA not inoculated was used as negative control (C-).

4.4.3. Staphylococcal enterotoxins

With the Staph Enterotoxins ID Test it was possible to determine that *S. saprophyticus* DPC5671 was not able to produce A, B, C, D and E enterotoxin (Fig. 4.2). The test showed that the control positive (provided with the kit), *S. aureus* SA45, FRI13, R963, R4071, and 178, were positive for the enterotoxin production (Fig. 4.2).

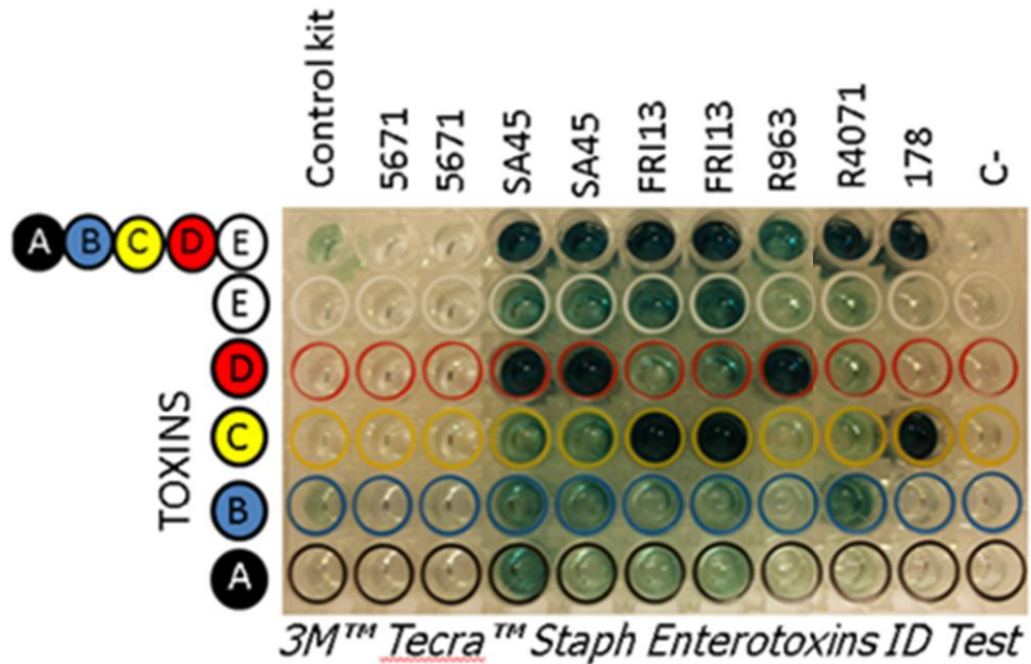


Figure 4.2. Staph Enterotoxins ID Test performed on *S. saprophyticus* DPC5671 (5671). *S. aureus* SA45 (SA45), *S. aureus* FRI13 (FRI93), *S. aureus* R963 (R963), and *S. aureus* R4071 (R4071) were used as positive controls, while sterile skim milk was used as negative control (C-). A green colour in the wells denotes a positive reaction.

4.4.4. Haemolytic activity

The growth of *S. saprophyticus* DPC5671 on TSA, containing 5 % rabbit blood (v/v) (Difco), did not show any halo around the colonies, suggesting the strain was non-haemolytic (Fig. 4.3). The strains used as positive controls, *S. aureus* SA45 and *S. aureus* FRI13, displayed a transparent halo around the colonies, derived from the lysis of the red blood cells (Fig. 4.3).

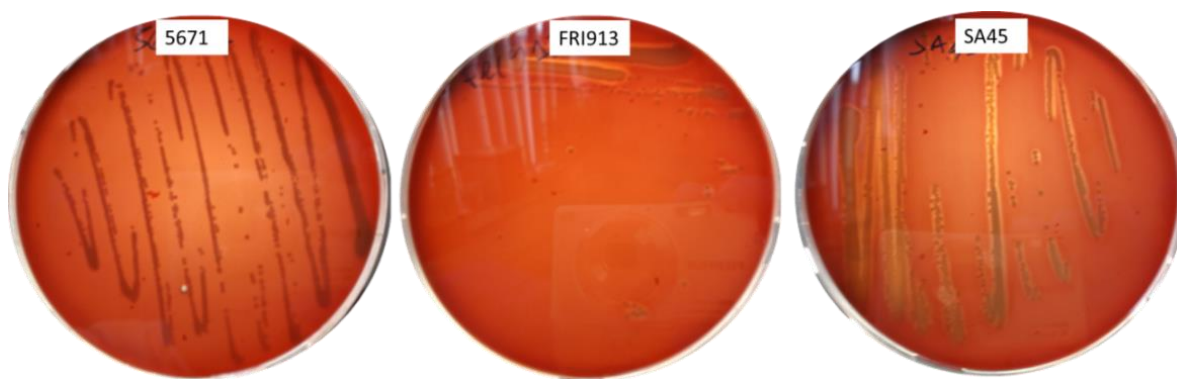


Figure 4.3. Haemolytic activity on TSA containing 5 % rabbit blood v/v (Difco), of *S. saprophyticus* DPC5671 (5671). *S. aureus* SA45 (SA45) and *S. aureus* FRI13 (FRI13) were used as positive control.

4.4.5. Antibiotic resistance

The minimum concentrations which inhibited the growth of *S. saprophyticus* DPC5671 were 0.5 $\mu\text{g mL}^{-1}$ for ampicillin, 0.5 $\mu\text{g mL}^{-1}$ for vancomycin, 0.5 $\mu\text{g mL}^{-1}$ for gentamycin, 2 $\mu\text{g mL}^{-1}$ for kanamycin, 0.5 $\mu\text{g mL}^{-1}$ for streptomycin, 0.25 $\mu\text{g mL}^{-1}$ for tetracycline, 0.25 $\mu\text{g mL}^{-1}$ for erythromycin, 0.12 $\mu\text{g mL}^{-1}$ for clindamycin, and 2 $\mu\text{g mL}^{-1}$ for chloramphenicol. The antibiotic test showed that the minimum inhibitory

concentrations were lower than the European Food Safety Authority (EFSA) cut-off values for Gram-positive organisms other than lactic acid bacteria (EFSA 2012) (Table 4.1).

Table 4.2. Results of the minimum inhibitory concentrations test for antibiotics, on *S. saprophyticus* DPC5671, and related cut-offs published by EFSA for Gram-positive organisms other than lactic acid bacteria.

Antibiotic	MIC ($\mu\text{g mL}^{-1}$)	EFSA cut-off ($\mu\text{g mL}^{-1}$)
Ampicillin	0.5	≥ 1
Vancomycin	0.5	≥ 2
Gentamycin	0.5	≥ 4
Kanamycin	2	≥ 16
Streptomycin	0.5	≥ 8
Tetracycline	0.25	≥ 2
Erythromycin	0.25	≥ 0.5
Clindamycin	0.12	≥ 0.25
Chloramphenicol	2	≥ 2

¹NA: not applicable

4.4.6. Draft Genome analysis

The draft genome of *S. saprophyticus* DPC5671 (GenBank assembly accession: GCA_002009035.1) includes 2,676,318 bp, with an average G+C content of 33.1 %. It consists of a single circular chromosome and does not appear to harbour any plasmids (Fig. 4.4). Within the draft genome there are 2,647 coding regions predicted, in addition to 4 rRNA and 59 tRNA genes. The genome sequence also includes two putative novel prophages of ~43.6 kb and ~42.9 kb within the chromosome (Fig. 4.5). Automated annotation performed with RAST showed the predicted functions of the annotated protein-coding sequence (Fig.4.6).

Overall, the genome of *S. saprophyticus* DPC5671 shows high similarity to the *S. saprophyticus* ATCC 15305 in terms of genome size, G+C content, and gene synteny (Kuroda et al., 2005).

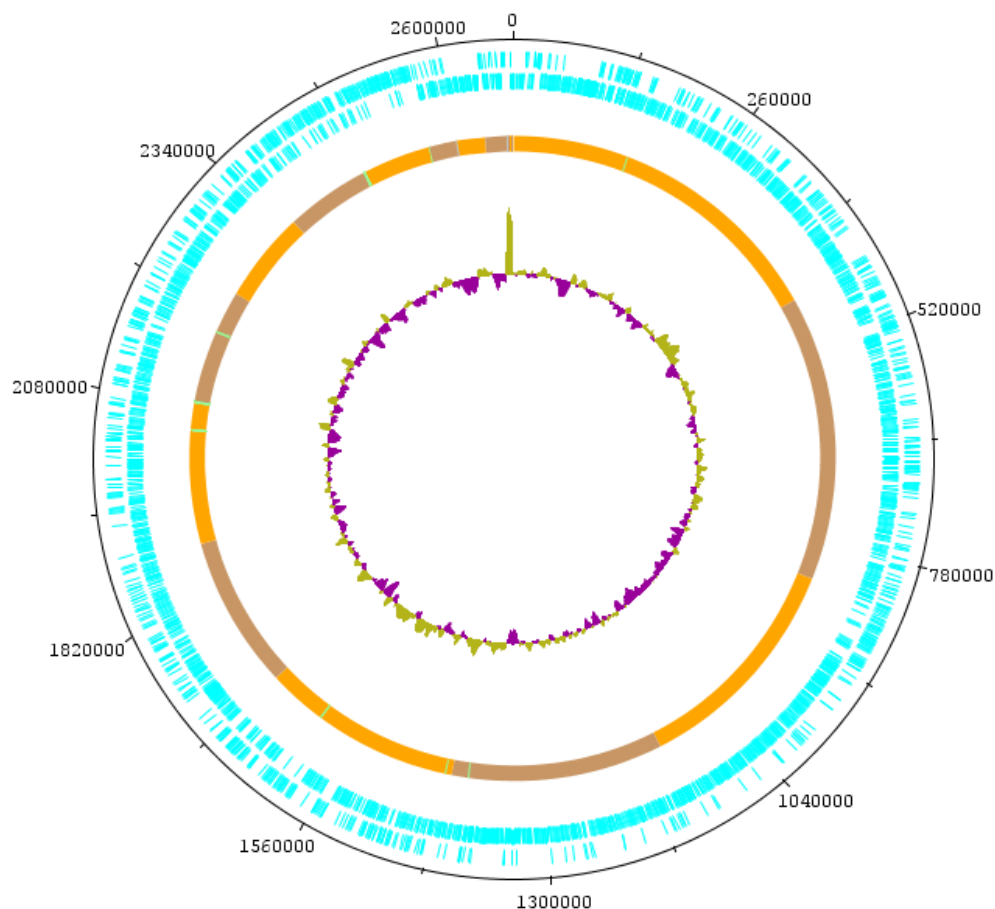


Figure 4.4. Genome plots realized with ARTEMIS genome browser. The map displays the features of the circular chromosome of *S. saprophyticus* DPC5671. From the outer to the inner concentric circle of the chromosome (a): circle 1, genomic position in kb; circles 2 and 3, predicted CDSs on the forward (outer part) and the reverse (inner part); circle 4, genomic contigs; circle 5, GC content.

Contig all_bases from *Staphylococcus saprophyticus* MR

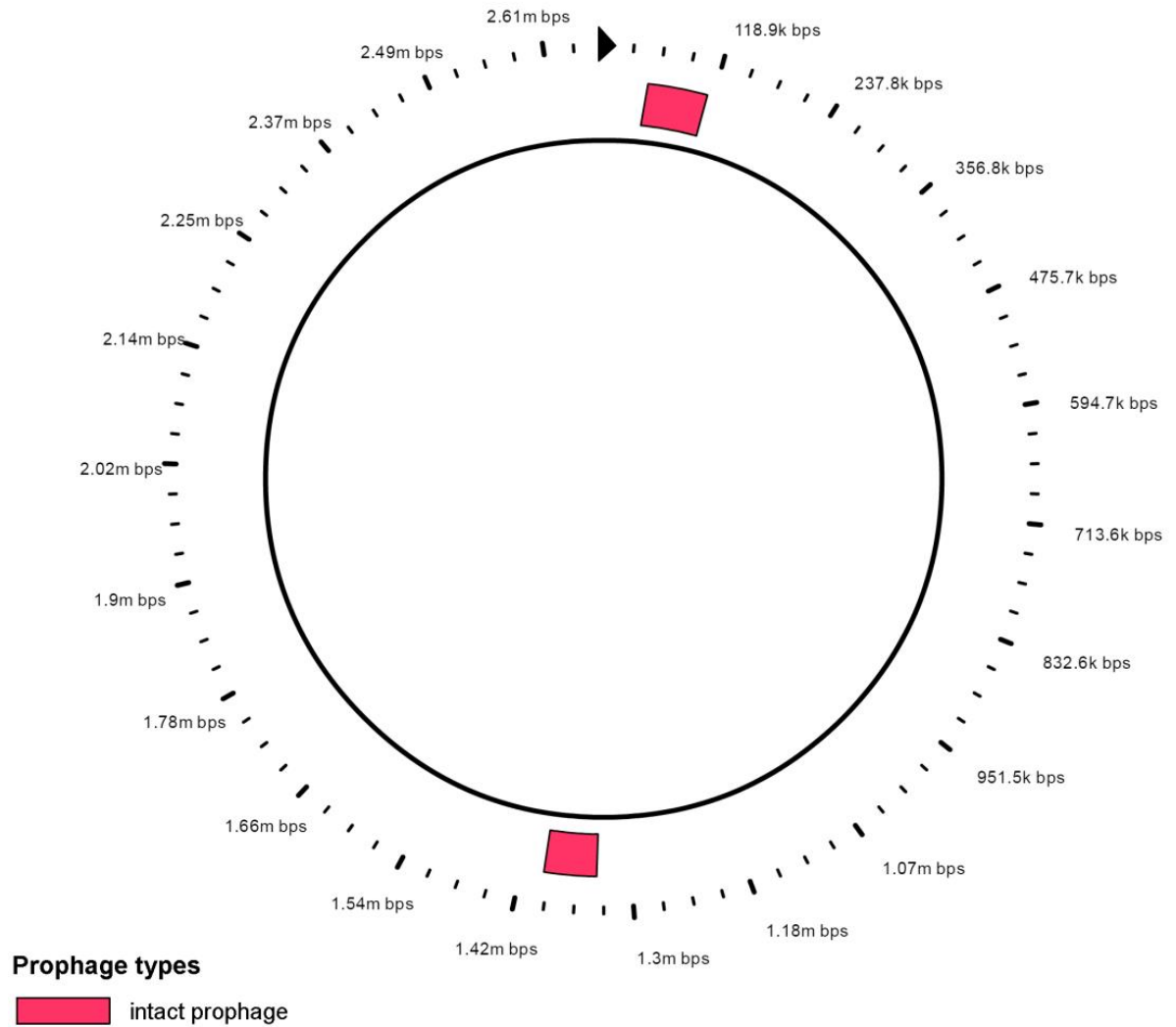


Figure 4.5. Putative phage within the genome of *S. saprophyticus* DPC5671 (based on Phast PHAST web server).

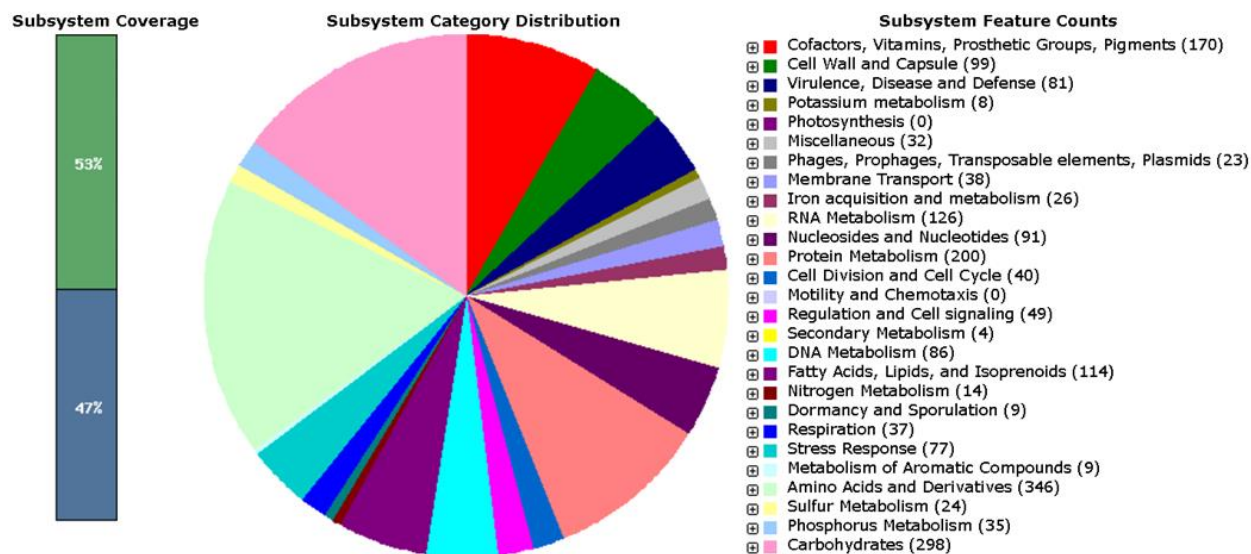


Figure 4.6. Subsystem distribution of *S. saprophyticus* DPC5671 (based on RAST annotation server).

4.4.7. Investigation of virulence and flavour genes

By the methods used in this study, genomic analysis revealed no obvious transferable antibiotic resistance genes, decarboxylases genes involved in the biosynthesis of biogenic amines, and toxigenic genes. Previous studies showed an involvement of some *S. saprophyticus* strains in urinary tract infection (UTI), showing a specific adhesin *uafA*, associated with adherence to the eukaryotic cell in the urinary tract (Kuroda et al., 2005). The *S. saprophyticus* DPC5671 genome was found to have the predicted CDSs for adhesion *uafA* (2236218-2242553), with similarities examined with BLASTP web server to *uafA* in *S. saprophyticus* ATCC 15305 of 39 % of query cover and 97% of protein identity.

The genome of *S. saprophyticus* DPC5671 was also investigated for the presence of CDSs encoding for enzymes involved in the proteolytic or lipolytic, processes which lead to the formation of volatile compounds (Table 4.3). The genome of *S. saprophyticus* DPC5671 was equipped with a large set of CDSs encoding for putative triacylglycerol esterase/lipase (EC 3.1.1.3; 452825-453700; 1445522-1446325; 2336285-2338546) and carboxylesterase (EC 3.1.1.1; 141578-142318; 280742-281773; 495623-496345; 559524-560126; 611875-612849; 636868-637560; 1451623-1452360; 1843057-1843959; 2197243-2197989). Also detected were CDSs encoding for subunits of proteinase Clp ATPase (EC 3.4.21.92; 15471-154955; 441093-443555; 1910855-1912117; 2648299-2650398) and for various peptidases, such as PepB (EC 3.4.11.1; 33604-35085; 2452644-2453882), PepF (EC 3.4.24.-; 881855-883666; 2211807-2213615), PepM (EC 3.4.11.18; 2446177-2446929), PepP (EC 3.4.11.9; 1026611-1027669; 1950805-1951857), PepT (EC 3.4.11.4; 186173-187405), pyroglutamyl-peptidase (EC 3.4.19.3; 480016-480657), and some putative glutamyl aminopeptidases (EC 3.4.11.7; 512089-513165; 864836-865873; 1999046-2000122). Other CDSs involved in the FAAs degradation were found, such as genes encoding for glutamate dehydrogenase (GDH) (EC 1.4.1.2; 17836-19080; 381290-382534), histidinol-phosphate aminotransferase (AT) (EC 2.6.1.9; 209075-210130), branched-chain amino acid AT (EC 2.6.1.42; 369574-370650), 4-aminobutyrate AT (EC 2.6.1.19; 601413-602750), and cystationie β -lyase (EC 4.4.1.8; 703070-704215; 1548097-1549272).

Table 4.3. Results of the best hit with BLASTP of the protein-coding sequences, for enzymes responsible for the flavour development.

	EC number	Genome location	Query AA Length	Domain hits	BLASTP Hits	Query cover	Query identity
Proteinase							
Clp subunit	3.4.21.92	15471-154955	194	ATP-dependent Clp protease proteolytic subunit (1-194)	ATP-dependent Clp protease proteolytic subunit	100%	100%
Clp subunit	3.4.21.92	441093-443555	820	Clp protease ATP binding subunit (3-815)	ATP-dependent Clp protease ATP-binding subunit ClpC	100%	100%
Clp subunit	3.4.21.92	1910855-1912117	420	ATP-dependent protease ATP-binding subunit ClpX (3-411)	ATP-dependent Clp protease ATP-binding subunit ClpX	100%	100%
Clp subunit	3.4.21.92	2648299-2650398	699	ATP-dependent Clp protease ATP-binding subunit ClpA (69-697)	ATP-dependent Clp protease ATP-binding subunit	100%	100%
Aminopeptidase							
PepB	3.4.11.1	33604-35085	493	PepB - Leucyl aminopeptidase (13-492)	Leucyl aminopeptidase family protein	100%	100%
PepB	3.4.11.1	2452644-2453882	412	Leucyl aminopeptidase (6-410)	Aminopeptidase	100%	100%
PepF	3.4.24.-	881855-883666	603	Oligoendopeptidase F (8-593)	Oligoendopeptidase F	100%	100%
PepF	3.4.24.-	2211807-2213615	602	Oligoendopeptidase F (9-602)	Oligoendopeptidase F	100%	100%
PepM	3.4.11.18	2446177-2446929	250	Methionine aminopeptidase (1-250)	Type I methionyl aminopeptidase	100%	100%
PepP	3.4.11.9	1026611-1027669	352	PepP - Xaa-Pro aminopeptidase (1-352)	Aminopeptidase P family protein	100%	100%
PepP	3.4.11.9	1950805-1951857	350	PepP - Xaa-Pro aminopeptidase (2-350)	Aminopeptidase P family protein	100%	100%
PepT	3.4.11.4	186173-187405	410	Peptidase T (2-402)	Peptidase T	100%	100%
Pyroglutamyl-peptidase	3.4.19.3	480016-480657	213	Pcp - Pyrrolidone-carboxylate peptidase (1-206)	Pyrrolidone-carboxylate peptidase	100%	100%
Glutamyl aminopeptidases	3.4.11.7	512089-513165	358	Glutamyl aminopeptidase (8-356)	M42 family peptidase	100%	100%
Glutamyl aminopeptidases	3.4.11.7	864836-865873	345	M42 glutamyl aminopeptidase (46-334)	Aminopeptidase	100%	100%
Glutamyl aminopeptidases	3.4.11.7	1999046-2000122	358	M42 glutamyl aminopeptidase (49-339)	M42 family peptidase	100%	100%
Aminotransferase (AT)							
Histidinol-phosphate aminotransferase	2.6.1.9	209075-210130	351	Histidinol-phosphate aminotransferase (1-349)	Histidinol-phosphate aminotransferase	100%	100%
Branched-chain amino acid aminotransferase	2.6.1.42	369574-370650	358	Branched-chain amino acid aminotransferase (1-356)	Branched-chain amino acid aminotransferase	100%	100%

4-aminobutyrate aminotransferase	2.6.1.19	601413- 602750	445	4-Aminobutyrate aminotransferase (1-445)	Aspartate aminotransferase family protein	100%	100%
Glutamate dehydrogenase (GDH)							
NAD-specific GDH	1.4.1.2	17836- 19080	414	Glutamate dehydrogenase/leucine dehydrogenase (6-413)	Glu/Leu/Phe/Val dehydrogenase	100%	100%
NAD-specific GDH	1.4.1.2	381290- 382534	414	Glutamate dehydrogenase/leucine dehydrogenase (6-413)	Glu/Leu/Phe/Val dehydrogenase	100%	100%
Lyase							
cystationie β -lyase	4.4.1.8	703070- 704215	381	Cystathionine beta-lyase (1-378)	Cystathionine gamma-synthase	100%	100%
cystationie β -lyase	4.4.1.8	1548097- 1549272	391	Cystathionine beta-lyase (1-374)	PLP-dependent transferase	100%	100%
Lipase and esterase							
Triacylglycerol esterase/lipase	3.1.1.3	452825- 453700	291	Triacylglycerol esterase/lipase EstA (4-280)	Triacylglycerol lipase	100%	100%
Triacylglycerol esterase/lipase	3.1.1.3	1445522- 1446325	267	Triacylglycerol esterase/lipase EstA (1-261)	Truncated lipase precursor	100%	99%
Triacylglycerol esterase/lipase	3.1.1.3	2336285- 2338546	753	Triacylglycerol esterase/lipase EstA (376- 753)	Lipase	100%	100%
Carboxylesterase	3.1.1.1	141578- 142318	246	Esterase/lipase (1-241)	Carboxylesterase	100%	100%
Carboxylesterase	3.1.1.1	280742- 281773	343	Acetyl esterase/lipase (65-320)	Alpha/beta hydrolase	100%	100%
Carboxylesterase	3.1.1.1	495623- 496345	240	Putative esterase (47-236)	Tributyryn esterase	100%	100%
Carboxylesterase	3.1.1.1	559524- 560126	200	Predicted esterase (1-197)	Carboxylesterase	100%	100%
Carboxylesterase	3.1.1.1	611875- 612849	324	Acetyl esterase/lipase (88-324)	Alpha/beta hydrolase	100%	100%
Carboxylesterase	3.1.1.1	636868- 637560	230	Putative esterase (13-225)	Alpha/beta hydrolase	100%	100%
Carboxylesterase	3.1.1.1	1451623- 1452360	245	Esterase/lipase (4-244)	Carboxylesterase	100%	100%
Carboxylesterase	3.1.1.1	1843057- 1843959	300	Acetyl esterase/lipase (54-300)	Alpha/beta hydrolase	100%	100%
Carboxylesterase	3.1.1.1	2197243- 2197989	248	Putative esterase (16-241)	Esterase family protein	100%	100%

4.5. Discussion

CNSs are commonly detected at high numbers in fermented foods, such as cheese and meat, and include species such as *S. saprophyticus*, *S. equorum*, *S. hominis*, *S. epidermidis* and *S. capitis* (Coton et al., 2010; Rea et al., 2007). While they are routinely used as adjunct cultures they can also arise from the starting food material, equipment or the environment during manufacture and ripening (Coton et al., 2010).

S. saprophyticus is a ubiquitous species, commonly detected on the surface of various types of cheese (Cogan et al., 2014; Larpin et al., 2011). *S. saprophyticus* DPC5671 was originally isolated from the surface of a commercial Cheddar cheese and used in cheese trials in conjunction with *Debaryomyces hansenii*, becoming well established on the cheese surface during ripening (35 days), and modifying the flavour and aroma profiles of the initial cheese curd (Bertuzzi et al., 2017). Mounier *et al.* (2006) observed that *S. saprophyticus* inoculated with *D. hansenii* in aseptic cheese curd, was able to reach high numbers ($\sim 10 \log \text{CFU g}^{-1}$) after 20 days. However, in a complex environment such as the cheese surface, the growth of *Staphylococci*, including *S. saprophyticus*, is probably limited, in the later stage of ripening, by competition for the nutrients with the other microbial components (Mounier et al., 2008).

Considering its presence in numerous fermented foods, it is clear that the role of *S. saprophyticus* in flavour development requires special attention, and for this reason its metabolic activity has been studied in the past to understand the pathways which lead to the biosynthesis of volatile flavour compounds. Within the genome of *S. saprophyticus*

DPC5671, CDSs encoding for CEP (cell envelope proteinase) were not detected, which is the specific caseinolytic proteinase, commonly found within the genome of *Lactobacillus sp.* and *Streptococcus sp.* (Stefanovic et al., 2017). However, CDSs were detected within the genome, encoding for subunits of a HSP (heat shock protein) Clp ATPase, that is active also on albumin and casein. Casaburi *et al.* (2006) observed that strains of *S. saprophyticus* displayed peptidase activities on different substrates, especially containing L-methionine and L-valine, and esterase activities on butyric (C4:0) and caprylic acid (C8:0). In a previous study, *S. saprophyticus* DPC5671 showed the highest activity, among the smear bacteria tested, for the hydrolysis of tributyrin (Chapter 2). This was in agreement with the CDSs encoding for peptidases and esterases/lipases found within the genome of *S. saprophyticus* DPC5671.

The presence of numerous CDSs associated with lipase/esterase was consistent also with the study on the esterolytic activity by Talon and Montel (1997), who observed high levels of hydrolysis for *S. saprophyticus* strains on butyric acid, and tributyrin. In addition, *S. saprophyticus* strains have shown the ability to esterify ethanol with short/medium FFAs to produce ethyl esters in aqueous media, to further highlight the involvement of esterases (Talon et al., 1998).

The genome of *S. saprophyticus* DPC5671 was characterized also by the presence of CDSs, encoding for GDH, and AT, which are two enzymatic classes important for the triggering of FAAs catabolism. ATs are the responsible enzymes for the removal of the amino group from FAAs to 2-oxoglutarate, producing the relative α -keto acid and L-glutamate; while GDH produces 2-oxoglutarate from L-glutamate,

which is the substrate necessary for AT activity (Ardö, 2006). Other CDSs encoding for enzymes related to FAAs degradation, were observed within *S. saprophyticus* DPC5671 genome, such as cystathionine β -lyase, which might be involved in the elimination of methionine, with the production of sulphur compounds (Yvon and Rijnen, 2001).

Although CNSs are particularly valued for their role in flavour development in fermented food, the fact that they have been associated with nosocomial infections and UTI has raised concerns about the safe use of CNSs in foods (Kuroda et al., 2005). In addition, Irlinger (2008) suggested that some CNSs could now emerge as exceptional opportunistic pathogens. The characteristics discussed by Irlinger (2008) as safety risks included virulence factors, such as enterotoxins (Podkowik et al., 2013; Vernozy-Rozand et al., 1996) and antibiotic resistance (Silva et al., 2014). The results presented here have clearly shown that *S. saprophyticus* DPC5671 was coagulase negative, non-toxigenic and non-haemolytic, and its antibiotic resistance profiles were below the MIC cut offs for the panel of antibiotics published by EFSA for Gram-positive organisms other than lactic acid bacteria. This was confirmed with the genomic analysis where no predicted protein-coding sequences were detected for enterotoxin production, or indeed for transferable antibiotic resistance using the methods described in this study. Genomic analysis also did not show the presence of decarboxylases genes, responsible for the development of biogenic amines in fermented food within the genome.

While *S. saprophyticus* DPC5671 did contain a protein-coding sequence with high similarities to a specific adhesin *uafA* gene, associated in some cases of UTI, there are no reported cases linking *S. saprophyticus* to food poisoning or human pathology

following ingestion of dairy, or meat products (Coton et al., 2010; Irlinger, 2008). However, new studies are needed to investigate the role of *S. saprophyticus* and CNSs in nosocomial infections, especially in immune-compromised hosts (Becker et al., 2014).

4.6. Conclusion

It is clear that *S. saprophyticus* species, which is a ubiquitous component of surface ripened cheese, is involved in the process of flavour development during cheese ripening. In this study, *S. saprophyticus* DPC5671 did not show any involvement in enterotoxin and antibiotic resistance, thus this strain has to be considered safe for the food use. It is possible that *S. saprophyticus* could be involved in UTI, even if its infection mechanism is most likely not related to fermented food consumption. Although, more research studies are needed to clarify its mechanisms of pathogenicity, it is clear that *S. saprophyticus* DPC5671 contributes to the biosynthesis of flavour volatile compounds, considering its genes content is characterized by CDSs encoding for putative proteinases, peptidases, AT, GDH, and esterase/lipase. It is believed that the availability of the genome sequence of DPC5671 will allow for further investigations regarding its growth on the cheese surface and its role in flavour development in cheese ripening.

4.7. Acknowledgements

Thanks to Dr Fiona Crispie for 454 DNA sequencing of *S. saprophyticus* DPC5671, and Dr Caitriona Guinane for initial DNA quality analysis and contig assembly.

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Chapter 5

Omics-based insights into flavour development and microbial succession
within surface-ripened cheese

This chapter has been published in *mSystems* (2018), 3, e00211-17. DOI:
10.1128/mSystems.00211-17

In this chapter Aaron Walsh performed the metagenomic analysis, and Dr Fiona Crispie performed whole-shotgun metagenome sequencing.

5.1. Abstract

In this study, a young Cheddar curd was used to produce two types of surface-ripened cheese, using two commercial smear-culture mixes of yeasts and bacteria. Whole-metagenome shotgun sequencing was used to screen the microbial population within the smear-culture mix, and on the cheese surface, comparing microorganisms both at species and strain level. The use of two smear mixes resulted in the development of distinct microbiota on the surface of the two test cheeses. In one case, most of the species inoculated on the cheese established themselves successfully on the surface during ripening; while in the other, some of species inoculated were not detected during ripening and the most dominant bacterial species, *Glutamicibacter arilaitensis*, was not a constituent of the culture mix. Generally, yeast species, such as *Debaryomyces hansenii* and *Geotrichum candidum*, were dominant during the first stage of ripening, but were overtaken by bacterial species, such as *Brevibacterium linens* and *G. arilaitensis*, in the later stages. Using correlation analysis, it was possible to associate individual microorganisms with volatile compounds detected by GCMS in the cheese surface. Specifically, *D. hansenii* correlated with the production of alcohols and carboxylic acids, *G. arilaitensis* with alcohols, carboxylic acids and ketones and *B. linens* and *G. candidum* with sulphur compounds. In addition, metagenomic sequencing was used to analyse the metabolic potential of the microbial population on the surface of the test cheeses, revealing a high relative abundance of metagenomic clusters associated with the modification of colour, variation of pH and flavour development.

5.2. Introduction

Recent studies, involving both metabolomic and metagenomic analysis, have begun to address the relationship between the microbiota and biochemical pathways, that take place during the fermentation process (Dugat-Bony et al., 2015; Walsh et al., 2016; Wolfe et al., 2014; Wolfe and Dutton, 2015). It is clear that, in fermented food, the metabolic interactions, which regulate the composition of the microbial population, influence the taste, shelf life and safety of the subsequent product (Montel et al., 2014). The ability to manipulate the microbiota of fermented food represents an important avenue for the food industry to develop new food products with precise characteristics.

Surface-ripened cheese (e.g., Münster, Tilsit, Livarot, Limburger and Comté) is characterized by the growth of a heterogeneous microbiota on the cheese surface, with the consequent development of a strong flavour. The flavour and the appearance of these types of cheese are related to the metabolic activities of bacteria and yeasts, which comprise the smear consortium. Generally, the cheese is brined or surface-salted, which also influences the growth of surface microbiota. In some traditional procedures, young cheese is smeared by transferring the smear from older cheese to younger curd (“old-young” technique) (Desmasures et al., 2015; Mounier et al., 2017). However, today, commercial mixtures of smear bacteria and yeasts are more commonly used to produce a more standardized product.

So far, metagenomic sequencing represented a valid method to investigate the microbial population on the exterior of the surface-ripened cheese (Bokulich and Mills,

2013; Delcenserie et al., 2014; Quigley et al., 2012; Wolfe et al., 2014) In studies of complex microbial communities in fermented foods, such as kefir, the information gained through whole-metagenome shotgun sequencing allowed the variations of the microbial population, and also the metabolic pathways involved in the fermentation process, to be monitored (Walsh et al., 2016).

The aim of the current study was to investigate, at both the species and strain level, the succession of the microbial populations present on the rind of a surface-ripened cheese, produced with young Cheddar cheese curd as a base, using two different commercial smear-culture mixes. Studies were performed, over the course of 30 days of ripening, to correlate volatile analysis with data generated through whole-metagenome shotgun sequencing, in order to understand how microbial composition related to flavour development. Moreover, metagenomic analysis allowed for the screening of metagenomic clusters during cheese ripening, showing the involvement of the surface microbiota in a variety of biochemical processes.

5.3. Materials and methods

5.3.1. Smearing of cheese blocks

A block of commercial Cheddar cheese, < 24 hours after manufacture, was aseptically cut into smaller blocks ($\sim 8 \times 6.5 \times 30$ cm), and washed with smearing solutions, as described in our previous study (Bertuzzi et al., 2017). Two commercial smear-culture mixes comprising of *Geotrichum candidum*, *Debaryomyces hansenii*, *Brevibacterium linens*, *Glutamicibacter arilaitensis* (previously classified as *Arthrobacter arilaitensis*) and *Staphylococcus xylosus* (S5 mix) (Sacco, Cadorago, Italy) and *D. hansenii*, *Cyberlindnera jadinii*, *Brevibacterium casei* and *B. linens* (D4 mix) (DuPont™ Danisco®, Beaminster, Dorset, UK), were used to inoculate the surface of the cheese curd. D4 mix was a liquid product which was provided ready-to-use, while S5 mix was a powder which was rehydrated (5 g L^{-1}) in NaCl solution (0.75%). The blocks of cheese were washed with the smearing solutions and placed in sterile racks inside a sterile plastic bags (Südpack Verpackungen, Ochsenhausen, Germany), as previously described (Bertuzzi et al., 2017). The cheese was ripened for 30 days at 15°C, with a relative humidity of $\sim 97\%$. At days 7, 10 and 15 of ripening, the cheese blocks were brushed with a sterile sponge, soaked in a sterile brine solution (5% NaCl), to uniformly spread the smear microbiota on the cheese surface. As a control, un-smear cheese blocks were vacuum-packed in sterile bags and incubated at 15°C, similarly to the test cheeses.

5.3.2. Sampling cheese

Three replicate cheese trials were performed at different times during Cheddar cheese making season. All data presented are the results of the analysis performed on samples taken from the cheese surface (at a depth of $\sim 0.5\text{cm}$). All analyses were performed in triplicate.

5.3.3. Compositional analysis and pH

The moisture and total fat content was calculated by using Fast trac II (CEM, Dublin, Ireland). The salt content was analysed by a potentiometric method (International Dairy Federation, 1988). Standard methods were used to determine total protein (International Dairy Federation, 1993, 1996). pH level was measured on day 0, 18, 24 and 30 using a standard pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland), while the other compositional parameters were measured only at the end of the ripening (day 30). The data were analysed by one-way analysis of variance (ANOVA) using SAS 9.3.

5.3.4. Determination of colour

At day 0, 18, 24 and 30 of ripening the colour was measured on the cheese surface at room temperature, using a Minolta Colorimeter CR-300 (Minolta Camera, Osaka, Japan). The instrument was calibrated on white tile and the colour of the cheese

surface was measured using L*, a* and b*-values. L*-value measures the visual lightness (as values increase from 0 to 100), a*-value measures from the redness to greenness (positive to negative values, respectively) and b*-value from the yellowness to blueness (positive to negative values, respectively).

5.3.5. Total DNA extraction from cheese surface

The total DNA was extracted from the smear culture mixes and the cheese samples using the PowerSoil DNA Isolation kit as described in the manufacturer's protocol (Cambio, Cambridge, United Kingdom). For the DNA extraction from the cheese surface, at day 0, 18, 24 and 30, a pre-treatment step was included as follows: samples were removed from different parts of the cheese block and pooled to give a representative sample of 5 g. The cheese was placed in a stomacher bag with 50 mL of 2% trisodium citrate and homogenized using a masticator mixer (IUL S.A., Barcellona, Spain) for 5 min.

Fifteen mL of the smear-culture mix, or the cheese solution, were placed into sterile falcon tubes and centrifuged for 30 min at 4,500×g. After centrifugation, the supernatant was discarded and the pellet was placed in a 2mL eppendorf tube. The pellet was washed several times with sterile phosphate buffered saline (PBS) by centrifuging at 14,500×g for 1 min, until the supernatant was completely clear. The pellet was then added to PowerBead tubes (Cambio, Cambridge, United Kingdom) provided with the kit as described in the protocol and homogenized by shaking on the TissueLyser II (Qiagen, West Sussex, United Kingdom) at 20 Hz for 10 min. The DNA was then purified

according to the protocol of the standard PowerSoil DNA Isolation kit (Cambio, Cambridge, United Kingdom).

Total DNA was initially qualified and quantified by gel electrophoresis and the NanoDrop 1000 (BioSciences, Dublin, Ireland) before more accurate quantification with Qubit High Sensitivity DNA assay (BioSciences, Dublin, Ireland).

5.3.6. Whole-metagenome shotgun sequencing

Whole-metagenome shotgun libraries were prepared in accordance with the Nextera XT DNA Library Preparation Guide from Illumina (Clooney et al., 2016). Samples were sequenced on the Illumina MiSeq sequencing platform in the Teagasc sequencing facility, with a 2300 cycle V3 kit, in accordance with standard Illumina sequencing protocols.

5.3.7. Bioinformatic analysis

Raw whole-metagenome shotgun sequencing reads were processed, on the basis of quality and quantity, using a combination of Picard Tools (<https://github.com/broadinstitute/picard>) and SAMtools (Li et al., 2009). Kaiju (Menzel et al., 2016) was used to determine the species-level microbial compositional of samples. SUPER-FOCUS (Silva et al., 2016) was used to characterize the microbial metabolic potential of samples.

5.3.8. Accession number

Sequence data have been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB15423.

5.3.9. Proteolysis

Proteolysis levels at day 0, 18, 24 and 30 of ripening were determined by the ratio between non-casein nitrogen content, soluble at pH 4.6 (pH4.6-SN) and total nitrogen (TN). The pH 4.6-SN fraction was extracted as described by Fenelon and Guinee (2000). The Macro-Kjeldahl method was used to determine the nitrogen content in pH4.6-SN and TN (International Dairy Federation, 1993). The levels of proteolysis were expressed as a percentage of the ratio (% pH4.6-SN/TN).

5.3.10. Free amino acids analysis

FAAs analysis was performed at the end of the ripening (day 30) on the soluble N extracts using a Jeol JLC-500V AA analyser fitted with a Jeol Na⁺ high performance cation exchange column (Jeol Ltd., Garden city, Herts, UK) (McDermott et al., 2016). The chromatographic analyses were conducted at pH 2.2. Results were expressed as $\mu\text{g mg}^{-1}$ of cheese.

5.3.11. Free fatty acids analysis

FFAs extracts were performed at the end of the ripening (day 30) according to the method outlined by De Jong and Badings (1990). The FFAs extracts were derivitized

as methyl esters as described by Mannion *et al.* (2016). Fatty acid methyl esters extracts were analysed using Varian CP3800 gas chromatograph (Aquilant, Dublin 22, Ireland) with a CP84000 auto-sampler and flame ionization detector and a Varian 1079 injector (Aquilant, Dublin 22, Ireland). Results were expressed as $\mu\text{g mg}^{-1}$ of cheese.

5.3.12. Volatile analysis

The volatile compounds were analysed at days 0, 18, 24 and 30. The surface of the cheese was removed, wrapped in foil and stored vacuum-packed at -20°C until analysis. Before analysis the samples were defrosted, grated and 4 g of cheese surface were used. Analysis was carried out as outlined by Bertuzzi *et al.* (2017) (see “Volatile analysis” p 138-139).

5.3.13. Statistical analysis

Statistical analysis was done with SAS 9.3 and R-3.2.2 (Team, 2014). The R packages ggplot2 and pheatmap were used for data visualization. The vegan package was used to calculate the Bray-Curtis dissimilarity between samples, while the Hmisc package was used for correlation analysis.

5.4. Results

5.4.1. Microbial composition of the smear-culture mixes

Two smear-culture mixes D4 and S5 were used for the cheese trials, and contained, as outlined in the supplier specification sheet, *B. linens*, *D. hansenii*, *C. jadinii* and *B. casei*, or *S. xylosus*, *B. linens*, *D. hansenii*, *G. candidum* and *G. arilaitensis*, respectively. Using metagenomic analysis, performed with Kaiju (Menzel et al., 2016), the relative abundances of the individual species within the mixes were determined (Fig. 5.1).

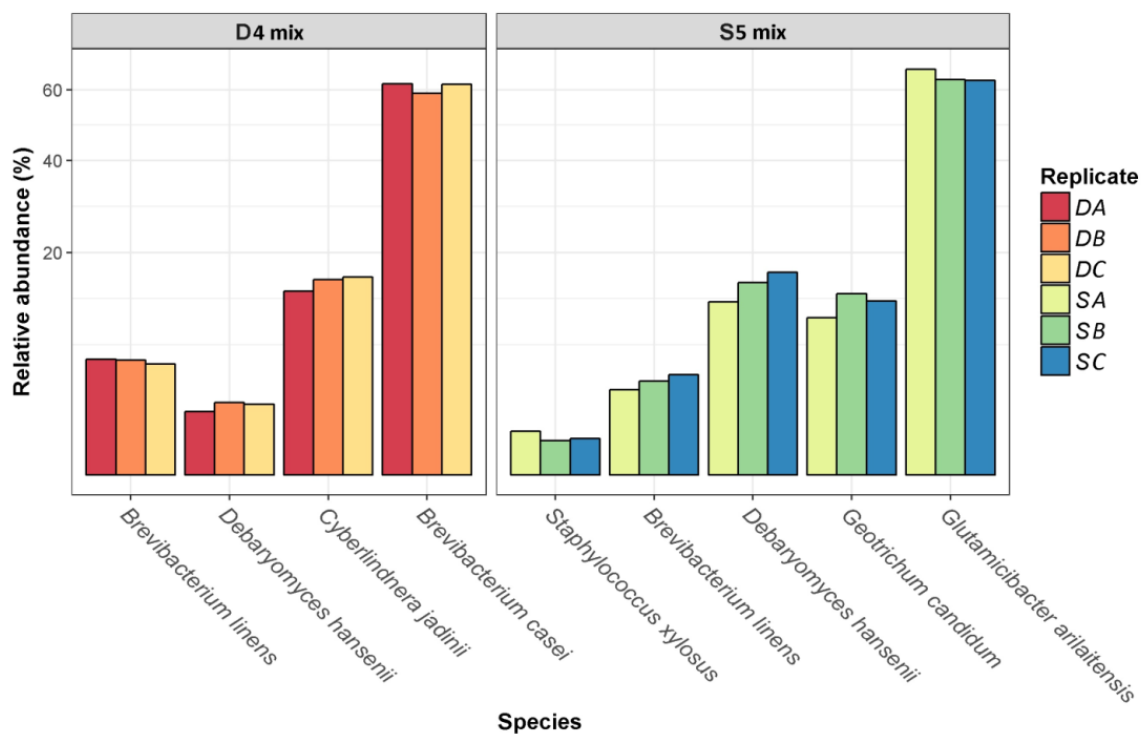


Figure 5.1. Relative abundances of the species (%), which were indicated as being present by the supplier, within the smear-culture mixes D4 and S5 (replicates of three analyses DA, DB, DC, and SA, SB, SC).

B. casei (60.83%) and *C. jadinii* (15%) were the most abundant bacterial and yeasts species in D4, while *B. linens* and *D. hansenii* were minor components in the smear-culture mix with relative abundances of 5.25% and 1.92%, respectively (Fig. 5.1; Table 5.1). In the S5 mix, *G. arilaitensis* (64.25%) together with *D. hansenii* (14.56%) and *G. candidum* (11.83%) were the most abundant bacterial and yeasts; *S. xylosus* (0.59%) and *B. linens* (3.52%) were present at lower relative abundances. Other species, not specified by the suppliers, were identified at low relative abundance in the smear-culture mixes D4 and S5, and are reported in Table 5.1.

Table 5.1. Relative abundance (%) of the microbial species within D4 and S5 mix. Data are the mean of three replicates. Species highlighted in bold were stated as present by the culture provider.

Species in the smear-culture mixes (%)	D4 mix	S5 mix
<i>Brevibacterium casei</i>	61.09	- nd
<i>Brevibacterium linens</i>	5.26	3.53
<i>Glutamicibacter arilaitensis</i>	-	64.03
<i>Staphylococcus xylosus</i>	-	0.57
<i>Cyberlindnera jadinii</i>	14.84	-
<i>Debaryomyces hansenii</i>	1.88	14.66
<i>Geotrichum candidum</i>	-	12.12
<i>Brevibacterium</i> sp. VCM10	12.82	
<i>Brevibacterium siliguriense</i>	1.41	-
<i>Brevibacterium epidermidis</i>	1.10	-
<i>Brevibacterium sandarakinum</i>	0.59	-
<i>Arthrobacter</i> sp. NIO-1057	-	1.27
<i>Debaryomyces fabryi</i>	-	1.07
<i>Arthrobacter</i> sp. W1	-	0.38
<i>Glutamicibacter mysorens</i>	-	0.24
<i>Arthrobacter</i> sp. EpRS66	-	0.16
<i>Paeniglutamicibacter antarcticus</i>	-	0.14
<i>Others</i>	1.00	1.81

ndNot detected

5.4.2. Microbial composition of the cheese surface

Two test cheeses, D4 and S5, were prepared by smearing young Cheddar cheese curd with the two aforementioned commercial smear-culture mixes and ripened for 30 days at 15°C. Kaiju was used to determine the bacterial and yeast composition of the cheese surface at day 0, 18, 24 and 30, for both the control cheese (un-smeared and ripened under vacuum) and the two test cheeses (Menzel et al., 2016). Compositional data of the cheese surface were analysed by a one-way analysis of variance (ANOVA), designed with SAS 9.3 to determine the significant differences in the proportions of the individual species present over time. The metagenomic sequences of the bacteria used as starter cultures in the Cheddar cheese curd (*L. lactis* and *S. thermophilus*), and as smearing cultures (*B. linens*, *S. xylosus*, and *G. arilaitensis*), were compared at strain level, using PanPhlAn, to determine the presence/absence of the inoculated bacterial strains on the cheese throughout ripening (Scholz et al., 2016).

As expected, lactic acid bacteria dominated the surface of all samples at day 0, and their relative abundance on the surface of the control did not significantly change throughout the 30 days of ripening (Fig. 5.2; Table 5.2).

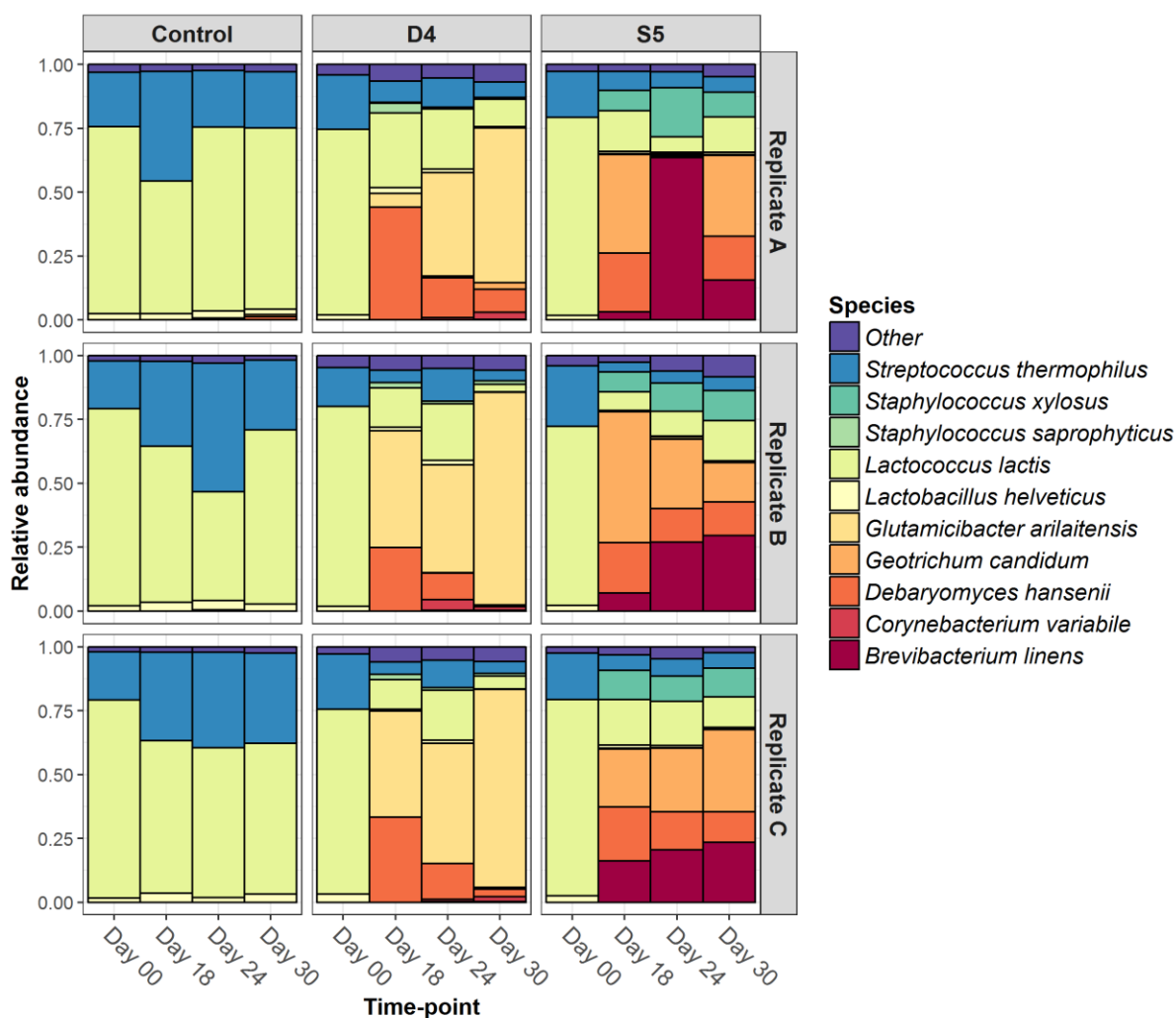
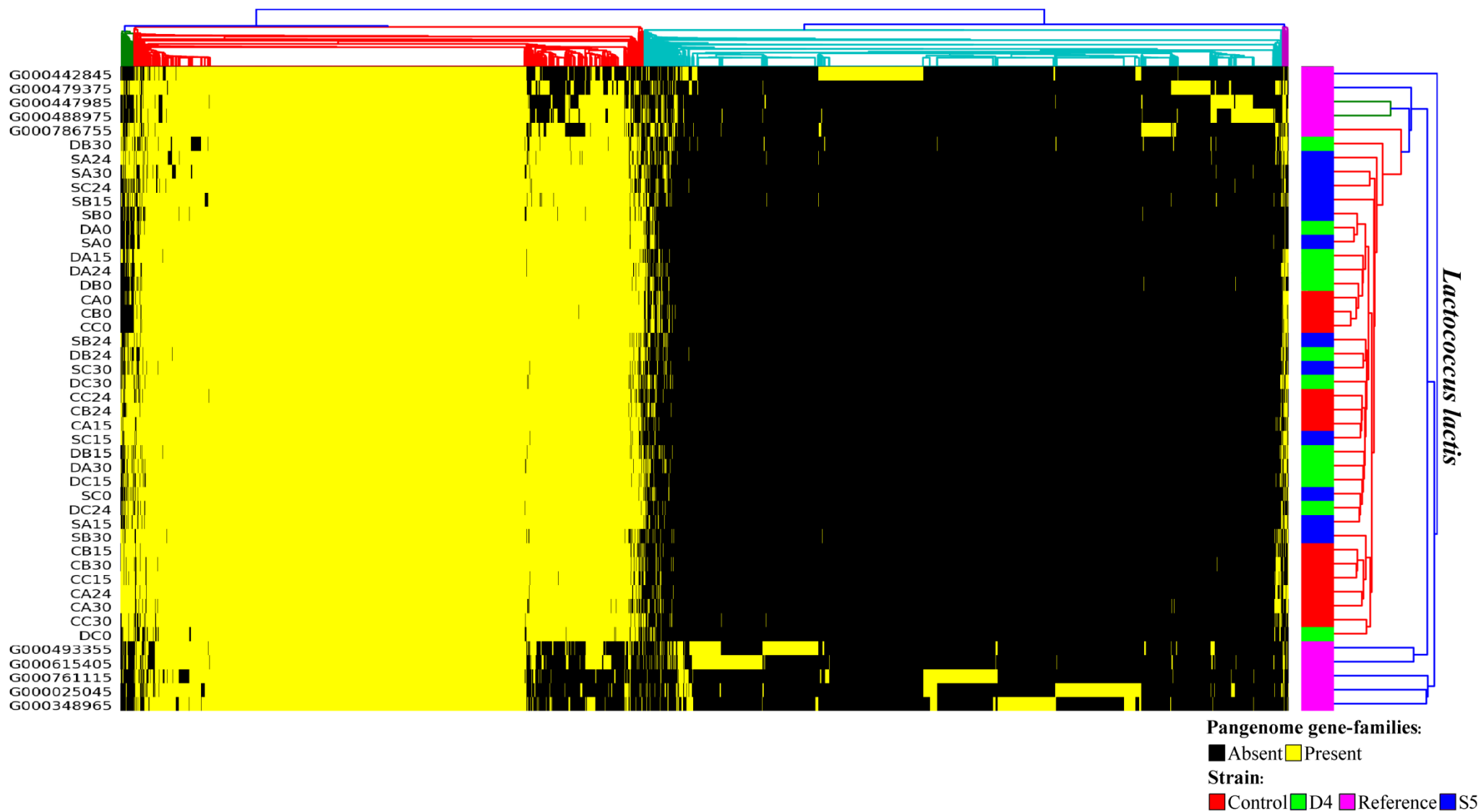
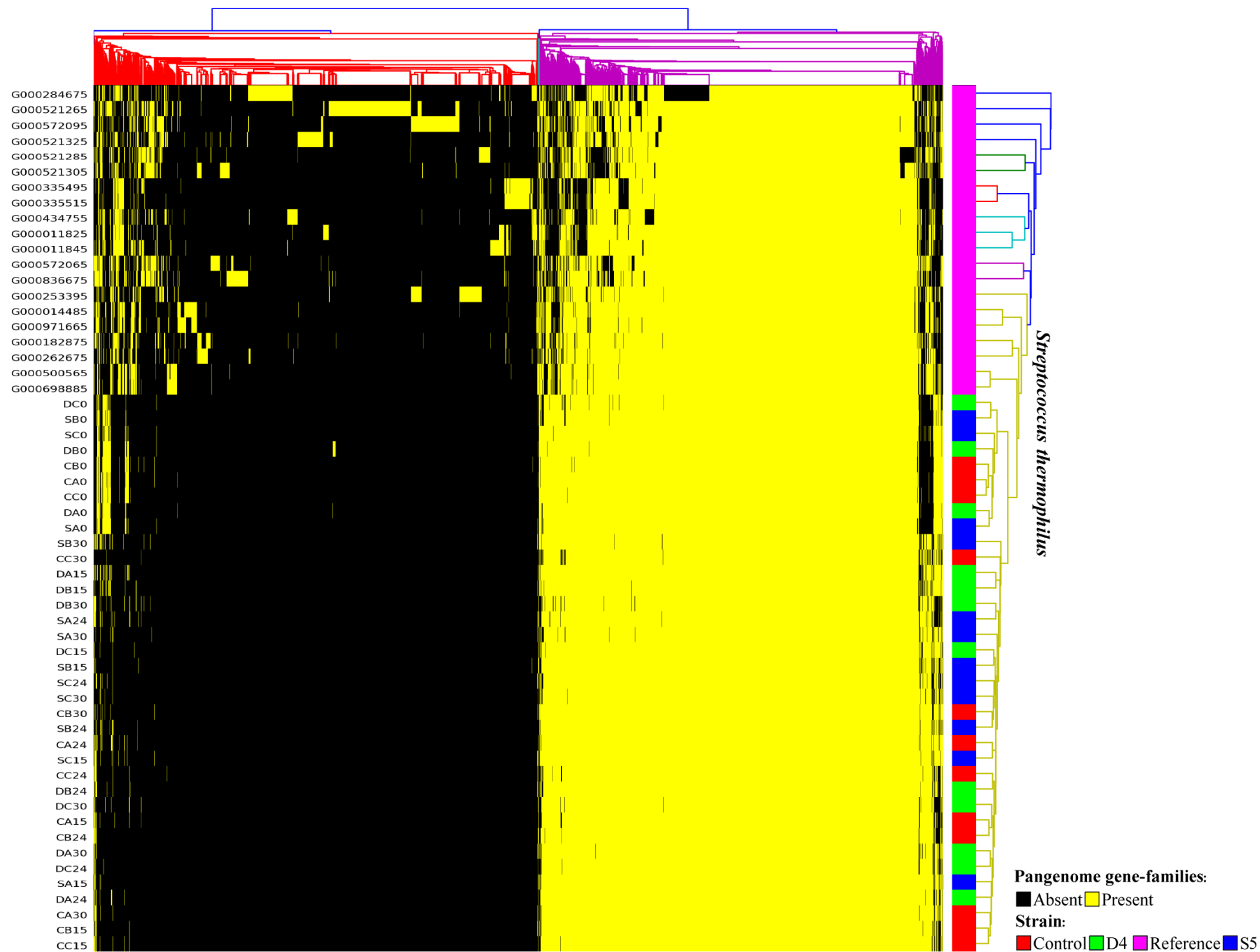
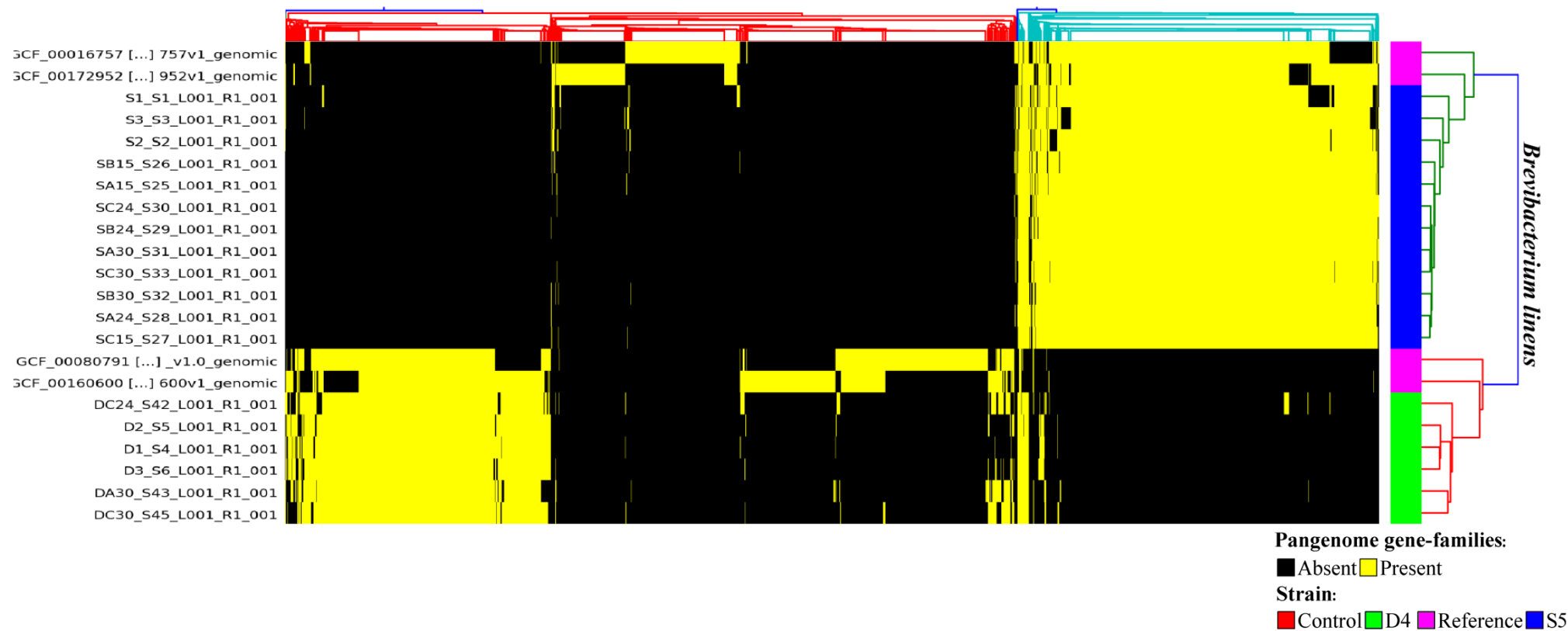


Figure 5.2. Relative abundance at the species-level of the microbiota on the cheese surface of control, D4 and S5 at day 0, 18, 24 and 30. Data shown for the three replicate trials (A, B and C).

L. lactis and *S. thermophilus* were identified in all samples analysed (D4, S5 and control) (Fig. 5.3; Fig. 5.4). *L. lactis* was the dominant species in the control, constituting 75.85% of the initial population at day 0, decreasing to 65.99% at day 30. *S. thermophilus* increased from 19.65% at day 0 to 28.21% at day 30, while the relative abundance of *Lb. helveticus* was low throughout the ripening period (2.12% at day 0, and 2.72% at day 30) (Table 5.2).









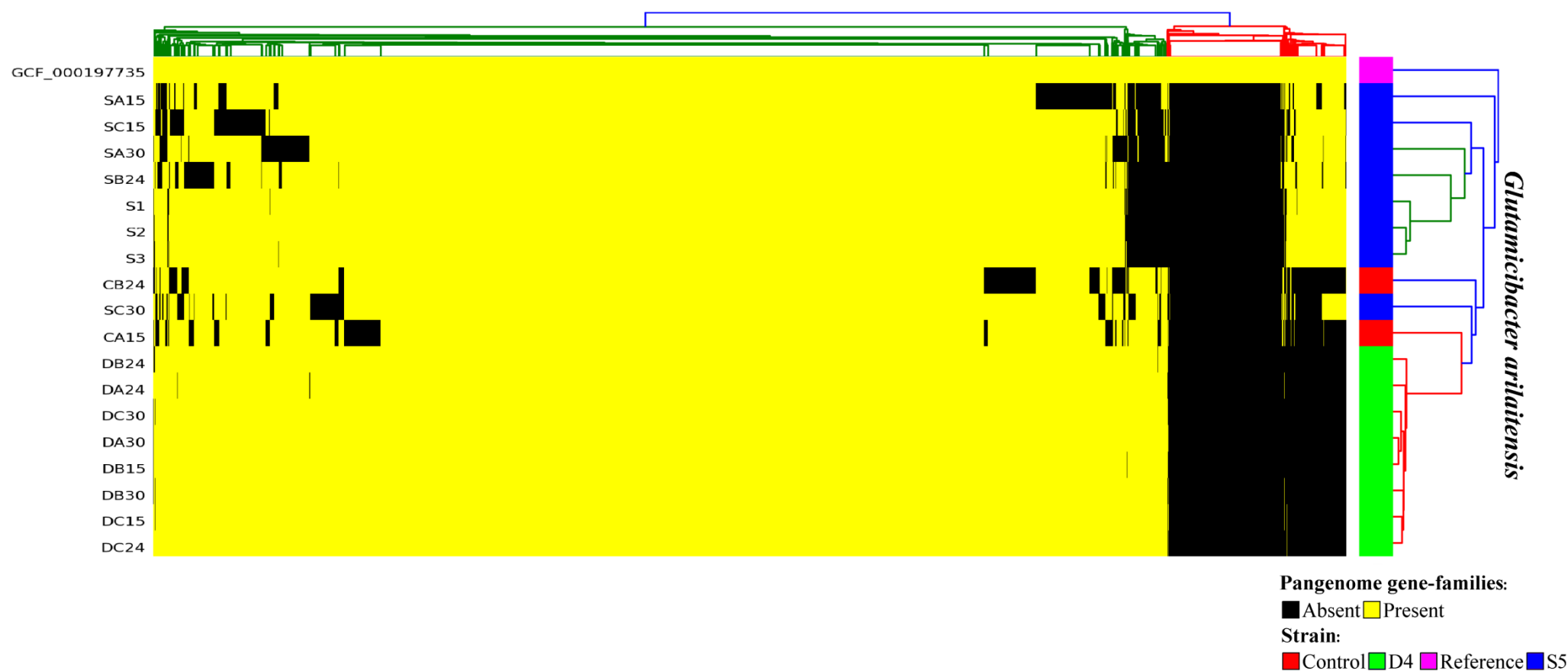


Figure 5.3. Heatmaps showing the relatedness of strains detected in cheese samples to reference strains, as determined by PanPhlAn, based on the presence/absence of pangenome gene-families in their respective genomes. The term “reference” refers to the reference genome sequence of strains present in NCBI databases, and highly similar to the genome sequences of the strains identified in the smear mixes, or on the cheese surface.

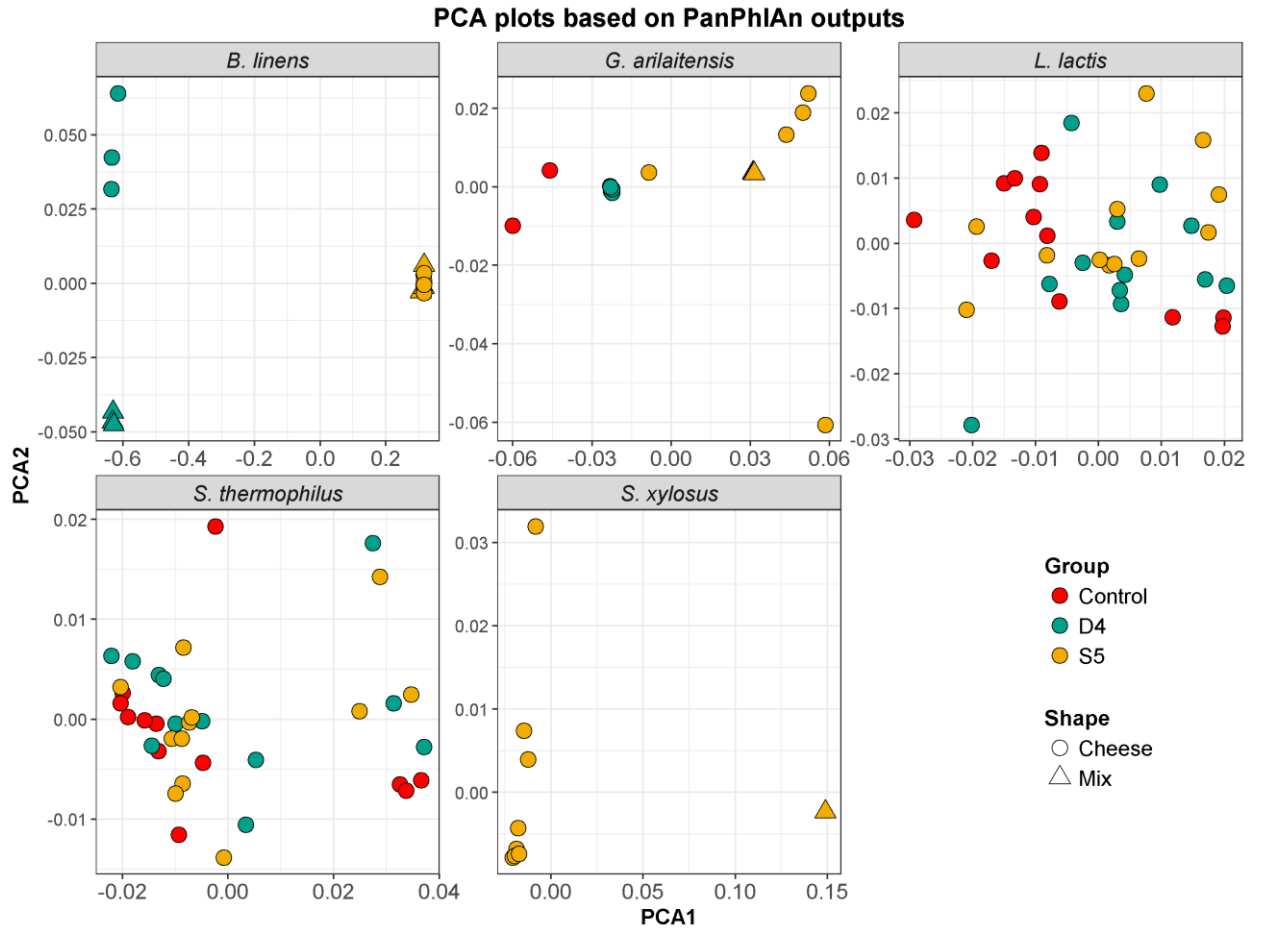


Figure 5.4. Principal component analysis (PCA) plot of the profiles of the strains determined by PanPhlAn.

However, over the course of 30 days of ripening, the smearing processes clearly influenced the microbial population of the cheese surface of both test cheeses, D4 and S5, causing a significant reduction in the relative abundance of *Lb. helveticus* ($P < 0.03$) and *L. lactis* ($P < 0.0001$). At day 18, the microbial populations of the two test cheeses were dissimilar from the control cheese and also from each other, clustering in three distinct groups (Fig. 5.5). As expected, the microbiota of the control cheese clustered

together throughout the 30 days of ripening (Fig. 5.5). From day 0 to day 18, the population on the surface of D4 changed from predominately LAB to *Debaryomyces hansenii* and *Glutamicibacter arilaitensis* (Fig. 5.2).

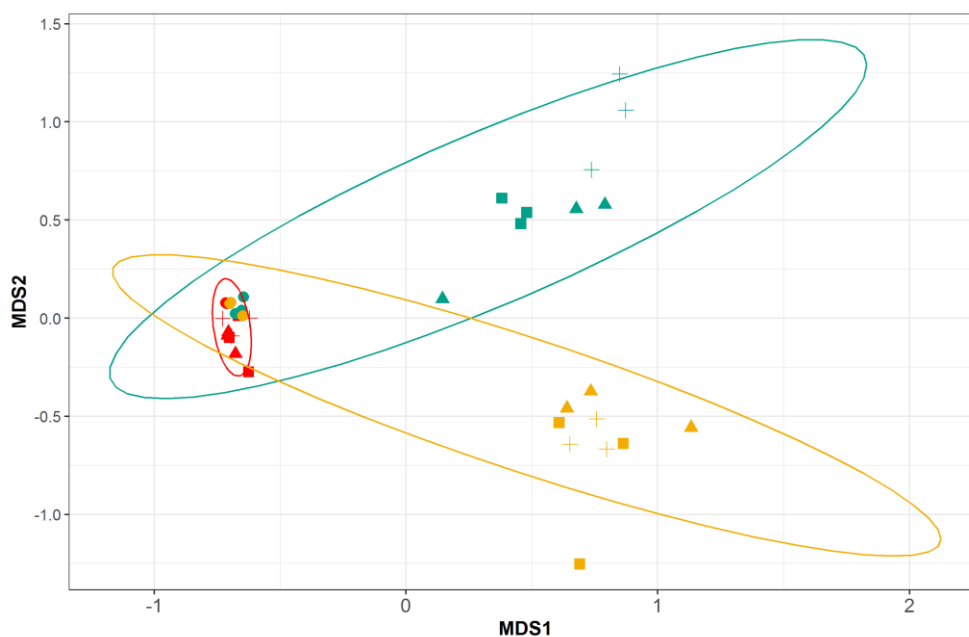


Figure 5.5. Bray-Curtis MDS plot based on the different microbial populations of the cheese surface of control (red), D4 (green) and S5 (yellow), at day 0 (○), 18 (△), 24 (□) and 30 (+), resulting from the metagenomic analysis performed with Kaiju.

Table 5.2. Relative abundance of the microbial species on the cheese surface of control, D4 and S5 at day 0, 18, 24 and 30. Data are the mean of three replicates.

Species	Control				D4				S5			
	0	18	24	30	0*	18	24	30	0*	18	24	30
<i>Lactococcus lactis</i>	75.8 5	57.5 8	57.7 6	65.9 9	74.3 5	18.7	21.7 2	6.18	74.7 4	13.5 9	11.0 2	13.9 4
<i>Streptococcus thermophilus</i>	19.6 5	36.9 3	36.5 3	28.2 1	19.4	6.03	11.6 6	4.98	20.0 4	5.79	5.91	5.82
<i>Glutamicibacter arilaitensis</i>	- nd	-	0.24	-	-	30.9	43.2 7	73.7 5	-	0.26	0.44	0.42
<i>Debaryomyces hansenii</i>	-	-	0.07	0.45	-	34.1 2	13.2 9	4.14	-	21.2	9.57	14.0 9
<i>Geotrichum candidum</i>	-	-	0.08	0.28	-	-	0.31	1.11	-	37.5 4	17.6	26.4 4
<i>Brevibacterium linens</i>	-	-	-	-	-	-	0.17	0.26	-	8.84	37.0 5	22.8 4
<i>Staphylococcus xylosus</i>	-	-	-	-	-	0.11	-	-	-	9.08	13.3 6	10.8 3
<i>Lactobacillus helveticus</i>	2.12	3.1	2.78	2.72	2.38	1.39	1.41	0.34	2.19	0.86	0.48	0.46
<i>Acinetobacter baumannii</i>	0.82	0.12	0.17	0.37	0.63	-	-	-	0.95	-	-	0.15
<i>Streptococcus pneumoniae</i>	0.56	0.79	0.74	0.66	0.95	-	0.29	0.06	0.74	0.12	0.06	-
<i>Streptococcus salivarius</i>	0.5	0.93	0.93	0.71	0.52	-	0.3	-	0.5	0.11	0.06	0.05
<i>Arthrobacter sp. NIO-1057</i>	-	-	-	-	-	0.57	0.72	1.29	-	-	-	-
<i>Staphylococcus equorum</i>	-	-	-	-	-	1.32	0.43	0.45	-	-	-	-
<i>Staphylococcus saprophyticus</i>	-	-	-	-	-	2.69	0.93	1.06	-	-	-	-
<i>Penicillium camemberti</i>	-	-	-	-	-	0.37	0.4	0.63	-	-	-	-
<i>Corynebacterium variabile</i>	-	-	-	-	-	-	2.04	2.08	-	-	-	-
<i>Debaryomyces fabryi</i>	-	-	-	-	-	1.47	0.56	0.13	-	1.64	0.71	1.09
<i>Psychrobacter sp. P11F6</i>	-	-	-	-	-	-	-	-	-	-	0.4	0.5
<i>Psychrobacter glacincola</i>	-	-	-	-	-	-	-	-	-	-	0.83	1.13
<i>Psychrobacter sp. JCM 18903</i>	-	-	-	-	-	-	-	-	-	-	0.52	0.65
<i>Stenotrophomonas maltophilia</i>	-	-	0.2	0.37	-	-	-	-	-	0.17	-	0.18
<i>Brevibacterium sandarakinum</i>	-	-	-	-	-	-	-	-	-	0.2	0.94	0.58
<i>Anaplasma phagocytophilum</i>	0.22	-	-	-	0.74	-	-	-	0.45	-	-	-
<i>Other</i>	0.27	0.54	0.49	0.24	1.04	2.33	2.5	3.56	0.39	0.59	1.03	0.86

*Cheese D4 and S5 at day 0, before the smearing process

nd Not detected

Subsequently, over the course of ripening, the relative abundance of *D. hansenii*, significantly decreased ($P<0.0001$) from 34.12% at day 18 to 4.14% at day 30 (Table 5.2). In parallel, the relative abundance of *G. arilaitensis* significantly increased ($P<0.0001$) from 30.9% at day 18, to become the dominant population on the cheese surface (73.75%) at day 30 (Table 5.2). Using PanPhlAn, it was determined that the strain of *G. arilaitensis* detected on the cheese surface of D4, was different from the *G. arilaitensis* strain used in the smear-culture mix inoculated onto the surface of S5, confirming that the growth of this strain on D4 did not result from cross contamination of the two cheeses during inoculation or ripening (Fig. 5.3; Fig. 5.4). The secondary microbial population (individually between 1% and 3% of the population) of the D4 surface was composed of species not included in the initial smear-culture mix, and included *Arthrobacter* sp., *Corynebacterium variabile*, *Debaryomyces fabryi*, *G. candidum*, *Staphylococcus equorum* and *Staphylococcus saprophyticus* (Table 5.2). In addition, some species present in the initial smear-culture mix (*C. jadinii* and *B. casei*) were not detected during ripening, while the inoculated *B. linens* strain was detected at only at a very low relative abundance on the cheese surface of D4 throughout ripening (Fig 5.2; Table 5.2).

By comparison, the microbiota was more diverse in cheese S5 (Fig 5.2; Table 5.2). On the cheese surface of S5, the relative abundance of the LAB decreased, while *B. linens* increased significantly ($P<0.004$) from day 18 to day 24, reaching 37.05 %, before decreasing, but not significantly, to 22.84% at day 30 (Table 5.2). The strain detected was confirmed by PanPhlAn to be that inoculated within the S5 mix (Fig. 5.3;

Fig. 5.4). The yeasts *D. hansenii* and *G. candidum* (components of the S5 mix) were the most abundant population on the cheese surface at day 18, comprising 21.2% and 37.54% of the microbiota, respectively, but their relative abundance significantly decreased ($P<0.04$) by day 24 to 9.57% and 17.6%, respectively, without showing further significant reductions at day 30 (Table 5.2). *S. xylosus* did not correspond to the strain present in S5 mix (Fig. 5.3; Fig. 5.4), and was detected at 9.08% at day 18, and did not change significantly throughout the ripening period (Table 5.2). In addition, a secondary microbial population, comprising of *D. fabryi* (detected in the S5 mix; Table 5.1) and *Psychrobacter sp* (not detected in the S5 mix; Table 5.1), developed at low relative abundance (1-2%) on the surface of the cheese S5 (Table 5.2) over the course of the ripening period. However, some inoculated species were either not detected (*S. equorum*) at any stage throughout ripening, or detected at very low relative abundance (*G. arilaitensis* ~0.44%) on the cheese surface during ripening (Table 5.2).

5.4.3. Volatile compounds detected on the cheese surface

Headspace solid phase micro-extraction (HS-SPME) gas chromatography-mass spectrometry (GCMS) was used to analyse the development of volatile compounds at day 0, 18, 24 and 30 of ripening, for both control and test cheeses. In total, 53 volatile compounds that could potentially contribute to the flavour development were detected on the cheese surface. These compounds are predicted to arise from a variety of substrates, and consisted of 8 alcohols, 6 aldehydes, 10 carboxylic acids, 10 esters, 13 ketones, 2 S-thioesters and 4 sulphur compounds (i.e. a total of 53 compounds; Fig. 5.6).

As expected, given the microbial diversity on the surface there was a greater variety and intensity of volatile compounds detected compared to the control cheese, in which only 23 of the aforementioned 53 compounds were detected (Fig. 5.6). In all cheeses, all volatiles detected increased throughout the ripening period, apart from 2,3-butanediol, hexanal, heptanal, octanal, nonanal, 2,3-butanedione and dimethylsulphone (Fig. 5.6).

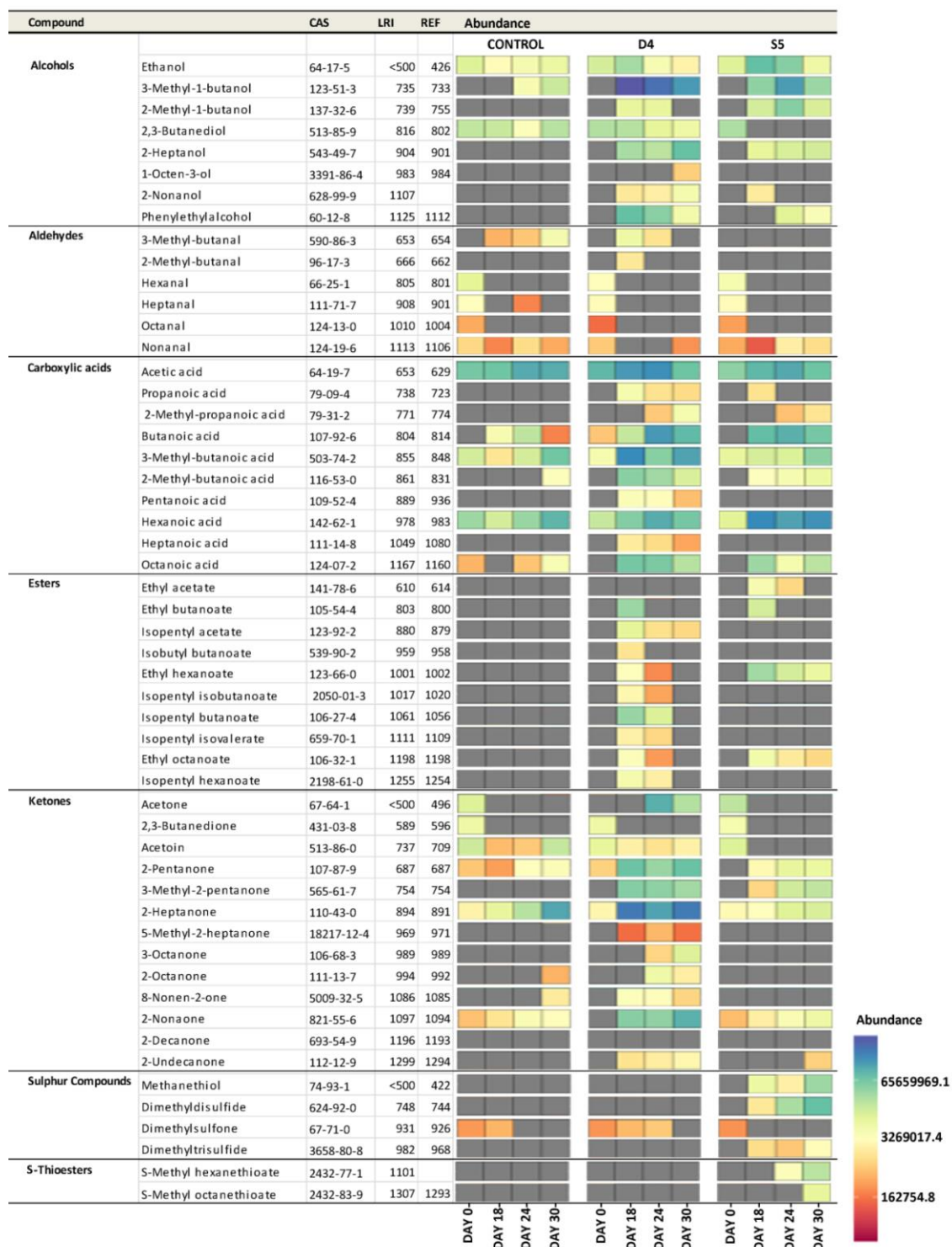


Figure 5.6. Volatile compounds detected in cheese by GCMS and faceted heat map showing the variation of volatile compounds between the cheeses at day 0, 18, 24 and 30. The grey tiles are used when the volatile compounds were not detected. The linear retention index (LRI) was calculated and compared with the reference linear retention index (REF) to confirm the identification. Values are the mean of three replicates.

5.4.4. Correlations between microbial taxa and volatile compounds

The correlation analysis between the relative abundance of microbial species and the abundance of volatile compounds detected on the cheese surface was performed using the Spearman correlation test, as described previously by Walsh *et al.* (2016). From the results of the metagenomic analysis (performed with Kaiju) and the volatile analysis, it was possible to associate both yeasts and bacteria, at species level, with specific volatile compounds. Figure 5.6 demonstrates the degree of correlation between the volatile compounds and the organisms detected.

There was a strong correlation between *B. linens* and *G. candidum* with sulphur compounds and 2-methyl-1-butanol; *S. xylosus* was correlated with sulphur compounds, 2-methyl-1-butanol and some ethyl esters; *C. variabilis* was correlated with ketones; *D. hansenii* was correlated with acids and alcohols; *G. arilaitensis* was correlated with ketones and alcohols and acids, and *S. saprophyticus* with ketones, esters, acids and alcohols (Fig. 5.7; Table 5.3).

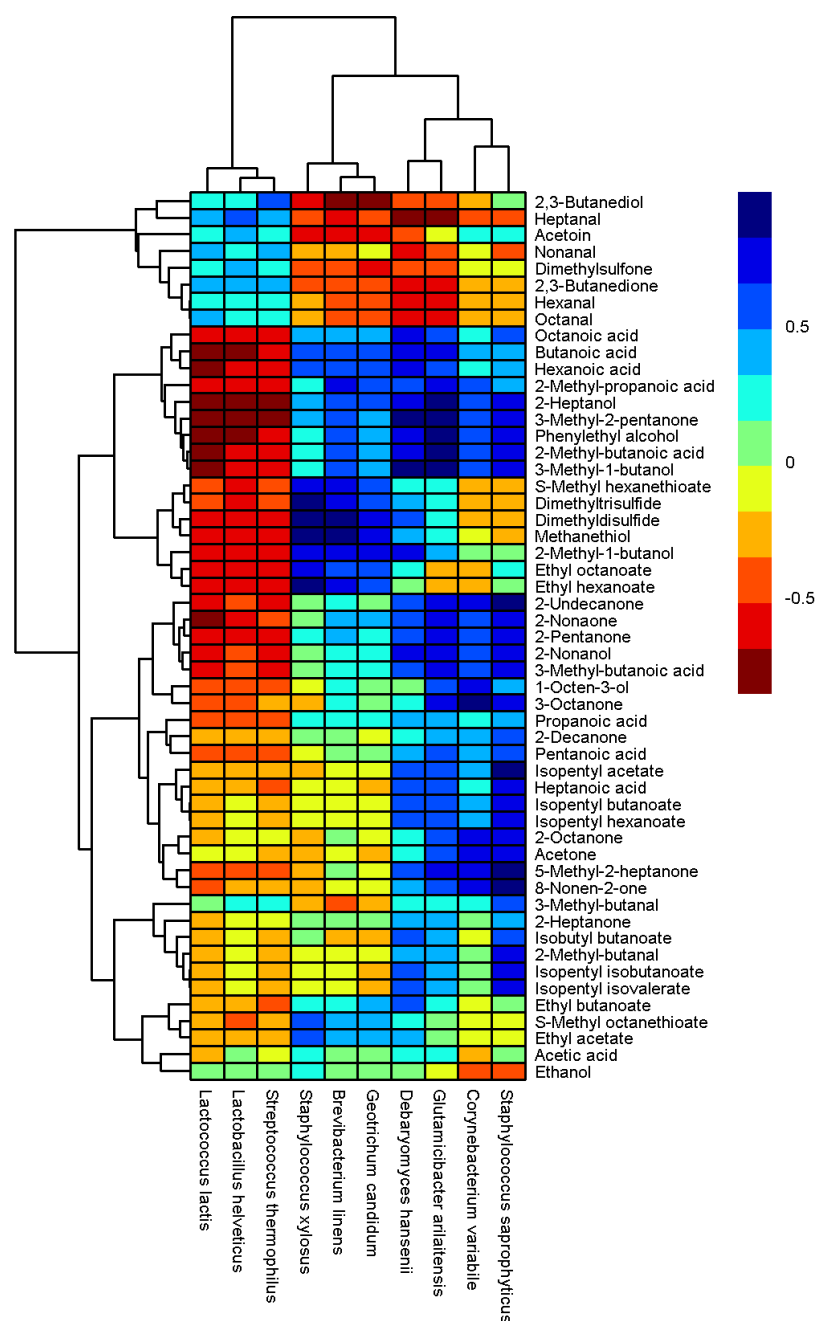


Figure 5.7. Hierarchically clustered map showing the correlation between the relative abundance of the microbial species and the levels of volatile compounds detected on the cheese surface. Clustering was performed by using the hclust function in R. The colour of each tile of the heat map indicates the level of correlation for a given species-compound combination, as indicated by the colour key.

Table 5.3. List of strong positive correlations^a between the levels of volatile compounds and the relative abundance of species on the cheese surface.

Correlations species-compounds	Potential precursor	R value
<i>Debaryomyces hansenii</i>		
2-Methyl butanoic acid	Isoleucine	0.81
3-Methyl-1-butanol	Leucine	0.85
Octanoic acid	Lipolysis	0.76
Hexanoic acid	Lipolysis	0.81
2-Heptanol	2-Heptanone (fatty acid oxidation)	0.8
<i>Glutamicibacter arilaitensis</i>		
2-Methyl butanoic acid	Isoleucine	0.9
3-Methyl-1-butanol	Leucine	0.86
3-Methyl butanoic acid	Leucine	0.77
Phenylethyl alcohol	Phenylalanine	0.83
3-Methyl-2-pentanone	Fatty acid oxidation	0.89
2-Undecanone	Fatty acid oxidation	0.82
5-Methyl-2-heptanone	Fatty acid oxidation	0.78
2-Pentanone	Fatty acid oxidation	0.77
2-Nonaone	Fatty acid oxidation	0.76
2-Heptanol	2-Heptanone (fatty acid oxidation)	0.86
<i>Geotrichum candidum</i>		
2-Methyl-1-butanol	Isoleucine	0.76
Methanethiol	Methionine	0.76
Dimethyldisulphide	Methanethiol	0.79
<i>Brevibacterium linens</i>		
2-Methyl-1-butanol	Isoleucine	0.81
Methanethiol	Methionine	0.82
Dimethyldisulphide	Methanethiol	0.85
Dimethyltrisulphide	Methanethiol	0.77
<i>Staphylococcus xylosus</i>		
2-Methyl-1-butanol	Isoleucine	0.77
Methanethiol	Methionine	0.84
Dimethyldisulphide	Methanethiol	0.95
Dimethyltrisulphide	Methanethiol	0.86
Methylthio hexanoate	Methanethiol + Hexanoic acid	0.78
Ethyl hexanoate	Ethanol + Hexanoic acid	0.85
Ethyl octanoate	Ethanol + Octanoic acid	0.77

Staphylococcus saprophyticus

2-Methyl-butanoic acid	Isoleucine	0.76
3-Methyl-1-butanol	Leucine	0.77
Heptanoic acid	Lipolysis	0.76
5-Methyl-2-heptanone	Fatty acid oxidation	0.98
2-Undecanone	Fatty acid oxidation	0.88
8-Nonen-2-one	Fatty acid oxidation	0.87
3-Methyl-2-pentanone	Fatty acid oxidation	0.77
2-Nonanol	2-Nonaone (fatty acid oxidation)	0.78
Isopentyl acetate	3-Methyl-1-butanol + Acetic acid	0.87
Isopentyl butanoate	3-Methyl-1-butanol + Butanoic acid	0.8
Isopentyl hexanoate	3-Methyl-1-butanol + Hexanoic acid	0.8

Corynebacterium variabile

3-Octanone	Fatty acid oxidation	0.99
2-Octanone	Fatty acid oxidation	0.78
5-Methyl-2-heptanone	Fatty acid oxidation	0.77

^aCorrelations with P value < 0.001 , (calculated as unadjusted, false discovery rate and Berroni) and R value > 0.75 .

5.4.5. Gene content of cheese surface microbiota

Using SUPER-FOCUS (<https://edwards.sdsu.edu/SUPERFOCUS>) (Silva et al., 2016), whole-metagenome shotgun sequencing was used to characterize the functional potential of the whole microbial community on the cheese surface at different stages of ripening. The functional clusters analysed were initially organized into three different levels, in relation to the specificity of the metabolic pathways. It became apparent that the samples clustered into three groups, i.e., control cheese at all time points formed a group with the test cheeses D4 and S5 at time 0; D4 cheese and S5 cheese formed separate groups throughout the ripening, with respect to their metagenomic profiles by Bray-Curtis MDS plot (Fig. 5.8).

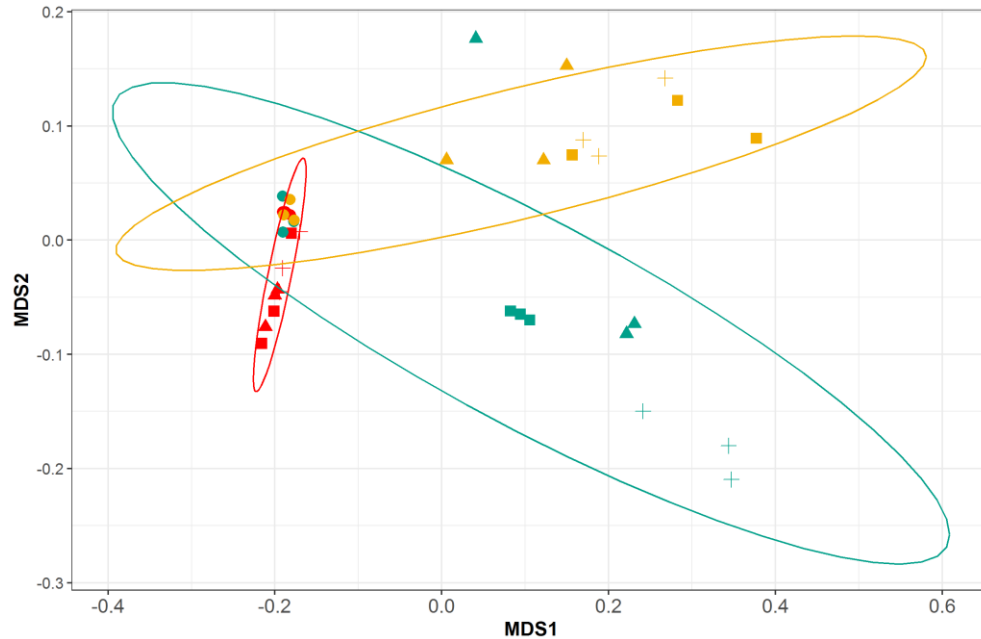


Figure 5.8. Bray-Curtis MDS plot based on the metabolic clusters associated with metabolic pathways of the cheese surface of control (red), D4 (green) and S5 (yellow), at day 0 (○), 18 (△), 24 (□) and 30 (+), resulting from the analysis performed with SUPER-FOCUS.

Pathway data was analyzed to determine the significant differences of the individual metabolic clusters by one-way analysis of variance (ANOVA), using SAS 9.3, with the selection of sixteen specific functional clusters with relative abundance significantly higher ($P < 0.05$) on the cheese surface of S5 and D4, compared to the control (Fig. 5.9).

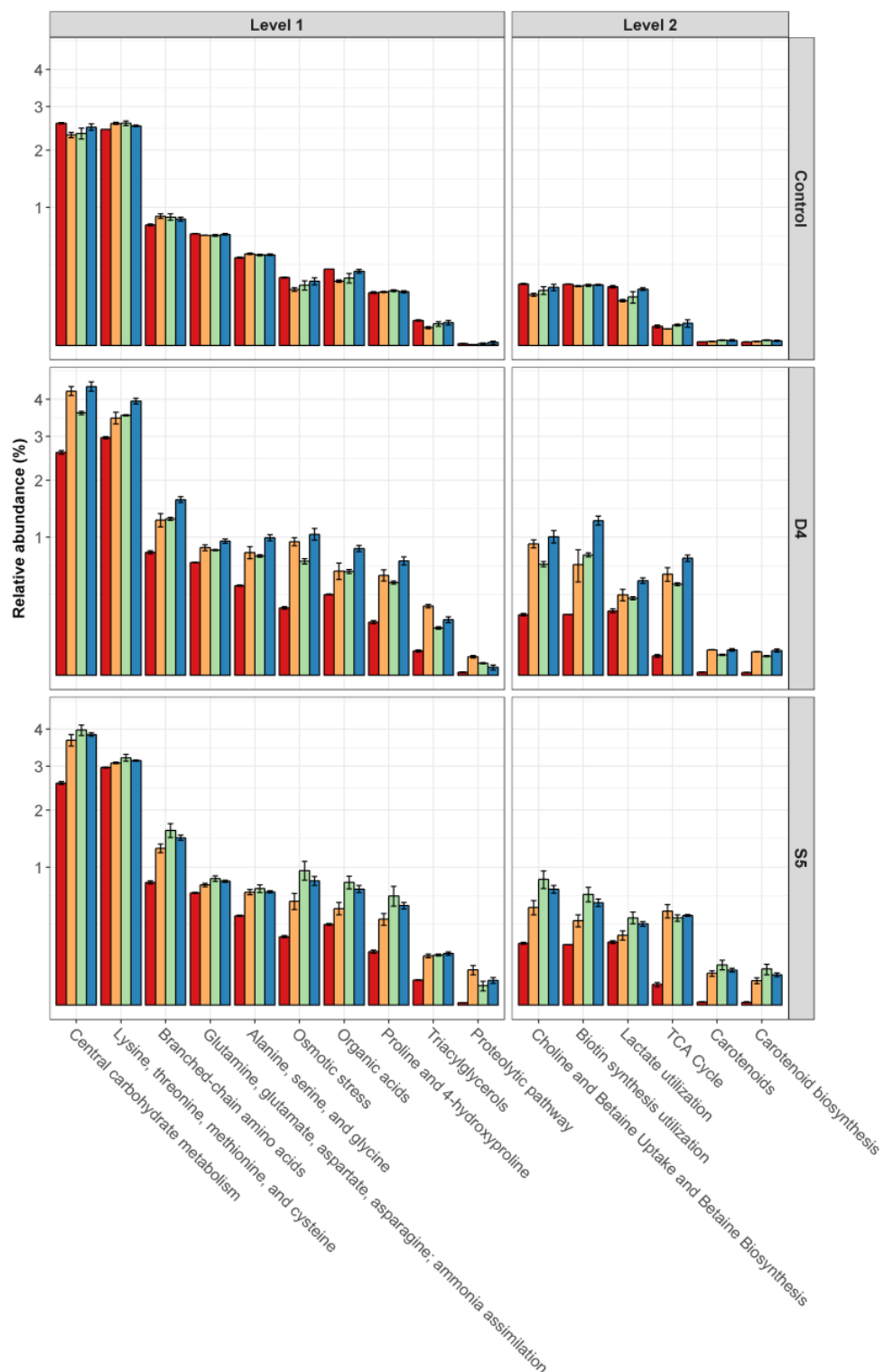


Figure 5.9. Means and SD between the three replicate trials of the relative abundance of significantly different ($P < 0.05$) metagenomic clusters detected with SUPER-FOCUS at day 0 (red), 18 (orange), day 24 (green) and 30 (blue), for the cheese surface of control, D4 and S5.

5.4.6. Colour and pH variation

pH and colour analysis was performed on the three cheese types and the resultant data was examined using a split-plot test, designed with SAS 9.3. A significant interactive effect ($P<0.0001$) between smear treatments and ripening time was observed for pH. At days 18, 24 and 30, the pH was significantly higher ($P<0.0001$) on the surface of S5 and D4, compared to the control. In addition, the pH was significantly higher ($P<0.0001$) on the surface of S5, compared to D4, from day 18 onwards (Fig. 5.10).

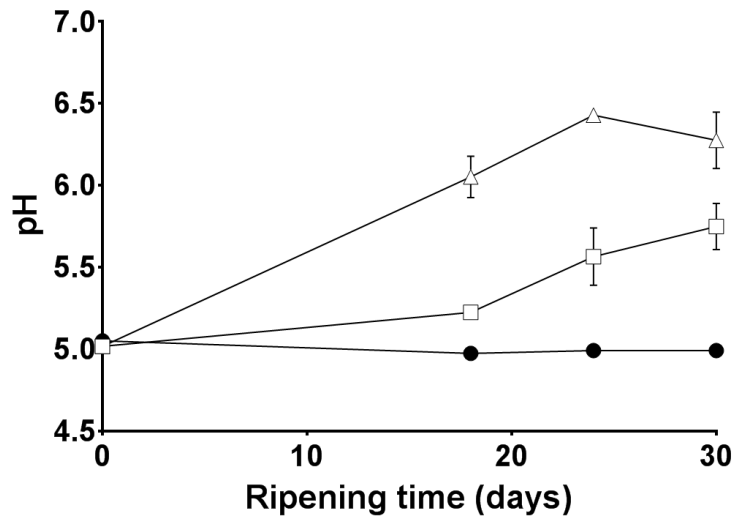


Figure 5.10. Change in pH of the cheese surface of control (●), D4 (□) and S5 (△). Data show the means and standard deviation of three replicate trials.

A significant interactive effect ($P<0.0001$), between time and smear treatments, was observed on L^* , a^* and b^* values. At days 18, 24 and 30, the a^* value was significantly higher ($P<0.0001$) for the surface of S5 and D4, compared to the control. At day 30, the a^* value was also significantly higher ($P<0.02$) on the surface of D4 compared to S5 (Fig. 5.11).

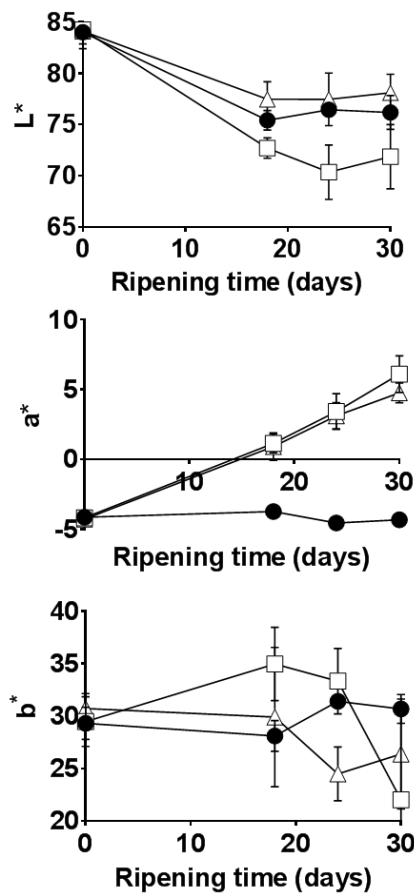


Figure 5.11. Colour development on the cheese surface of control (●), D4 (□) and S5 (△). Data show the means and standard deviation of three replicate trials.

5.4.7. Free amino acids and free fatty acids

Free amino acids (FAAs) and free fatty acids (FFAs) analysis was performed on the three cheese types and the experimental results were examined by one-way analysis of variance (ANOVA), using SAS 9.3. The concentrations of total FAAs on the surface of S5 ($15158 \pm 1683 \mu\text{g mg}^{-1}$) and D4 ($11914 \pm 1769 \mu\text{g mg}^{-1}$) were significantly higher ($P < 0.05$) than those on the control surface ($6605 \pm 819 \mu\text{g mg}^{-1}$). In addition, some individual FAAs, such as tyrosine, proline and histidine, were significantly higher ($P < 0.05$) on the surface of S5, compared to the surface of D4 and the control (Fig. 5.12).

The concentrations of total FFAs on the surface of S5 ($22069 \pm 3875 \mu\text{g mg}^{-1}$) and D4 ($26562 \pm 2606 \mu\text{g mg}^{-1}$) were significantly higher ($P < 0.05$) compared to the control ($1336 \pm 70 \mu\text{g mg}^{-1}$). Some individual FFAs, such as C4:0, C8:0, C10:0, C12:0, C14:0 and C18:0, were significantly higher ($P < 0.05$) on the surface of D4, compared to S5, or the control (Fig. 5.12).

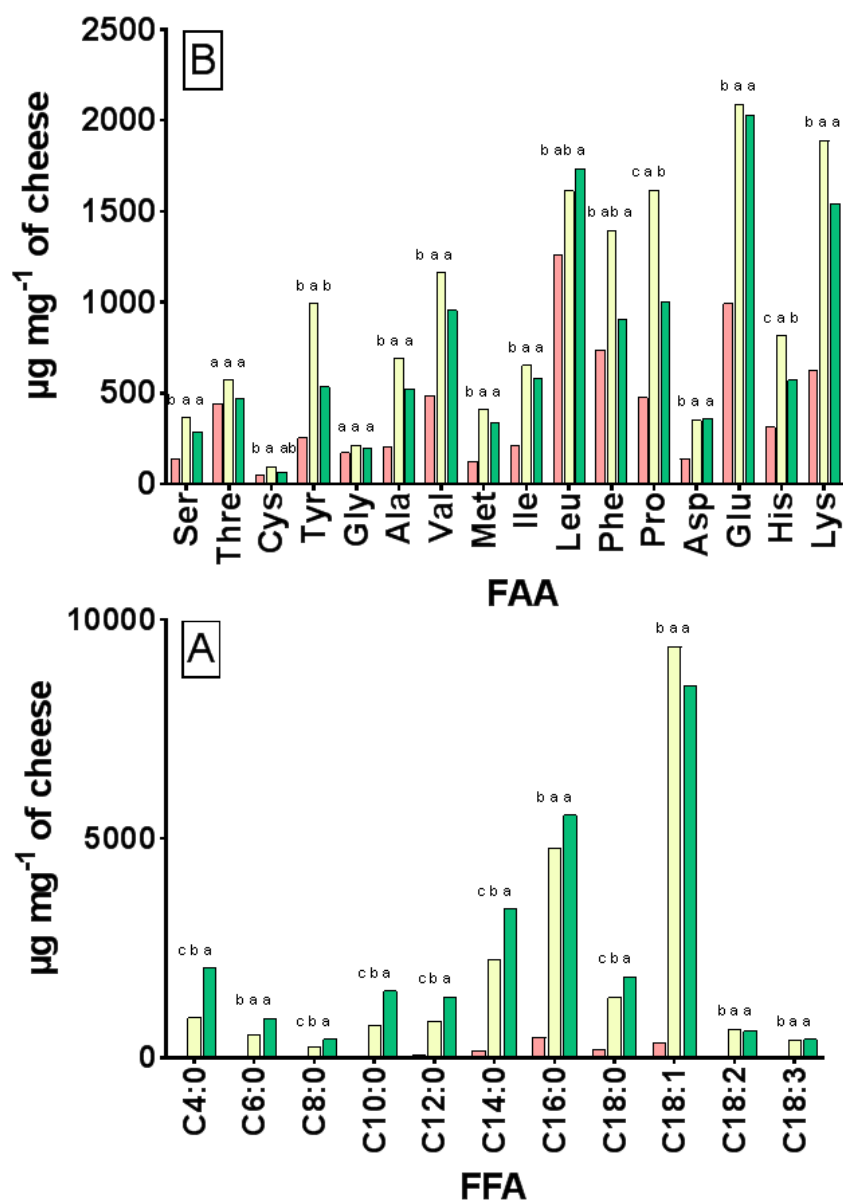


Figure 5.12. Free amino acid (A) and free fatty acid (B) concentration ($\mu\text{g mg}^{-1}$) on the cheese surface of the control (red), D4 (green) and S5 (yellow), at day 30. Data shows the means of three replicate trials. The significant differences ($P < 0.05$) are indicated with a, b, c.

5.4.8. Proteolysis

The proteolysis was particularly high on the cheese surface of S5 and D4 (Fig. 5.13), compared to the control. A significant interactive effect ($P<0.05$) between time and smear treatments was observed on the level of % pH 4.6-SN/TN. From day 24, the level % pH 4.6-SN/TN significantly higher ($P<0.05$) on the cheese surface of S5 and D4 compared to the control (Fig. 5.13).

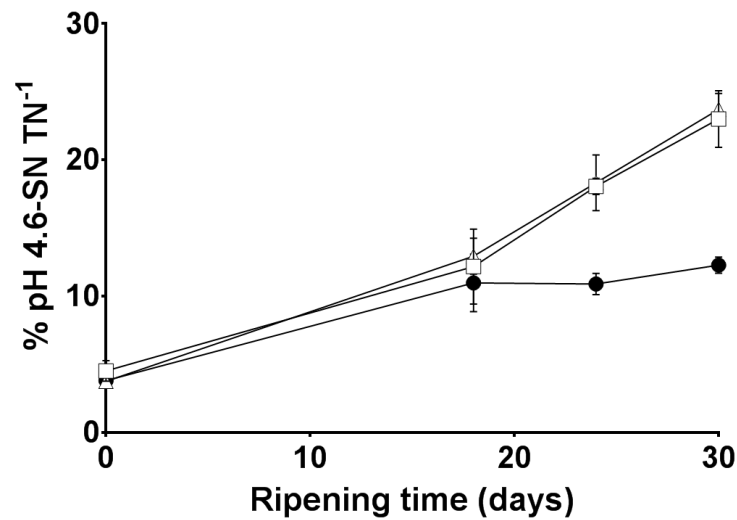


Figure 5.13. Proteolysis, expressed as % pH4.6-SN/TN during ripening of the cheese surface of control cheese (●), D4 (△) and S5 (□). Data show the means and standard deviation of three replicate trials.

Table 5.4. Means and standard deviation between the three replicate trials of the compositional parameters of the cheese surface of control, S5 and D4 at day 30.

Composition	Control d30	S5 d30	D4 d30
Moisture (% w/w)	35.54±0.08 ^a	35.13±0.72 ^a	33.46±0.84 ^b
Fat (% w/w)	31.14±0.08 ^a	28.22±0.27 ^b	26.63±1.68 ^c
Protein (% w/w)	25.69±0.15 ^a	26.00±0.37 ^a	25.62±0.37 ^a
MNFS (% w/w)	51.60±0.12 ^a	48.94±0.90 ^b	45.63±1.69 ^c
FDM (% w/w)	48.31±0.12 ^a	43.51±0.40 ^b	40.03±2.67 ^c
S/M (% w/w)	4.14±0.13 ^a	3.38±0.22 ^b	3.92±0.08 ^{ab}
Salt (% w/w)	1.47±0.05 ^a	1.19±0.10 ^b	1.31±0.01 ^{ab}

The compositional values for moisture, fat, protein, MNFS (moisture in non fat substances), FDM (fat in dry matter), S/M (salt in moisture) and salt of control at day 30 (Control d30), for the surface of cheese S5 at day 30 (S5 d30), and for the surface of cheese D4 at day 30 (D4 d30). Values presented are the means±standard deviations of three replicate trials. The significant differences ($P<0.05$) are indicated with ^{a, b, c}.

5.5. Discussion

In this study, the use of whole-metagenome shotgun sequencing facilitated the study, at species and strain level, of microbial succession among smear microorganisms (both bacteria and yeasts) on the cheese surface, and the analysis of the metabolic potential of the whole microbial community at different stages of ripening. Volatile flavour compounds were analysed over time, using HS-SPME GCMS, and correlated with the microbial species that developed during ripening.

Cheddar cheese curd, < 24h post manufacture, was inoculated with two different smear-culture mixes and incubated at 15°C, for 30 days. Un-smearied Cheddar cheese curd, vacuum packed to prevent the growth of spoilage moulds on the cheese surface, was used as a control. This model was chosen to investigate the microbial succession and flavour development as it had been shown in a previous study that yeasts and bacteria establish themselves satisfactorily on the surface of young Cheddar cheese curd, producing cheese with modified flavour and appearance (Bertuzzi et al., 2017).

On the cheese surface of S5 and D4, a very heterogeneous microbial consortium developed during ripening, triggering an array of biochemical processes. Yeasts are considered the responsible of the deacidification of the cheese surface (observed on S5 and D4; Fig. 5.10) by the degradation of lactate (to CO₂ and H₂O) (Ferreira et al., 2015) together with the formation of alkaline metabolites (from metabolism of FAAs) (Zikánová et al., 2002), and the secretion of growth factors (vitamins and amino acids) which support the growth of bacteria (Corsetti et al., 2001; Mounier, 2015). As

expected, in parallel with the growth of the yeasts, the relative abundance of the metagenomic clusters related to lactate utilization, and the biosynthesis and uptake of biotin, was higher for the cheese surface of D4 and S5, compared to the control (Fig. 5.9). During ripening, the surfaces of D4 and S5 were washed with a 5% salt solution, causing a hyperosmotic stress on the microbial population of the cheese surface (Hickey et al., 2018). This correlated with a higher relative abundance for the metagenomic clusters related to osmotic stress resistance and metabolism of choline and betaine (osmoprotectants) (Monnet et al., 2015), for the washed cheeses compared to the unwashed control (Fig. 5.9).

The development of a red/orange colour on the surface is an important characteristic of many smear ripened cheeses. This colour development is usually derived through the metabolism of carotenoids (Krubasik and Sandmann, 2000; Mounier et al., 2006), and correspondingly higher relative abundance of metagenomic clusters, involved in the metabolism of the carotenoids (carotenoids and carotenoid biosynthesis), was observed on the surface of the cheese S5 and D4, compared to the control (Fig. 5.9).

Surface-ripened cheeses are also characterized by a strong flavour which is driven by the biochemical metabolism of the microbial consortium which develops on the cheese surface over time. These are associated with proteolytic and lipolytic pathways, driving the increase in the levels of FAAs and FFAs. These pathways, together with lactose and citrate metabolism, are considered to be responsible for the main precursors of flavour compounds in cheese. During ripening, the relative

abundance of the metagenomic clusters associated with the proteolytic pathway, and the metabolism of triacylglycerols, was higher for D4 and S5, compared to the control, which was consistent with proteolysis (Fig. 5.13), and FAAs and FFAs related data (Fig. 5.12). During ripening, the relative abundance of metagenomic clusters directly related to the formation of volatile compounds, such as carbohydrate metabolism, organic acids (including FFAs) and FAAs (except aromatic amino acids), and indirectly related, such as TCA cycle (important for the α -ketoglutarate production), was significantly higher ($P < 0.05$) for the cheese surface of both D4 and S5, compared to the control (Fig. 5.9). Correspondingly, numerous volatile compounds (alcohols, aldehydes, carboxylic acids, ketones, sulphur compounds, esters and S-thioesters) (Fig. 5.6) were produced on the cheese surface of S5 and/or D4, conferring an intense flavour to the cheese surface of D4 and S5.

During ripening, on the cheese surface of S5 and D4, a microbial succession, involving various inoculated, and indeed some non-inoculated, microorganisms, was apparent. Consistent with other studies, specific smear strains, added as adjunct cultures to the milk, or on the exterior of surface-ripened cheese during manufacture, have not been detected at the end of ripening (Feurer et al., 2004; Goerges et al., 2008; Gori et al., 2013; Larpin et al., 2011; Rea et al., 2007). In this study, the species detected on the cheese surface by metagenomic analysis did not fully correspond with the components of the smear-culture mixes. Different contaminant populations developed on the cheese surface of both test cheeses, especially on D4, probably due to the different interactions and competition with the cultures of the two mixes (Fig. 5.2; Table 5.2).

D. hansenii was part of the inoculum used for both S5, and D4 surface. *D. hansenii* is a component of the surface microbiota of many surface-ripened cheeses, and is very tolerant to high salt, and low pH conditions (Cholet et al., 2007; Ferreira and Viljoen, 2003). Presumably due to these characteristics, *D. hansenii* was present at high relative abundance in both test cheeses, mainly in the early stage of ripening (at day 18), and then decreased gradually in the later stages (day 24 and 30) (Table 5.2). Volatile compounds significantly associated ($P < 0.001$) with *D. hansenii* were mainly alcohols and carboxylic acids (Fig. 5.7; Table 5.3). The biosynthesis of branched chain alcohols and carboxylic acids, from FAAs metabolism, and the biosynthesis of medium-long carboxylic acids, from FFAs metabolism, are processes attributed mainly to yeast and mould metabolism, including *D. hansenii* (Arfi et al., 2002; Collins et al., 2003; Gori et al., 2012; Leclercq-Perlat et al., 2004; Martin et al., 2001; Yvon and Rijnen, 2001).

On the cheese D4, the relative reduction of *D. hansenii* with time, corresponded to an increase of Gram-positive bacteria. *G. arilaitensis*, a component of S5 mix, did not grow on the cheese surface of S5, and, though not inoculated as part of the culture mix, was the dominant bacteria on the surface of D4 (Fig. 5.2; Table 5.2). Through the use of PanPhlAn, which uses metagenomic data to achieve strain-level microbial profiling resolution, we have demonstrated that the *G. arilaitensis* strain, present on D4, was not the same strain as inoculated onto S5 (Fig. 5.3; Fig. 5.4). The inability of the inoculated *G. arilaitensis* strain to grow on the S5 cheese is most likely due to the different interactions within the microbiota on the cheese surface. Other studies on the microbial composition of the surface of Limburger cheese observed that *G. arilaitensis* behaved in

a similar manner, showing high relative abundance when it was co-inoculated only with *D. hansenii*, while showing low relative abundance when combined with both *D. hansenii* and *G. candidum* (Mounier, 2015). That *G. arilaitensis* contributes to cheese flavour has been shown previously in model cheese media (Deetae et al., 2007) (producing alcohols, and especially ketones), and in the current study, where it was significantly ($P<0.001$) associated with 3-methyl-1-butanol and phenylethyl alcohol, branched carboxylic acids (from FAAs metabolism), 2-heptanol and ketones (from FFAs metabolism) (Fig. 5.7; Table 5.3). In addition, a genomic study showed numerous genes encoding for protein degradation and fatty acid oxidation in *G. arilaitensis* (Monnet et al., 2010).

On the cheese surface of S5, *G. candidum* was co-inoculated with *D. hansenii* and established itself to become the most abundant yeast population by day 18. The successful cohabitation of *G. candidum* and *D. hansenii* may be explained by the fact that they do not compete for energy sources in the same way in cheese. *D. hansenii* uses lactate, or the limited amount of lactose present in the cheese post manufacture (0.8-1%), while *G. candidum* preferentially uses only lactate (Monnet et al., 2015; Mounier et al., 2008). During ripening, sulphur compounds were significantly associated ($P<0.001$) with *G. candidum* (Fig. 5.7; Table 5.3), which is in agreement with other studies which have shown that *G. candidum* is able to catabolize methionine in one-step degradation, with the biosynthesis of sulphur compounds (Arfi et al., 2002; Boutrou and Guéguen, 2005; Jollivet et al., 1994).

The production of sulphur compounds is an important characteristic of many surface ripened cheese and *B. linens* is considered one of the main species responsible for the development of the strong flavour of many surface-ripened cheese through the biosynthesis of sulphur compounds derived from methanethiol. In this study, *B. linens* was present at relatively low abundance in the original culture mixes (5.26% and 3.53% for D4 and S5, respectively; Table 5.1). However, while detected at very low relative abundance on the cheese surface of D4, was one the most dominant bacteria detected on S5 (37.05% at day 24; Table 5.2). While this may be due to inter-strain differences, it is most likely due to the different interactions within the microbiota of S5 and D4. Studies have shown that *B. linens* does not always establish itself on the cheese surface during ripening, even if it is present in the initial culture mix (Brennan et al., 2002; Goerges et al., 2008; Larpin et al., 2011; Mounier et al., 2005; Rea et al., 2007). However, in previous studies, *G. candidum* has been shown to stimulate the growth of *B. linens* in co-culture (Lecocq and Gueguen, 1994), suggesting the hypothesis that in S5, *G. candidum*, present at high relative abundance, was likely producing growth factors that supported the growth of *B. linens*; while in D4, it was out-competed by *G. arilaitensis*, which established itself very quickly on the surface of S5 and made up 75% of the microbiota at the end of ripening. *B. linens* was significantly associated ($P<0.001$) with methanethiol and its derivatives (dimethyldisulphide and dimethyltrisulphide) (Fig. 5.7; Table 5.3), which likely originated from the one-step degradation of methionine (Deetae et al., 2007; Jollivet et al., 1992; Rattray and Fox, 1999; Yvon and Rijnen, 2001).

Other species, while present at lower relative abundance on the cheese surface of S5 and D4, were also responsible for the biosynthesis of some volatile compounds. *S. xylosus*, present in the S5 mix, was not as successful as *B. linens* at establishing itself on the cheese surface, and was present at only at 10.83-13.36% of relative abundance, during ripening (Table 5.2). This is most likely due to competition for nutrients within the microbiota, as suggested by Mounier *et al.* (2008). Members of the genus *Staphylococcus* can establish themselves on surface ripened cheese in the early stages of ripening but are regularly overtaken by other bacteria at the later stages (Irlinger et al., 1997; Mounier et al., 2006; Rea et al., 2007).

In this study, specific species detected in low relative abundances in S5, such as *S. xylosus* (9.08-13.36%), and in D4, such as *S. saprophyticus* (1.06-2.69%), and *C. variable* (2.04-2.08%) (Table 5.2) were significantly associated ($P < 0.001$) with a range of flavour compounds important in surface-ripened cheese (Fig. 5.7; Table 5.3), and interestingly, while *S. xylosus* has been previously shown to produce sulphur compounds only in fermented meat (Stahnke, 1999; Tjener et al., 2004), in this study was correlated with specific sulphur compounds in cheese. This data would suggest that some smear bacteria though present at relatively low abundance in cheese are likely contributors to the release of FFAs and to their degradation, due to esterase activity and hence contribute to the aroma and flavour in the final cheese product (Casaburi et al., 2006; Curtin et al., 2002).

5.6. Conclusion

In the study reported here, whole-metagenome shotgun sequencing was employed as a novel method for the analysis of a fermented product with a complex microbiota. Metagenomic analysis was an efficient tool to understand the variations of the microbial population of the cheese surface over time and the related metabolic potential. Moreover, the association between the volatile compounds and the species represents a novel system to study the flavour development in cheese. In conclusion, the approach used in this study enabled us to determine the microbial succession during ripening, and also to begin to unravel the contributions of the various components of the surface microbiota when present within a complex microbial environment. The method proposed in this study can be utilised within industry to control the microbiota of fermented food and helping to drive the production of food products with specific flavor characteristics.

5.7. Acknowledgements

Thanks to Aaron Walsh for the metagenomic analysis. Thanks to Dr Fiona Crispie for whole-shotgun metagenome sequencing of cheese.

5.8. References

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6. General discussion

While numerous studies have focused on the flavour development in cheese by the metabolic activity of lactic acid bacteria, this research focused on surface-ripened cheese. In this type of cheese, the flavour development is a process which occurs during ripening, through the metabolism of a complex microbial population, which establishes on the cheese surface, comprising mainly yeasts and Gram-positive bacteria (Desmasures et al., 2015; Mounier et al., 2017). The aim of this research was to investigate the potential flavour activity of the surface microbiota in cheese. The compounds which contribute for the formation of the cheese flavour can be volatiles, mainly responsible for aroma, such as methyl ketones, lactones, aldehydes, alcohols, carboxylic acids, phenols, sulphurs, S-methylthioesters, esters, terpenes, and pyrazines, and non-volatiles responsible for taste, such as free amino acids (FAAs), and peptides.

Commonly, extraction techniques associated with gas chromatography mass spectrometry (GCMS) are routinely used for the characterization of the cheese volatile compounds. However, to date, only a limited number of studies have been undertaken to characterize the volatile flavour compounds of surface-ripened cheese, and the techniques used are, in the main, outdated (Dumont et al., 1974a; Dumont et al., 1974b; Guichard et al., 1987). In this study, a newer extraction technique, solid phase microextraction (SPME) was chosen, due to its high efficiency in the characterization of the volatiles of Tilsit cheese and other varieties (Fuchsmann et al., 2015; Mondello et

al., 2005). SPME was selected for the analysis (for cheese and model systems), due to its proven reliability, and ease of use. In addition, a triple phase-coated SPME fiber (DVB/CAR/PDMS) was chosen, as it is considered the most efficient fiber for the extraction of cheese volatiles (Mondello et al., 2005); and the stationary phase used for volatile separation presented an intermediate polarity (DB5) which guaranteed good affinity with a wide range of compounds.

It is known that the type of extraction technique used greatly influences the selectivity for volatile analysis, and it is proven that the use of different techniques can provide more information regarding the volatile compounds which characterize the flavour of surface-ripened cheese (Valero et al., 2001). The nature of the stationary phase of the GC column can also affect the sensitivity for specific compounds (Imhof and Bosset, 1994), and the simultaneous use of different columns, separately or in combination (two dimensional gas chromatography), can provide more complete results (Arora et al., 2006). In the future, a simultaneous use of different extraction techniques and stationary phases to give a fuller description of the volatile compounds which characterize the cheese flavour may be advantageous.

While this research mainly focused on the volatile compounds, the non-volatile compounds were also partially investigated. FAAs, derived from microbial metabolism, were analysed due to their important role as precursors for flavour volatile compounds. The FAAs analysis was achieved through the use of cation exchange high pressure liquid chromatography (HPLC), as described by McDermott *et al.* (2016), enabling both quantitative and qualitative characterization. Although in this research

FAAs analysis was successfully performed, the peptides content was not evaluated (either in cheese trials, or in cheese model system). The peptides which originate from the proteolytic activity of the microbial population, might have the potential to contribute to the cheese taste, considering particular peptides isolated from cheese display specific tastes (Zhao, Schieber, and Gänzle, 2016). To date, only Roudot-Algaron *et al.* (1994) have studied flavour peptides in surface-ripened cheese, identifying γ -glutamyl peptides from Comté. Nowadays, different techniques are available for the detection of these peptides in cheese, and usually the analysis is performed through gel permeation chromatography to separate the peptides fractions on the basis of size, and liquid chromatography–mass spectrometry (LC-MS) for identification. The characterization of flavour active peptides could be a new potential topic for further studies on the metabolism of the surface microbiota, and the flavour development in surface-ripened cheese.

In this study, microbial species of surface-ripened cheese (yeasts and bacteria), were investigated within cheese model system, and when were then applied to the cheese surface. A cheese model system was used as a quick and simple approach to screening for the biosynthesis of volatile compounds from individual bacteria inoculated. The results showed clearly that Gram-positive bacteria were more active in a cheese model system where less complex substrates were readily available, such as FAAs and peptides. These were seen as ideal substrates to investigate their contributions for the cheese flavour diversification. While most of the studies in cheese model system explore the individual ability of the bacteria, or yeasts, to produce volatile compounds,

few studies have been performed on the combination of yeasts and bacteria. Specific combinations can potentially show a lack of, synergistic, or inhibitory effect on the production of volatile compounds, in relation to the nature of their metabolic interaction, as shown by Martin *et al.* (2001) who have studied flavour generation, by coculturing several yeasts, moulds, and bacteria in a cheese model medium. To gain a better understanding of flavour development in surface-ripened cheese, it could be advantageous to continue the study on complex mixes of microorganisms within such systems.

The flavour-forming abilities were determined by the multiple enzymatic pathways displayed by the various microorganisms inside the genome. The microbial genome contains protein-coding sequence for enzymes involved in flavour development. The genome of *Staphylococcus saprophyticus* DPC5671, isolated from a Cheddar cheese surface was sequenced to investigate its potential metabolic activity. Analysis of volatiles produced by *S. saprophyticus* DPC5671 was performed when the strain was inoculated into cheese model systems, or when applied in combination with *D. hansenii*, to the cheese surface. The enzymatic activities (esterase, lipase, aromatic aminotransferase, and glutamate dehydrogenase) of *S. saprophyticus* DPC5671 and other Gram-positive bacteria were also tested with the use of chromogenic and agar-based assays. Numerous genes encoding for enzymes involved in the cheese flavour development were identified successfully, confirming the data from the volatile and enzymatic analysis. This study showed how genome sequencing is a valid and robust tool to reveal the metabolic potential of a specific strain. In the future the use of more

accurate high-throughput biochemical tests (e.g., Phenotype MicroarraysTM) could be combined with genome sequencing, to facilitate the correlations between the genomic and metabolic data (Shea et al., 2012).

As mentioned before, the metabolism of the smear microbiota was also studied directly in cheese. Complex culture mixes were applied to the Cheddar cheese surface, modifying the flavour and appearance. The surface microbiota was highly metabolically active, and volatile analysis was performed to better understand its role in the flavour development. Volatile analysis clearly showed how the metabolism of the surface microbiota influenced the flavour development.

In addition, whole-genome shotgun sequencing was used to study of the microbiota on our surface-ripened cheese. The microbial variations were statistically correlated with the volatile analysis in order to identify the species responsible for the volatile flavour development. This was a novel and important study in cheese, which has combined volatilomic and metagenomic data. Metagenomic analysis allowed the full characterization of the microbiota during ripening, showing the microbial variations both at species and strain level, and providing more information about the microbial interactions on the cheese surface. Specific strains were able to establish on the cheese surface in cohabitation with others, or did not survive due the competition for the nutrients. This also represents an efficient method to screen the complete metabolic potential of the cheese microbiota during ripening, providing an overview of the metagenomic clusters associated with specific metabolic pathways. Unfortunately, the complexity of the surface microbiota has not allowed for the identification of the

individual species responsible for specific metabolic pathways. Other studies on Kefir, showed through the use of HUMAnN2 (bioinformatics software), that it was possible to link individual pathways to microbial species (Walsh et al., 2016), and this should be the next approach to adopt also for cheese. Some recent studies have shown also that nuclear magnetic resonance (NMR) spectroscopy can be a potential tool to differentiate cheeses manufactured with different starter cultures in relation to the metabolites produced (Piras et al., 2013; Rodrigues et al., 2011). In future, metabolomic analysis with NMR might help to identify better the metabolites from the microbial metabolism, responsible for the diversification of surface-ripened cheese.

Overall, this thesis has largely and exhaustively explored the role of surface microbiota in relation to cheese flavour development, through the use of GCMS analysis on different substrates (cheese and model systems), genome sequencing (on an individual strains), and whole-genome shotgun sequencing (on the cheese microbiota). Further research is required to gain a more in-depth analysis of cheese flavour development, with this thesis providing a useful approach for future research in cheese and fermented foods, where volatilomic, metabolomic and metagenomic data could be combined to gain a better insight into flavour development.

The results of this study have showed a potential way to change the flavour of fermented food in a targeted way, by applying the appropriate smear treatment to the product.

6.1. Discussion references

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7. Appendix

Detection of Volatile Compounds of Cheese and Their Contribution to the Flavor Profile of Surface-Ripened Cheese

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Abstract: The volatiles responsible for the typical aroma of cheese are produced mainly by lipolytic and proteolytic pathways and by the metabolism of lactose, lactate, and citrate. The volatile profile of cheese is determined by gas chromatography (GC), which includes the extraction, separation, and detection of volatiles. A wide range of extraction techniques is available, and technological improvements have been developed in GC separation and detection that enhance our understanding of the role of individual key volatiles to cheese flavor. To date, for surface-ripened cheese, the main volatiles detected that contribute to flavor include acids, ketones, alcohols, and sulfur compounds. However, based on the limited number of studies undertaken and the approaches used, it appears that a significant degree of bias possibly exists that may have over- or underestimated the impact of specific chemical classes involved in the flavor of these types of cheese.

Keywords: cheese, flavor, gas chromatography, surface-ripened cheese, volatiles

Introduction

Flavor development of cheese is related to milk quality, processing operations, and, most importantly, microbial activity. The cheese environment is characterized by a complexity of microbial populations which contribute to numerous biochemical reactions leading to the formation of flavor volatile compounds (Fox, Guinee, Cogan, & McSweeney, 2017a; McSweeney, 2017).

The combination of volatile compounds and their interactions contributes primarily to the formation of aroma and taste, which together constitute cheese flavor (Niimi et al., 2015; Zehentbauer & Reineccius, 2002). Cheese flavor, in combination with the overall appearance of cheese and its texture, is decisive for consumer selection and preference (Drake & Delahunty, 2017; Kilcawley, 2017). Consequently, the importance of studying cheese flavor is related principally to both the acceptance of a cheese within the marketplace and the perception of a cheese's flavor by the consumer (Drake & Delahunty, 2017). It is known that the flavor preference of the consumer is motivated by the stimulation of human chemical senses, particularly those for odor (aroma) and taste (D'Acampora Zellner, Dugo, Dugo, & Mondello, 2008). Specifically, the perception of flavor is mainly driven by the combination of active volatile compounds perceived in the orthonasal

and/or retronasal cavity. Olfactory perception is a complex biological phenomenon triggered by certain volatile molecules, generally hydrophobic ones, which interact with odorant receptors (G protein-coupled receptors) in the olfactory epithelium of the nasal cavity (Breer, Fleischer, & Strotmann, 2006; Dunkel et al., 2014). A precise chemical characterization of the mixture of potential stimulants in food plays an important role in investigating olfactory perception and to evaluate food flavor.

The analysis of volatile compounds in food is commonly performed using gas chromatographic (GC) techniques, employing very sensitive technologies capable of detecting trace levels of volatile compounds. It is fundamental that GC detection is of high efficiency, especially as volatile compounds, which are present at trace levels, can be easily perceived during sensory analysis (low perception threshold) and can contribute significantly to the flavor profile of cheese (Hummel, Sekinger, Wolf, Pauli, & Kobal, 1997). The complete analysis of the volatile aroma components of cheese is a complex procedure which includes extraction/concentration techniques, chromatographic separation, and identification/quantification. However, it is necessary to concentrate the volatiles extracted from the food matrix, because the techniques available for the detection of volatiles are still less sensitive than the human nose, which is able to perceive odors at extremely low concentrations (parts per billion to parts per trillion) (Bartlett, Elliott, & Gardner, 1997; Mackie, Stroob, & Varel, 1998). The techniques and analytical tools available can be adapted to optimize results for any particular cheese variety, including surface-ripened cheeses (Lecanu, Ducruet, Jouquand, Grataudoux, & Feigenbaum, 2002; Mondello et al., 2005; Salun, Erbay, Kelebek, & Selli, 2017; Valero, Sanz, & Martínez-Castro, 2001).

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Surface-ripened cheese (such as Münster, Munster, Tilsit, Livarot, Limburger, and Comté) is characterized by the growth of surface bacteria and yeasts on the cheese surface during ripening, which are responsible for the strong typical flavor and the characteristic "glistening" appearance (Desmaures, Bora, & Ward, 2015; Fox, Guinee, Cogan, & McSweeney, 2017b; Mounier, Cotton, Irlinger, Landaud, & Bonnamy, 2017). This cheese variety is characterized by an intense flavor, conferred by a wide variety of volatile compounds (acids, ketones, alcohols, esters, and sulfur compounds), which can be detected in different ratios, in relation to the techniques and methods used for volatile analysis (Lecanu et al., 2002; Valero et al., 2001).

This review describes the formation of the main volatile classes that contribute to cheese flavor, and it examines the analytical methodologies used to detect volatile compounds in dairy products, highlighting the advantages and potential shortcomings of specific techniques. Subsequently, the flavor of surface-ripened cheese is described, and the main volatiles which contribute to the characteristic flavor of the surface-ripened cheese are presented, focusing on their flavor contribution and the methods used for their detection.

Flavor development in cheese

The biochemical processes which lead to the synthesis of volatile compounds are very complex (Ardö, McSweeney, Magboul, Upadhyay, & Fox, 2017; Ganesan & Weimer, 2017; Kilcawley, 2017; McSweeney, 2017; McSweeney, Fox, & Ciocia, 2017; Thierry et al., 2017). It is known that the volatile compounds identified in cheese are mainly the products of lipolysis, proteolysis, metabolism of residual lactose, lactate, and citrate. They also include metabolism of free fatty acids (FFAs), and free amino acids (FAAs) (Fox et al., 2017a; McSweeney, 2017).

Lactose is the principal carbohydrate present in milk (~4.8% in raw cow milk). During cheese manufacture, the starter lactic acid bacteria (LAB) convert some lactose into lactate, which is responsible for the decrease in pH. Lactate can be further metabolized by LAB into formate, acetaldehyde, ethanol, and acetate. In surface-ripened cheese, the lactate is also converted by yeasts and molds into CO₂ and H₂O, while in Swiss-type cheese propionic acid bacteria metabolize lactate into propionate, acetate, and CO₂ (McSweeney et al., 2017). After cheese manufacture, the residual citrate present in the curd can be metabolized by citrate-positive LAB into acetate and lactate, also producing other important flavor compounds such as acetoin, 2,3-butanediol, diacetyl, and 2-butanone (McSweeney et al., 2017; Singh, Drake, & Cadwallader, 2003).

The catabolism of FAAs produces mainly aldehydes, alcohols, carboxylic acids, amines, and sulfur compounds (Figure 1) (Ganesan & Weimer, 2017; Kilcawley, 2017). Aromatic amino acids, branched-chain amino acids, methionine, and aspartic acid are converted into α -keto acids by a transamination reaction, catalyzed by amino acid aminotransferase. The resulting α -keto acids are then further degraded to branched-chain and aromatic aldehydes, acyl-CoA, hydroxy acids, and methanethiol (Ardö, 2006; Ganesan & Weimer, 2017; Smit, Smit, & Engels, 2005). The transamination of valine, isoleucine, and leucine leads to the production of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, respectively (Ardö, 2006; Singh et al., 2003). Aspartic acid can be converted by transamination into oxaloacetate and further into acetoin, diacetyl, or 2,3-butanediol (Ardö, 2006). Recently, Peralta, Wolf, Bergamini, Perotti, and Hynes (2014) observed that in *Lactobacillus paracasei*, the transamination of aspartic acid has

a fundamental role in the production of diacetyl. Previously, Morgan (1976) had observed that in *Lactococcus lactis* var. *multi-genes* enzymatic pathways exist for the reduction of phenylalanine and methionine to phenylacetaldehyde and methional, respectively. However, aromatic aldehydes are produced mainly through spontaneous chemical oxidation of α -keto acids, derived from tryptophan and phenylalanine, producing benzaldehyde, or from tryptophan, generating nitrogen-containing compounds such as indoleacetate, indol-3-aldehyde, and skatole (Gao et al., 1997; Yvon & Rijnen, 2001).

Aldehydes can be converted to their corresponding alcohols by alcohol dehydrogenase, or oxidized to their corresponding carboxylic acids by aldehyde dehydrogenase (Ganesan & Weimer, 2017; Kilcawley, 2017; Yvon & Rijnen, 2001). The biosynthesis of primary and aromatic alcohols, and relative carboxylic acids, is mainly attributable to the metabolism of molds and yeast. *Geotrichum candidum* and yeasts isolated from Camembert have the ability to produce alcohols and carboxylic acids through the metabolism of FAAs (Yvon & Rijnen, 2001). The presence of alcohols, such as 2-methylpropanol, 2-methylbutanol, 3-methylbutanol, and phenylethanol, together with the presence of carboxylic acids, such as 2-methylpropanoic, 2-methylbutanoic, 3-methylbutanoic, and phenylacetic acid, are indicators of the reduction of branched-chain and aromatic FAAs (Singh et al., 2003; Yvon & Rijnen, 2001).

The metabolism of FAAs by decarboxylation can produce amines, which are not associated with good quality cheese, due to their potentially adverse health effects and often poor flavor. Gram-positive bacteria, including LAB, are considered the principal producers of amines, due to their high decarboxylase activity. An intense and uncontrolled ripening could support extensive proteolysis, with the release of high amounts of FAAs, which are the fundamental substrates for the biosynthesis of amines (Linares, Martín, Ladero, Alvarez, & Fernández, 2011; Marino et al., 2013). The most important amines are histamine, tyramine, putrescine, cadaverine, and β -phenyl ethylamine, which are produced from the decarboxylation of histidine, tyrosine, ornithine, lysine, and β -phenylalanine, respectively (Linares et al., 2011; Marino et al., 2013).

In addition, catabolism of FAAs can be initiated by elimination reactions, catalyzed by amino acid lyase, which cleave the side chain of amino acids. This pathway leads to the synthesis of phenol and indole from the metabolism of aromatic amino acids, and to the production of methanethiol from methionine (Ardö, 2006; Ganesan & Weimer, 2017). *B. linens* (by methionine γ -lyase) and *L. lactis* (by cystathionine β - and γ -lyase) were reported to produce methanethiol, through elimination reactions, starting from methionine (Yvon & Rijnen, 2001). Yeasts, *Mirococcaceae*, and *Brevibacterium linens*, commonly found in surface-ripened cheese, have the ability to cleave the side chains of tyrosine and tryptophan, releasing phenol, and indole, respectively (Jollivet, Bézengher, Vayssier, & Belin, 1992; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994). For this reason, phenol is a flavor compound frequently detected in surface-ripened cheese (Urbach, 1997).

The further catabolism of methanethiol via oxidative reactions leads to the production of dimethyldisulfide and dimethyltrisulfide. Sulfur compounds are particularly important contributors to the flavor of surface-ripened cheese, due to their very low odor perception and their strong garlic, cabbage and very ripe cheese odors (Curioni & Bosset, 2002; Sablé & Cotteceau, 1999). Numerous LAB species are capable of degrading methanethiol, producing dimethyldisulfide and dimethyltrisulfide. However, typical

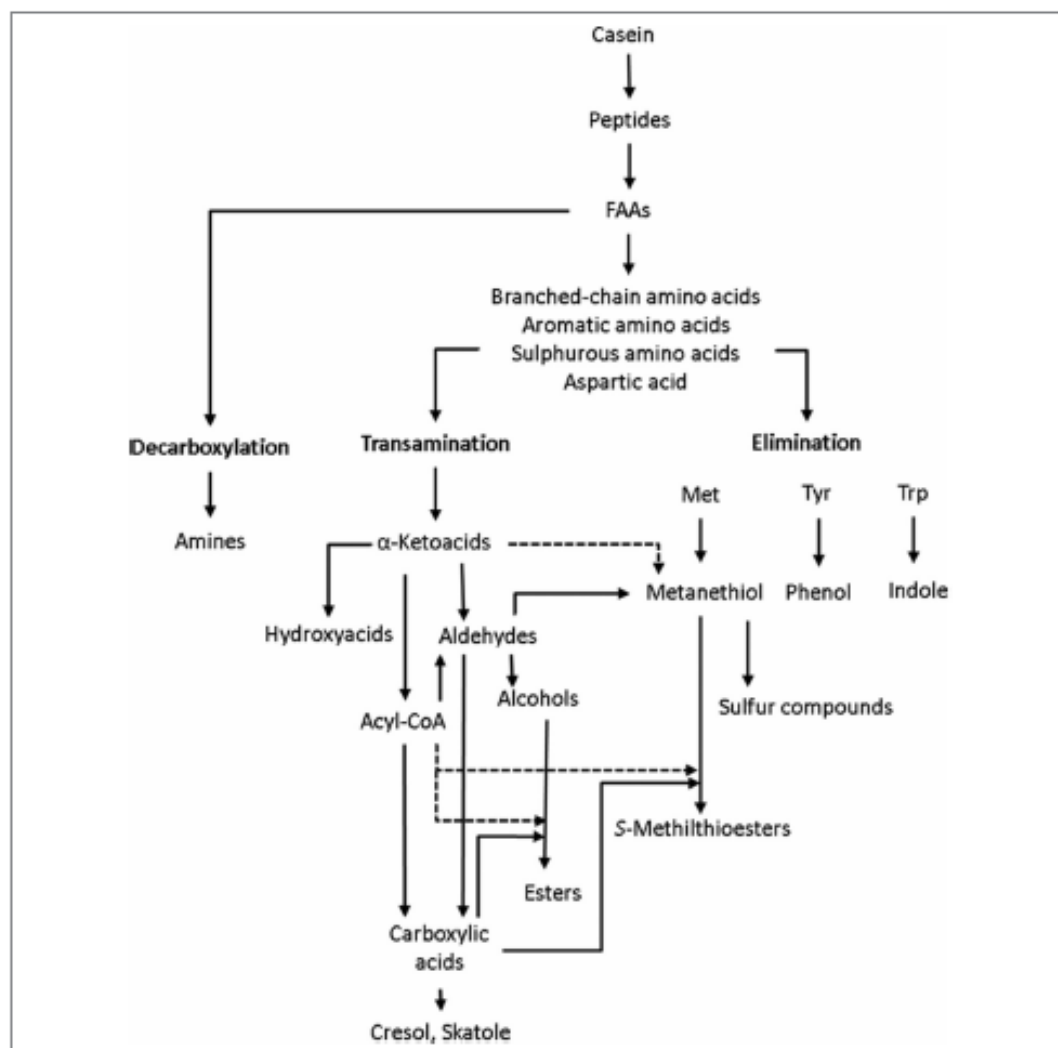


Figure 1—General pathways for catabolism of free amino acids in cheese, modified from Yvon and Rijnen (2001).

species found on surface-ripened cheese, such as *Micrococcaceae*, *B. linens*, and *C. candidum*, are considered the main producers of sulfur compounds, which are detected at high concentrations in these varieties (Jollivet et al., 1992, 1994).

Lipolysis of the triglycerides by microbial and indigenous milk enzymes, and also enzymes from added rennet pastes, results in the development of medium-chain (carbon chain lengths ≤ 10) and long-chain (carbon chain lengths > 10) FFAs (Collins, McSweeney, & Wilkinson, 2003; Thierry et al., 2017). The lipase enzymes catalyze the hydrolysis of triglycerides, with the formation of FFAs, di- and mono-glycerides, and glycerol. The abundance of FFAs directly affects the flavor properties of cheese, and it ranges from low levels in Edam cheese (total amount ~ 300 ppm) to very high levels in blue-veined cheese (total amount ~ 30000

ppm) (Woo & Lindsay, 1984). The flavor contribution of FFAs in cheese is mainly influenced by the pH. FFAs at high pH levels are less flavor active and are often perceived as “soapy” as they are converted to nonvolatile salts. At low pH FFAs exist in free form and are perceived as rancid at high concentrations (Alewijn, 2006; Singh et al., 2003).

FFAs contribute to the formation of cheese flavor not only directly, but also indirectly as they are precursors of methyl ketones, secondary alcohols, straight-chain aldehydes, lactones, esters, and S-thioesters (Figure 2) (Collins et al., 2003; Smit et al., 2005; Thierry et al., 2017). FFAs can be oxidized to β -ketoacids, and then decarboxylated to corresponding methyl ketones, with one carbon less (Collins et al., 2003; Kilcawley 2017; Thierry et al., 2017). The biosynthesis of methyl ketones is mainly attributed to

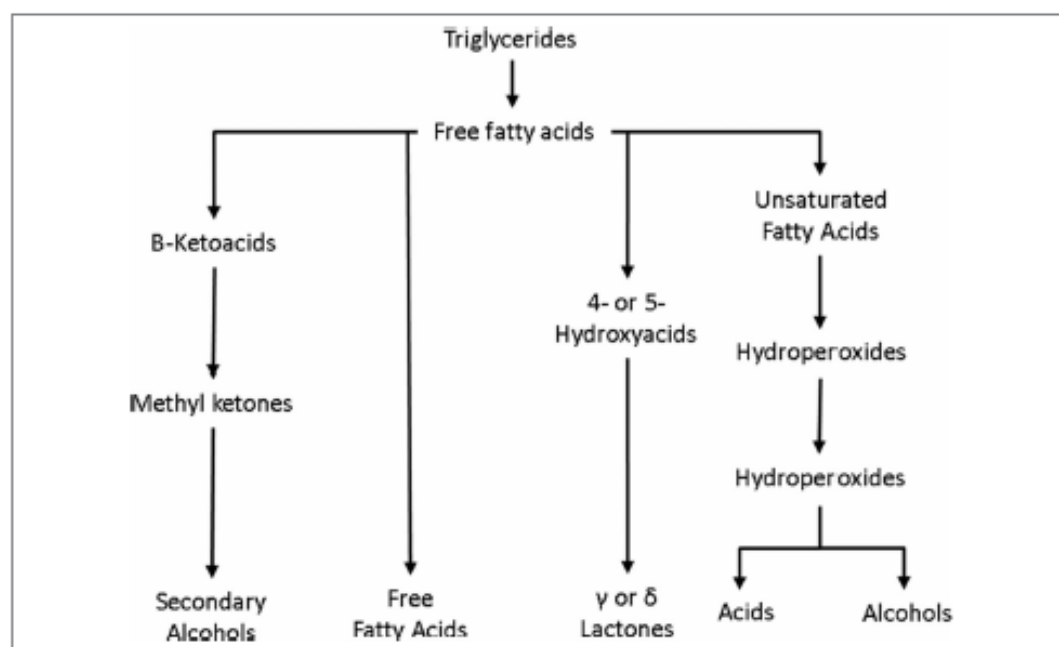


Figure 2—General pathways for the catabolism of free fatty acids in cheese, modified from McSweeney and Sousa (2000).

mold metabolism, such as *Penicillium roqueforti*, *Penicillium camemberti*, and *G. candidum*, and due to their typical odors and low perception thresholds, are most likely responsible for the characteristic flavor of surface-mold-ripened, and blue-veined cheeses (Collins et al., 2003; Curioni & Bosset 2002; Sablé & Cotteceau, 1999). However, it is also postulated that ketones can be produced by heating milk, or directly from esterification of β -keto acids (Alewijn, 2006). Urbach (1997) suggested that some ketones may be formed directly from β -ketoacids in the GC inlet, possibly resulting in an overestimation of ketones in the volatile flavor profile of the cheese sample.

Ketones can be further converted by reductase to secondary alcohols (Collins et al., 2003; Kilcawley, 2017). This metabolic pathway is mainly attributed to molds (such as *Penicillium* spp.), which are considered responsible for the production of secondary alcohols (such as 2-pentanol, 2-heptanol, and 2-nonanol) in blue-veined cheeses (Collins et al., 2003). However, secondary alcohols are thought not to strongly contribute to the cheese flavor (Singh et al., 2003), even if 2-heptanol was identified as a key odorant of Gorgonzola and Grana Padano cheese types (Curioni & Bosset, 2002).

Although unsaturated fatty acids, both free and esterified, can be involved in nonenzymatic autooxidation reactions, these reactions are not overly common in cheese, as cheese is a very reducing environment. However, when this reaction occurs it produces straight-chain aldehydes, which are characterized by "green grass-like" aromas, producing compounds such as propanal, hexanal, heptanal, octanal, nonanal, 2-decenal, and 2-undecenal, plus others (Alewijn, 2006).

Esters are another group of volatile compounds which are involved indirectly in the metabolism of FFAs, whereby the esterifi-

cation and alcoholysis reactions lead to the biosynthesis of esters (Collins et al., 2003; Liu, Holland, & Crow, 2004). Esterification is the formation of esters from alcohols and carboxylic acids by esterase, whereas alcoholysis is the production of esters from alcohols and acylglycerols, or acyl-CoA (derived from the metabolism of FFAs, FFAs, and/or carbohydrates), by acyltransferase. In alcoholysis, fatty acyl groups from acylglycerols and acyl-CoA derivatives are directly transferred to alcohols and are the major mechanism of ester biosynthesis by LAB (Liu et al., 2004). Some esters are characterized by low perception thresholds and are generally appreciated for their sweet, fruity, and floral notes, and for their ability to minimize the sharpness and bitterness of a cheese, usually associated with high levels of FFAs and amines (Curioni & Bosset 2002; Liu et al., 2004; Sablé & Cotteceau, 1999).

FFAs, or fatty acyl groups, can react with methanethiol to produce *S*-methylthioesters. Similarly, in ester biosynthesis, *S*-methylthioesters originate from esterification or alcoholysis reactions (Liu et al., 2004). The biosynthesis of *S*-methylthioesters is strictly dependent on the availability of methanethiol and, for this reason, is attributed mainly to those species which intensely contribute to methanethiol production (such as *Micromonospora*, *B. linus*, and *G. candidum*). *S*-Methylthioesters are frequently found in surface-mold-ripened and blue-veined cheeses, and they are characterized by potent odors with low aroma threshold perception (Liu et al., 2004).

Hydroxylated FFAs represent the precursors of lactones. Hydroxylated FFAs are incorporated in milk fat triglycerides and are released by enzymatic lipolytic activities or by any heating process (Alewijn, 2006). Hydroxylated FFAs can also be produced by the activities of microbial enzymes (lipoxygenase and hydratase) with the catabolism of unsaturated fatty acids. Lactones are produced

Table 1—Extraction techniques available for the volatile analysis of food and dairy products.

Extraction technique	Sample position	Operating principle	Trapping technology
Distillation/solvent-extraction	Flask	Flask under vacuum and heated to release the volatiles	Condenser (cold trap)
Solid-phase microextraction	Sealed vial	Phase-coated silica fiber exposed into the liquid phase or the headspace	Phase-coated fiber
Stir bar extraction	Sealed vial	Coated magnetic stirring bar in contact with the liquid phase or the headspace	Coated magnetic stirring bar
Purge & trap	U-shaped sparger	Inert gas is purged through the U-shaped glass sparger	Trap inside the unit
Thermal desorption	Sampling chamber	Inert gas is purged through the sampling chamber	Tube trap
Solid phase dynamic extraction	Sealed vial	Syringe with phase-coated needle sucks volatiles from the headspace	Phase coated needle
In-tube extraction	Sealed vial	Syringe made by a needle with an above trap sucks volatiles from the headspace	Trap above the needle

from hydroxylated FFAs by a one-step transesterification reaction (Alewijn, 2006).

Some other volatile classes of compounds, such as phenols and terpenes, can be detected in several cheese varieties because they can be naturally present in milk before cheese manufacture. Phenolic compounds were detected in higher concentrations in sheep and goat milk, when compared to cow milk, and they were considered responsible for the typical phenolic flavor of sheep milk cheese (Ha & Lindsay, 1991; Kilic & Lindsay, 2005). Phenolic compounds contribute positively to cheese flavor until a certain concentration is reached, but thereafter tend to give unpleasant notes, as their concentrations increase. The presence of phenolic compounds in milk is related to the process of conjugation, which is a detoxification mechanism that enables an animal to solubilize xenobiotics and to excrete them, usually in urine. It seems that the abundance of phenolic compounds in sheep milk is influenced by both feeding and breed (Kilic & Lindsay, 2005). Another class of compounds often identified in dairy products are terpenes, which are volatiles mainly detected in artisanal cheeses manufactured in Alpine regions. These compounds primarily, if not solely, originate from the differences in a cow's feeding regime in those areas (Karoui & De Baerdemaeker, 2007). It is likely that the content of terpenes in cheese produced in the Alpine region is high because of the diverse wild flora on which cows graze (Faulkner et al., 2018). However, Aprea et al. (2016) also reported an increase in terpene content of Montasio cheese during ripening, which could be attributed to the ability of LAB to modify and biosynthesize terpenoids (Belviso, Giordano, Dolci, & Zeppa, 2011).

Extraction techniques of volatile compounds

As outlined above, there is a wide range of flavor compounds, predominantly volatile in nature, involved in the development of cheese flavor. As a result, there has been a wide array of extraction techniques employed to isolate and concentrate the volatiles from different cheese varieties. In this regard, the selection of the correct extraction technique is of importance, because it can significantly influence the type of chemical classes extracted and identified (Bicchi, Condero, Liberto, Rubiolo, & Sgorbini, 2004; Dumont & Adda, 1972; Lecanu et al., 2002). The extraction techniques most frequently used to isolate volatiles from cheese are distillation-extraction, simultaneous distillation-extraction (SDE), solvent-assisted flavor extraction (SAFE), purge and trap (P&T), thermal desorption (TD), and solid-phase microextraction (SPME). More recently, newer extraction techniques, such as solid-phase dynamic extraction (SPDE), in-tube extraction (ITEX), stir bar sorptive extraction (SBSE), and headspace sorptive extraction (HSSE), have been adopted to detect volatile compounds in food matrices (Table 1).

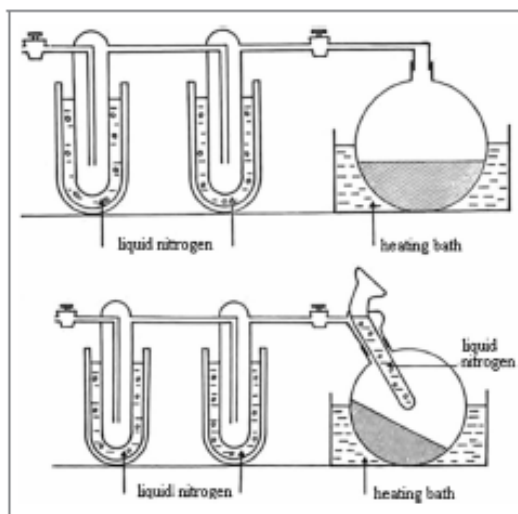


Figure 3—Distillation-extraction under vacuum from Dumont et al. (1974a). The cheese is placed in a flask connected to a group of vacuum pumps. The vacuum is maintained during extraction through the use of valves located at the beginning and at the end of the series of traps. The flask containing the cheese sample is heated by immersion in a heating bath and the traps are cooled by liquid nitrogen to collect the extract. The procedure is performed with a "finger condenser" to complete the extraction.

Distillation/Solvent extractions

Distillation-extraction techniques allow for the separation and concentration of volatile compounds from cheese samples through distillation. Distillation extraction is usually performed under reduced pressure ($<10^{-3}$ Pa), so-called "vacuum distillation," to avoid thermal degradation of volatile compounds. The cheese sample is mixed with water, flushed with nitrogen, and sealed. The resultant slurry is distilled under vacuum and the extract is collected in cooling traps (Figure 3). The extract may be concentrated to remove the water by solvent extraction. Usually, a solvent (acetonitrile, pentane, dichloromethane, or diethyl ether) is added to the extract and a further vacuum distillation is performed. The pH of the final extract can be adjusted to get different fractions (acidic, neutral, or alkaline) for GC injection. The adjustment of the polarity of the final extract causes separation of the volatile compounds. The acidic fraction contains primarily phenolic compounds and FFAs; the neutral fraction includes mainly alcohols, aldehydes, ketones, esters, and lactones; the alkaline

fraction presents mainly nitrogen-containing compounds, sulfur compounds, and terpenes. The distillation extraction technique was very common in the past, and numerous studies were performed on the analysis of the flavor of surface-ripened and Cheddar cheeses (Dumont, Roger, & Adda, 1974a; Guichard, Berdague, & Grappin, 1987; Libbey, Bills, & Day, 1963). Groux and Moiras (1974) analyzed the volatile compounds from Vacherin cheese by using a "gas stripping" technique combined with vacuum distillation. In this technique, the cheese sample was homogenized with distilled water, and the volatiles were extracted under reduced pressure by purging the system with an inert gas (argon) at low temperature.

It is possible to perform a distillation extraction of the volatiles directly in a solvent. This extraction is called SDE, and it is faster than a traditional distillation extraction. It requires a lower temperature for the distillation, considering the solvent usually has a low boiling point (Dacre, 1955; Godefroot, Sandra, & Verzele, 1981). This technique allows for the extraction of volatiles from the condensed solvent vapor (containing volatile compounds), which is separated from the condensed water vapor (containing nonvolatile compounds) in relation to their density, with subsequent injection of the extract directly onto the GC column. SDE guarantees a high extraction rate and it was used for a variety of cheese types, such as Edam, Swiss, Parmesan, Roncal, Pecorino, Gouda, Emmental, Cheddar, and Danish Blue, and it is an ideal extraction technique for components with low volatility (Aishima & Nakai, 1987; Dirinck & De Winne 1999; Alewijn, Sliwinski, & Wouters, 2003; Larráyoz, Addis, Gauch, & Bosset, 2001).

Solvent-assisted flavor extraction (SAFE) is another extraction technique which allows the separation and concentration of volatiles by vacuum distillation. The distillation system consists of a vacuum pump and usually 2 cooling traps of liquid nitrogen. The cheese sample is mixed with a solvent, and the volatiles are collected by distillation with the solvent in the first trap, while the impurities and the water condense in the second trap. Subsequently the extract, containing volatiles and solvent, is concentrated by purging with nitrogen, followed by injection onto the GC column (Engel, Bahr, & Schieberle, 1999). SAFE is frequently associated with GC-olfactometry analysis (Zabaleta, Gourrat, Barron, Albisu, & Guichard, 2016; Whetstone, Cadwalader, & Drake, 2005, 2006). In the GC-olfactometry analysis of Cheddar cheese, Drake, Miracle, and McMahon (2010) compared SAFE to headspace-SPME (HS-SPME) with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber. In this study it was clear that some highly volatile compounds were detected by HS-SPME, and not by SAFE, while some higher-molecular-weight aroma-active volatiles were detected by SAFE, and not by HS-SPME. SAFE is a valid method which allows the extraction of volatile compounds without extensive preparation, and it is particularly suitable for GC-olfactometry analysis, due to the preservation of the volatiles through extraction at low temperature. However, SAFE has some limits in the extraction of highly volatile compounds (Kilcawley, 2017; McGorin, 2007; Thomsen, Gourrat, Thomas-Danguin, & Guichard, 2014). Currently all distillation-extraction techniques, including SDE and SAFE, are not used as frequently as they were previously, due to the length of the procedure, the variable recovery rate of highly volatile compounds, and the risk of thermal degradation of heat-labile volatile compounds (Mariaca & Bosset, 1997; Sides, Roberts, & Helliwell, 2000; Tunick, 2014). The solvent used in these types of extraction can also create interference in the GC chromatogram, producing artifacts. Other techniques are now more

widespread, such as dynamic and passive extractions (discussed below), where the volatile compounds are captured by direct adsorption onto a solid-phase, avoiding time-consuming steps of distillation-extraction techniques (Bosset & Gauch, 1993; Tunick, 2014; Wampler, 1997).

Passive Extractions – Solid-Phase Microextraction, Stir Bar Sorptive Extraction, and Headspace Sorptive Extraction

SPME is a passive extraction technique, commonly used for a wide variety of applications. SPME can be performed as direct-SPME (DI-SPME), by exposing a phase-coated fiber directly to a liquid sample for the detection of semi- and nonvolatile compounds, or as HS-SPME by exposing a fiber in the headspace for the detection of medium or highly volatile compounds (Januszkiewicz, Sabik, Azarnia, & Lee, 2008; Mallia, Fernández-García, & Bosset, 2005) (Figure 4). HS-SPME is a passive extraction method which has become very popular for the detection of volatiles in cheese (Bertuzzi et al., 2017; Condurso, Verzera, Romeo, Ziino, & Conte, 2008; Januszkiewicz et al., 2008; Mondello et al., 2005; Yarlaga, Wilkinson, O'Sullivan, & Kilcawley, 2014).

In HS-SPME, the coated fiber is exposed to the headspace above a sample in a sealed vial, without direct contact with the sample, because the volatile compounds are in equilibrium between the gas and liquid/solid phases. The cheese sample (grated or as a slurry) in the vial is usually heated for a specified time (headspace-saturation time) to allow the volatiles to saturate the headspace. Subsequently, a coated silica fiber is inserted into the vial headspace for a precise time (equilibration/extraction time), at a specific temperature (equilibration/extraction temperature), to allow the volatile compounds to be captured by the polymer coating of the fiber (Pawliszyn, 1997). The sampling is commonly performed with a robotic autosampler, to reduce human error inherent in the manual injection. After the sorption, the fiber is thermally desorbed at a precise temperature, and preconditioned at a higher temperature before the next extraction, to avoid the carryover of analytes to the next analysis.

The type of fiber coating used for the extraction is the main factor which influences the efficiency and discrimination of the extraction. A wide variety of fibers are available, and selection and choice of fiber is critical (Mondello et al., 2005; Werkhoff, Brennecke, Bretschneider, & Bertram, 2001; Spietelun, Pilarczyk, Kloskowski, & Namiesnik, 2010). The fiber selection has to consider the polarity and thickness of the coating, in relation to the nature of the analytes of interest. The CAR/PDMS fiber is a common matrix used for cheese analysis, and it is particularly sensitive for the detection of highly volatile sulfur compounds, such as carbonyl sulfide, hydrogen sulfide, or methanethiol (Lecanu et al., 2002; Lestremieu, Andersson, Desautiers, & Fanlo, 2003; Januszkiewicz et al., 2008). In this double-phase fiber, the low-molecular-weight polar/apolar analytes are captured by the porous carbon structure of the CAR layer (Mondello et al., 2005). Salum et al. (2017) optimized HS-SPME for volatiles from white-brined Turkish cheese, comparing the efficiency of 2 different fibers (DVB/CAR/PDMS and CAR/PDMS) for the analysis of 10 key compounds. CAR/PDMS fiber was the most suitable fiber for the extraction of volatile compounds in white-brined cheese, especially for those analytes with low molecular weights (isoamyl alcohol, ethyl lactate, and butanoic acid). Mondello et al. (2005) evaluated 5 different fibers (DVB/CAR/PDMS,

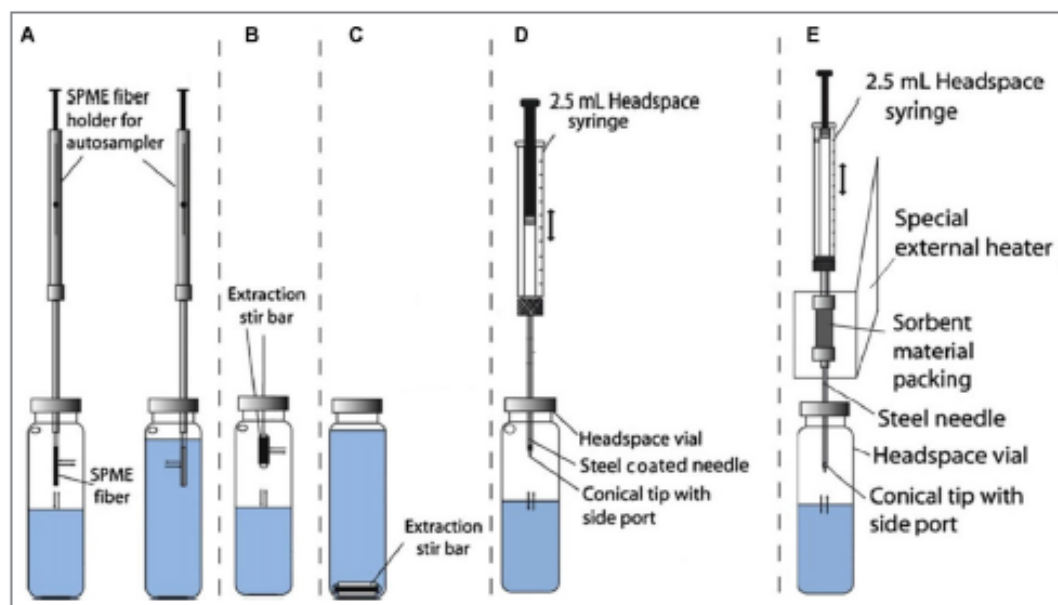


Figure 4—Solid-phase microextraction (direct and headspace) (A), headspace sorptive extraction (B), stir bar sorptive extraction (C), solid-phase dynamic extraction (D), and in-tube extraction (E), from Laaks, Jochmann, and Schmidt (2012).

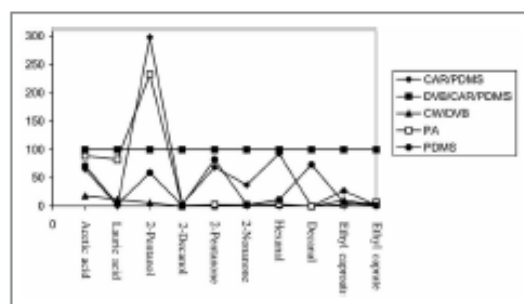


Figure 5—Optimization of HS-SPME for the analysis of goat cheese, from Mondello et al. (2005). Extraction capability of the most common polymeric fibers for the detection of 10 key compounds.

CAR/PDMS, carbowax/DVB, polyacrylate, and PDMS), suggesting that DVB/CAR/PDMS was the most efficient, with the highest extraction capability for the detection of the analytes of goat cheese (Figure 5). In the triple-phase fiber (DVB/CAR/PDMS), the larger compounds (less volatile) are captured by the porous DVB, while the smaller volatiles (highly volatile) pass through and are captured by the porous carboxen layer. However, the double-phase CAR/PDMS was efficient only for the extraction of low-molecular-weight polar/apolar analytes, with little affinity for compounds with high molecular weights. Also, Risticic and Pawliszyn (2013) analyzed the efficiency of different SPME fibers on a mixture containing a wide range of volatility and polarity compounds, confirming that DVB/CAR/PDMS was the most sensitive fiber for analytes with a molecular weight <185 g/mol. Moreover, they studied the different interactions of specific ana-

lytes with the DVB/CAR/PDMS fiber. Due to the limited surface capacity of the SPME fiber, competition occurs between the analytes for the adsorption sites, driven by their chemical characteristics (molecular weight and polarity) and the distribution constant (K_D), which indicates the ratio of the distribution of the analyte between the fiber coating and the sample. The analytes at high concentration, with higher K_D , can displace analytes with smaller K_D , thereby adversely affecting the ability to get a true reflection of the volatile profile of the sample. Risticic and Pawliszyn (2013) reported that interanalyte displacement was very limited by the use of DVB/CAR/PDMS fiber, which rarely occurred only for compounds with small K_D .

The extraction conditions, such as headspace-saturation time, extraction/equilibration temperature, extraction/equilibration time, sample volume, and sample agitation, can be controlled in relation to the nature of the sample, the analytes, and the type of fiber coating used (Harmon, 2002; Spietelun, Kloskowski, Chrzanoski, & Namiesnik, 2013). Mondello et al. (2005) optimized the conditions of HS-SPME associated with GC-FID and GC-MS for the analysis of 6 goat cheeses. They compared different headspace-saturation times at the same temperature (60 °C), suggesting 10 min as the optimal time. In general, low-molecular-weight volatiles presented the highest peak areas after 5 min saturation time, while high-molecular-weight compounds required a longer time (~10 min). Mondello et al. (2005) also showed that the optimal conditions were 60 °C for the extraction/equilibration temperature and 50 min for the extraction/equilibration time, which were the conditions shown to have the highest volatile extraction yields for both low- and high-molecular-weight volatiles. Burbank and Qian (2005) optimized the conditions of HS-SPME (with CAR/PDMS fiber), associated with GC-pulsed flame photometric detector, for the analysis of sulfur compounds in Cheddar

cheese. They compared different extraction/equilibration times at the same temperature (50 °C). The sulfur compounds detected increased their response over the course of 2 h, but 30 min was chosen as a good compromise between sensitivity and runtime efficiency for the instrument. Moreover, they compared different extraction/equilibration temperatures at the same equilibration time (30 min). The presence of methanethiol, dimethyldisulfide, and dimethyltrisulfide did not change between 30 and 70 °C, while methional was detected when the temperature was above 50 °C, and dimethyl sulfone reached the maximum response at 55 °C. Therefore, 50 °C was indicated to be the optimal extraction/equilibration temperature. Salum et al. (2017) optimized the conditions for GC-FID analysis of white-brined Turkish cheese, for both fibers tested (DVB/CAR/PDMS and CAR/PDMS). DVB/CAR/PDMS fiber presented an optimal performance with an extraction/equilibration time of 86 min at 54.8 °C, while CAR/PDMS fiber showed most effective extraction after 85 min, at 56.2 °C.

HS-SPME has been shown to be highly sensitive for the identification of low concentrations of odorous compounds such as sulfur compounds in different varieties of cheese (Frank, Owen, & Patterson, 2004). Lecanu et al. (2002) highlighted that HS-SPME was more sensitive in detecting a wide range of volatile compounds in surface-ripened cheese, which are important for odor perception (methanethiol, carbon disulfide, dimethyl sulfide, acetone, ethyl acetate, 2-butanone, and 3-methylbutanal), that were not detected using vacuum-distillation extraction, because of the overlap with a solvent peak or losses during the extraction procedure. In the analysis of Parmigiano-Reggiano cheese, Bellesia et al. (2003) compared HS-SPME with P&T, without noticing any substantial difference in the recovery of volatiles from the same sample, confirming the reliability of the 2 methods.

Overall, SPME is an excellent technique because of automated sampling, high sensitivity, and high throughput, without the use of extraction solvents (Harmon, 2002; Spietelun et al., 2010, 2013). The major drawback of SPME is the low entrapment capacity of the fiber coating; however, new fibers with thicker coatings have become available, including SPME Arrow technology that has much greater capacity and thus sensitivity than the original fibers.

Another method, successfully applied to food matrices, was SBSE (Huang, Qiu, & Yuan, 2009; Yu & Hu, 2009). The principle of SBSE is based on the sorption of volatile compounds in a sample (liquid or semi-liquid matrix), through the use of a magnetic stirring bar with a glass jacket, coated with a sorbent layer (Figure 4). The stirring bar is in direct contact with the sample to allow the full sorption of compounds, and the sample is typically stirred for between 30 and 240 min, depending on the size of the stirring bar. Once the volatiles are adsorbed, the stirring bar is rinsed in distilled water to remove nonvolatiles and thermally desorbed similarly to TD (Baltussen, Sandra, David, & Cramers, 1999). There are limited options for commercially available coatings, such as PDMS, ethylene glycol/silicone, and polyacrylate. However, newer technologies are now available to formulate special in-house coatings made of organic polymers and modified polymers (He, Chen, & Hu, 2014; Lancas, Queiroz, Grossi, & Olivares, 2009). The fiber most commonly used for food analysis is a PDMS fiber. SBSE performed with a PDMS fiber was used for analyzing flavor compounds in fresh and cooked milk, yogurt, and cream cheese by Hoffmann and Heiden (2000), using a polar column for the separation. In the analysis of cream cheese, the compounds detected were mainly ketones, long-chain

FFAs (C₁₀-C₁₆), lactones (δ-decalactone, δ-dodecalactone), and diethylphthalate. SBSE is a valid technique due to the high adsorption capacity, the minimal risk of thermal degradation of volatile compounds, and because the extraction does not require high temperatures (Lancas et al., 2009; Kilcawley, 2017). However, SBSE is not without its problems, with limited commercial availability of coatings and the difficulty of automating such a system (He et al., 2014). In addition, SBSE may be less suitable for the analysis of cheese and dairy products, as the high fat and protein contents may interfere with the extraction process (Licón et al., 2012).

HSSE represents a good alternative to SBSE, because it does not come into direct contact with the sample (Licón et al., 2012). HSSE is a technique recently adopted for cheese analysis with interesting results (Licón et al., 2012, 2015; Panseri et al., 2008). In HSSE, the coated stirring bar is placed in the headspace of a sealed vial containing the sample (Figure 4). The cheese sample is finely grated or mixed with water, and the volatiles are extracted similarly to HS-SPME, whereby the cheese sample is heated and stirred to ensure a uniform concentration of volatile compounds between the gas and liquid/solid phases. The time of extraction is longer than HS-SPME (usually 1 to 4 h) due to the volume of the coating material, to ensure maximum sorption of volatiles by the coating of the stirring bar (Licón et al., 2012; Panseri et al., 2008). However, the entrapment capacity is significantly higher than that of the original SPME fibers, which increases the sensitivity of the technique (Kilcawley, 2017). As in SBSE, after the sorption, the magnetic stirring bar is thermally desorbed. A PDMS stirring bar of 1 to 2 cm is preferred for cheese analysis (Licón et al., 2012, 2015; Panseri et al., 2008).

Licón et al. (2012) reported good linearity, recovery, precision, and reproducibility of data when using HSSE for cheese analysis. This technique showed good recovery of ketones in sheep milk cheese and aldehydes in Bitto cheese (Licón et al., 2012; Panseri et al., 2008).

Dynamic Extractions – Purge and Trap, Thermal Desorption, Solid-Phase Dynamic Extraction, and In-Tube Extraction

In dynamic methods, such as P&T, TD, SPDE, and ITTEX, the cheese sample is heated, and the volatile compounds are continuously removed and concentrated in a cold trap, or adsorbed onto an inert support before injection onto the GC capillary column.

TD is a common technique which has been used to differentiate the flavor compounds in different varieties of cheese (Lawlor, Delahunty, Wilkinson, & Sheehan, 2002; Valero et al., 2001; Villaseñor, Valero, Sanz, & Martínez Castro, 2000). Lawlor et al. (2002) used the TD technique for hard cheese types, such as Appenzeller, Dubliner, Emmentaler, Gähnel, Gruyère, Old Amsterdam, Raclette, and Tête de Moine. In TD, the cheese sample is heated in a sampling chamber to a desired temperature to release the volatile compounds. Using an inert carrier gas (helium or nitrogen), the stripped volatile compounds are transferred and trapped in a TD tube made of adsorbent trapping material, usually with low water affinity (such as Tenax TA) (Figure 6). Subsequently, the TD tube is purged to ensure that water is removed and then thermally desorbed to transfer the volatile compounds to a cooled trap and finally to the GC inlet by a flow of purging gas (helium or nitrogen) (Valero, Miranda, Sanz, & Martínez-Castro, 1997). A wide range of adsorbent and adsorbent trapping materials is available for the TD tube, based on the nature of the analytes. TD is an efficient technique, and the data obtained from

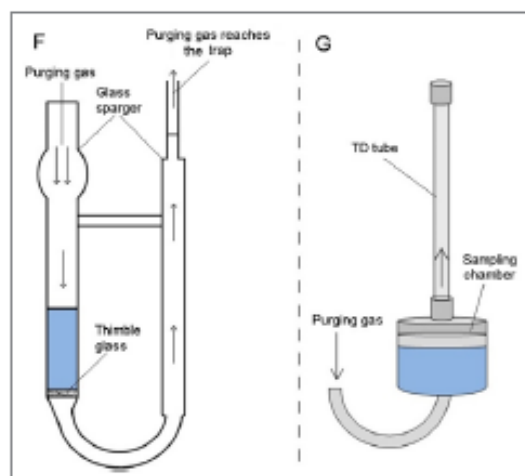


Figure 6—Purge and trap (F) and thermal desorption (G) apparatus.

this extraction are very representative, because a large amount of sample is analyzed due to the large loading capacity of the sampling chamber.

P&T is another dynamic extraction method commonly used in the determination of volatile compounds in cheese. P&T is a good technique for the detection of highly volatile compounds with lower boiling points, such as alcoholic compounds (Mallia et al., 2005; Thomsen et al., 2014). Bosset and Gauch (1993) used the P&T method to analyze different cheese varieties, such as Parmigiano, Mahón, Fontina, Comté, Beaufort, and Appenzeller, with numerous compounds detected, especially alcohols. There was a predominance of alcohols among the compounds detected when volatile analysis of Spanish sheep milk cheese was performed with P&T (Fernández-García, Carbonell, Gaya, & Nuñez, 2004; Barron et al., 2007; Gómez-Ruiz, Ballesteros, Viñas, Cabezas, & Martínez-Castro, 2002). Using the P&T technique, the cheese sample is usually homogenized with water, placed in a U-shaped glass sparger and heated. Subsequently an inert gas (nitrogen or helium) is purged through the sample to transfer the volatile compounds to an inert support of trapping material, which is thermally desorbed and concentrated once again in a cold trap (cryofocusing) before injection onto the GC-column (Figure 6) (Bellar, Lichtenberg, & Kroner, 1974). A wide range of trapping materials is available for this technique. P&T is a solvent-free and efficient technique, especially for the detection of highly volatile compounds (Mallia et al., 2005).

However, the high levels of moisture in some types of cheese can interfere with P&T and TD extractions, and even damage the MS-detector (Pillonel, Bosset, & Tabacchi, 2002). The moisture in the cheese samples can cause condensation in the TD tubes impeding the good desorption of volatiles, while in P&T the moisture can be purged from the sample to the trap, and injected onto the GC column, contributing to the production of artifacts (Kilcawley, 2017; Valero et al., 1997). Nowadays, numerous strategies are available to eliminate the moisture in the desorption process in cheese analysis. For example, before desorption, the solid trap can be flushed with an inert dry gas to remove part of the water (dry purge technique) (Canac-Arteaga, Viallon, & Berdagüe,

1999a). Another solution is to install a cold-water trap (condenser at $\sim -10^{\circ}\text{C}$) located before the trap to condense the residual water from the sample (condensation technique) (Canac-Arteaga, Viallon, & Berdagüe, 1999b). Other approaches include mixing a hygroscopic salt (such as sodium sulfate, sodium chloride, or potassium carbonate) with the sample (Canac-Arteaga, Begnaud, Viallon, & Berdagüe, 2000; Valero et al., 1997; Villaseñor et al., 2000), or placement of a cartridge with a hygroscopic salt (such as sodium carbonate, magnesium sulfate, or calcium chloride) in front of the trap to eliminate water (Guillot, Fernandez, & Le Cloirec, 2000). A drying tube made of permeable material such as Nafion has also been used to facilitate diffusion and to remove water from the sample (Pankow, 1991). Despite various strategies being adopted, these methods are not totally efficient due to partial removal of moisture and/or the losses of analytes (Pillonel et al., 2002). More modern systems contain built-in moisture control units, but moisture must still be managed for the effectiveness of these techniques.

SPDE is another dynamic technique that is now becoming commonly used (Bicchi et al., 2004; Gamero, Weselink, & de Jong, 2013). The innovative technology of SPDE involves a steel needle coated internally with a sorbent material, which extracts volatiles from the headspace. The needle is inserted inside the sample vial, and the plunger of the syringe is moved up and down several times to draw the volatiles inside the needle. Subsequently, the volatiles are thermally desorbed and a purging gas (helium or nitrogen) passes through the syringe with a direct injection onto the GC column (Lipinski, 2001) (Figure 4). Depending on the nature of the analytes, different needles, coated with various sorbent materials, are commercially available. To maximize the recovery of SPDE, the choice of coating materials and extraction conditions are fundamental. In flavor research, the most common sorbent materials used for the SPDE needles are PDMS and CAR/PDMS. During sampling, the major factors influencing the end result of SPDE are the sampling temperature and the number of extraction strokes, while the flow rate of the draw and eject steps were found not to be critical (Kamphoff, Thiele, & Kunz, 2007; Van Durme et al., 2007). For the analysis of volatiles in roasted coffee, Bicchi et al. (2004) compared SPDE to HS-SPME, maintaining the same extraction conditions and trapping material (PDMS) for both techniques. SPDE resulted in higher concentration factors, compared to HS-SPME for almost all analytes detected. Bicchi et al. (2004) suggested that the difference between the two methods was due to the fact that the extraction volume of the coating material of the needle in SPDE was higher than the volume of coating fiber of HS-SPME. However, dissimilar results were reported by Gamero et al. (2013), who stated that HS-SPDE (with PDMS/active carbon needle) had the least sensitivity, compared to HS-SPME (with DVB/CAR/PDMS fiber), DI-SPME (with DVB/CAR/PDMS fiber), and SBSE (with PDMS bar coating) for the analysis of volatiles in wine. The results presented in this study are not directly comparable, because different adsorbent material and extraction times were selected to compare different extraction techniques. Overall, in these studies, the efficiency of HS-SPDE was variable, and this extraction method should be further evaluated to understand its full potential in flavor analysis.

ITEX is a technique very similar to SPDE. It consists of a unique setup of a needle, a microtrap made of sorbent material, and a headspace syringe surrounded by an external heater (Figure 4). During extraction, the sample is heated under controlled conditions and the needle is inserted into the headspace of the sample vial. Similarly to SPDE, the plunger of the syringe is moved up

and down several times, to draw and concentrate the volatiles dynamically into the microtrap. The analytes are thermally desorbed in the GC injector with a purging gas (helium or nitrogen) which flows through the syringe. Different coating materials are available for different analytes. As with SPDE, the number of extraction strokes performed during the sorption step, is the most important parameter influencing the results (Laaks, Jochmann, Schilling, & Schmidt, 2015).

Together with the other dynamic extractions, SPDE and ITEX use large adsorption volumes and these techniques can be easily automated using a robotic autosampling system. Moreover, in SPDE and ITEX, the desorption step can be performed directly onto the injection port of the GC column. To date, very little research has been published on the use of ITEX or SPDE in food analysis.

Effect of salt and pH on the headspace extraction

The sample preparation, through the modification of pH levels or salt content can influence the yield of extraction of volatiles in the headspace. The practice of adding salt to the sample, or "salting out," is considered an efficient and low-cost method to increase the extraction rate of certain analytes. The added salt dissociates to its ions, reducing the solubility of specific analytes and forces these to move into the headspace. "Salting-out" facilitates the extraction of more polar and low-molecular-weight analytes, but it is not effective for high-molecular-weight analytes, which could adhere to the glass vial (Shirey, 2000). The most commonly used salt is sodium chloride, even though recent studies have highlighted new applications using bivalent or multivalent salts (Fiorini, Pacetti, Gabbianelli, Gabrielli, & Ballini, 2015; Pinho, Ferreira, & Ferreira, 2002). This technique showed positive effects on the extraction of isopropylamine, isopropanol (Shirey, 2000), carboxylic acids (Harmon, 2002; Fiorini et al., 2015), phenols (Buchholz & Pawliszyn, 1994), aromatic hydrocarbons (Djozan & Assadi, 1997), terpenoids (García, Magnaghi, Reichenbacher, & Danzer, 1996), amines (Müller, Fattore, & Benfenati, 1997), and Maillard reaction products (Coleman, 1996). Some adjustments may greatly influence the "salting-out" effect, as the headspace volume has to be kept as small as possible to increase the concentration of analytes. Also, stirring of the sample was observed to influence the extraction rate of specific analytes. The extraction/equilibration time of less volatile compounds was reduced by stirring, while having only a minimal effect on highly volatile compounds (Buchholz & Pawliszyn, 1994; Pillonel et al., 2002).

Moreover, variation in pH can influence the extraction rate of specific analytes in the headspace, particularly those weakly acidic or basic compounds, such as amines and phenols, which should be maintained in an undissociated form (Spietelun et al., 2013). The modification of pH facilitates the dissociation of the polar analytes, which become more easily extractable. Compounds with low pK_a (acid dissociation constant) are dissociated at acidic pH, while compounds with high pK_b (basic dissociation constant) are dissociated at alkaline pH. As expected, Shirey (2000) showed that acetic acid was best extracted at pH 2, while isopropylamine and propionitrile were best extracted at pH 11. In this study, it was not anticipated that the pH would have an effect on the extraction of nonpolar compounds; however, isopropanol and acetone were more efficiently extracted at high pH levels.

The modification pH and "salting out" are two methods recommended only for headspace extractions. In a direct immersion extraction, the adsorbent coating material cannot be resistant to strong alkaline/acidic pH, or at high salt concentration (Spietelun

et al., 2013). Prior to performing these techniques, it is recommended that a sample be completely homogenized with the salting agent, or with the basic/acidic solution, to ensure that the salt concentration, or the pH level, is uniform inside the matrix. Generally, in the analysis of cheese flavor, it is not a common practice to add salt, or modify pH, because there is no need to increase the extraction rate of certain chemical classes (nontargeted-analysis). However, acidification and the "salting out" effect are procedures used in the targeted-analysis of FFAs of cheese (Fiorini et al., 2015; Gonzalez-Cordova, & Vallejo-Cordoba, 2001; Pinho et al., 2002). Recently, Fiorini et al. (2015) analyzed the volatile FFAs of cheese with HS-SPME (with a DVB/CAR/PDMS), showing that the use of a mixture of multivalent salts, $(NH_4)_2SO_4/NaH_2PO_4$, improved the extraction of the short-chain FFAs (carbon chain lengths ≤ 5), while the monovalent salt NaCl performed better for the medium-chain FFAs (C8 to C10).

Gas chromatography

In flavor research, GC is the most commonly used analytical methodology to separate and identify volatile compounds. GC techniques use a mobile phase, which is an inert carrier gas (such as helium, hydrogen, nitrogen, or argon) to carry the extracted volatile compounds through a GC column. In volatile analysis, GC columns are heated in an oven following a set temperature program. The most volatile compounds elute first, and usually within the first 30 min; subsequently the column is heated to just below its maximum temperature to remove any volatile or lower volatile components, so that the column is uncontaminated for the next sample. Inside the GC column, the volatile compounds form weak electrostatic interactions with the stationary phase, based on the polarity of the compounds. The polarity of the stationary phase varies, usually depending on the analytes to be separated. Depending on the analyte volatility and degree of interaction with the stationary phase, the analytes elute from the column at different set times, during a fixed or isothermal temperature ramp. The volatile compounds are subsequently identified using a specific detector.

The factors which influence the performance of a GC system are inlet liners, type of injection (on-column, split, or splitless), type of column (stationary phase, length, diameter, or thickness), carrier gas (flow rate, pressure), and oven temperature (using cryotrap, ramping rates) (Kilcawley, 2017). The selection of the GC column is the most influential factor for the detection of the compounds (Imhof & Bosset, 1994). In the past, the analyses were performed on cheese using a packed GC column (Dumont et al., 1974a; Groux & Moinas, 1974), but fused silica open tubular capillary columns have become universally used; they have a much longer stationary phase, and thus offer better separation potential. GC capillary columns can have polar stationary phases (such as polyethyleneglycol, or nitroterephthalic-acid modified polyethyleneglycol), resulting in very efficient separation of polar compounds such as acids and some alcohols. Alternatively, columns can have nonpolar stationary phases (such as PDMS, phenyl-methylpolysiloxane), for the separation of compounds with low polarity such as aldehydes, ketones, esters, amines hydrocarbons, and phenols. Nonpolar columns tend to be much more stable than polar columns. However, in recent years, columns with intermediate stationary phases (phenyl-methylpolysiloxane, cyanopropylphenyl-methylpolysiloxane, or cyanopropylphenyl-dimethylpolysiloxane) are becoming more widely used, as they are more suitable for the detection of a wider range of polar and nonpolar volatile compounds.

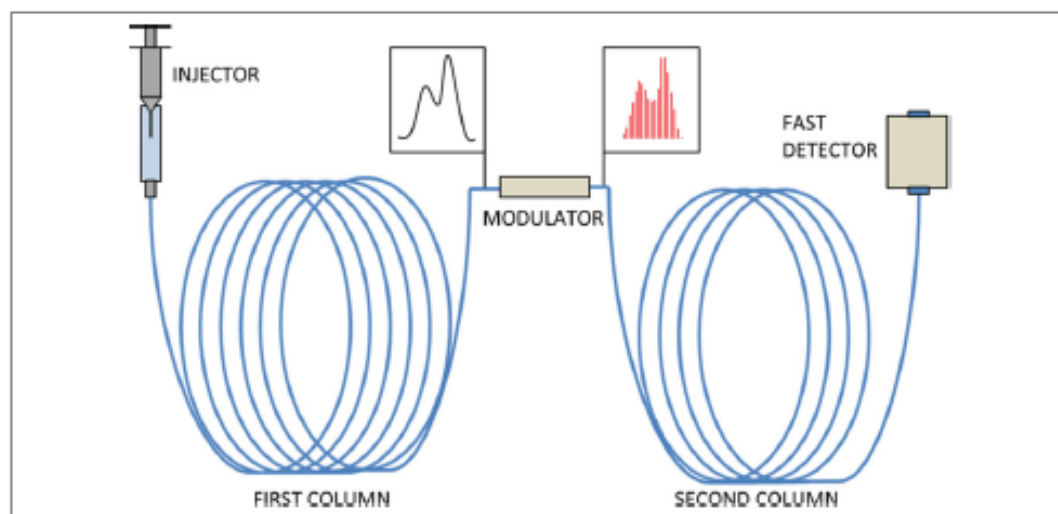


Figure 7—Two-dimensional gas chromatography system (GC $\times$ GC).

Imhof and Bosset (1994) compared different stationary phases for the GC analysis of volatiles of Swiss Emmentaler cheese. They stated that a GC capillary column, coated with a 4- μ m layer of 100% PDMS, had a better resolution, higher loading capacity, and higher number of volatiles detected than columns made of polyethyleneglycol and modified PDMS columns.

However, in a food matrix such as cheese both polar and non-polar compounds are present in considerable amounts; therefore it is advantageous to carry out the analysis with GC columns of different polarities or through a two-dimensional GC system.

Two-dimensional gas chromatography

In many cases one-dimensional GC cannot effectively separate co-eluting compounds. However, depending on the nature of the co-eluting compounds, these can still be identified and quantified successfully using advanced data processing deconvolution software. However, sometimes multiple compounds, or structurally very similar compounds, may co-elute, making identification and quantification more difficult or even impossible. Two-dimensional GC (GC $\times$ GC) is a powerful gas chromatographic technique that was developed to improve separation of co-eluting compounds in complex sample matrices. In GC $\times$ GC technology, the effluent passes through a first GC column and is then entirely (comprehensive GC $\times$ GC) or partially (heart-cutting GC $\times$ GC) transferred through a thermal, or flow modulator, to a second GC column, usually shorter and with a different polarity, and at the end reaches the detector (Figure 7). The sensitivity of the GC $\times$ GC systems is very high, because the volatiles which come from the first column are usually cryofocused in a trap and then released in the second GC column in a narrow band, generating a sharper signal (Kilcawley, 2017; Qian, Burbank, & Wang, 2007). The two GC columns, with different stationary phases, work independently and with different temperature settings, and thus have different interactions with the volatile compounds. The second column may also be set up in a separate column oven to provide more independent control of the temperature settings. Usually the first GC column is

selected to separate the compounds in relation to volatility, while the second GC column is used for the separation based mainly on differences in polarity (or hydrogen bonding with the stationary phase) (Qian et al., 2007; Pursch et al., 2002). The data from each GC column can be combined to have a resultant 2-dimensional chromatogram. In flavor analysis GC $\times$ GC is now widely applied to different food matrices, such as wine, fruit, coffee, and nuts (Dugo et al., 2014; Manzano, Diego, & Bernal, Nozal, & Bernal, 2014; Mommers, Plummakers, Knooren, Dutriez, & van der Wal, 2013; Samykanno, Pang, & Marriott, 2013). GC $\times$ GC can be used with single quadrupole mass spectrometry, but it is possibly best used with time-of-flight mass spectrometry as the acquisition rate is often higher thus providing more sensitivity.

Up to now, only a limited number of studies have been carried out using GC $\times$ GC for cheese analysis. For the detection of the volatiles of Cheddar cheese, Arora, Cormier, and Lee (1995) used a GC $\times$ GC system, using a nonpolar column, followed by a polar column, with a P&T extraction, while Gogus, Ozel, and Lewis (2006) selected a nonpolar column followed by a column of intermediate polarity, using a TD extraction. This technology increased the separation and identification power of the analysis of volatile components in cheese. It is clear that GC $\times$ GC has better sensitivity than those technologies with a single column. It is to be expected that GC $\times$ GC will be more widely applied to dairy products in the future to improve their flavor characterization.

Detection systems

After GC column separation it is necessary to identify and quantify the volatile compounds. The detection systems used are multiple and sophisticated and the selection of the correct technology depends on the product being analyzed and the range of analytes of interest. The detectors most commonly used in dairy research for the identification and quantification are: the mass spectrometry (MS) detector, flame ionization detector (FID), flame photometric detector (FPD), and pulsed flame photometric detector (PFPD). It is also possible to perform an olfactory analysis with the human nose, which is called GC-olfactometry (GC-O).

Flame ionization and flame detectors

In the past, flame ionization detectors (FIDs) were most frequently used for the detection of volatiles in food, but they now have been surpassed by MS detectors. FIDs have been the most commonly used detectors in GC analysis and are very popular for the detection of flavor compounds in cheese (Bergamini, Wolf, Perotti, & Zalazar, 2010; Peralta et al., 2014; Wolf, Peralta, Candiotti, & Perotti, 2016). In FID, the column effluent is combusted by an air-hydrogen flame, causing ionization of compounds and producing a current which is detected and converted to a signal. FIDs are widely used due to low cost, long-term reliability, and universal applicability for compounds with carbon-hydrogen bonds in their chemical structure (Mariaca, & Bosset, 1997; Rahman, El-Aty, & Shim, 2015).

A FPD or PFPD is characterized by the combustion of compounds which produce a flame emission passing across a photomultiplier window. Thereafter, the flame emission of each compound can be electronically separated and elaborated as a signal. In the PFPD, the flaming process is not continuous, and some classes of sulfur- or phosphorus-containing compounds are separated because they have a delay in the flame emission. These classes of compounds produce a flame emission also during the post-pulsed flame time, due to lower chemical bond energies. For this reason, a PFPD has more selectivity and sensitivity for sulfur, phosphorus, and nitrogen than a FPD, where the flaming is continuous. The PFPD is of low cost, easy to use, and highly selective for sulfur- or phosphorus-containing compounds. The main advantage of the PFPD for dairy products is that it is very sensitive for the detection of sulfur compounds, which are major contributors to the strong flavor associated with many cheese varieties, especially surface-ripened cheeses (Jing & Amirav, 1998; Mariaca & Bosset, 1997). For this reason, this detector is used for studies mainly focused on the detection of volatile sulfur compounds (O'Brien et al., 2017; Parliment, Kolor, & Rizzo, 1982). Burbank and Qian (2005) optimized HS-SPME with CAR/PDMS fiber, associated with PFPD, to investigate the volatile sulfur compounds of Cheddar cheese. Burbank and Qian (2005) found a predominance of certain types of compounds such as dimethyl sulfide, methanethiol and hydrogen sulfide when analyzing Cheddar cheese at various stages of maturity.

MS detectors

The principle of MS is based on the ionization of a molecule and the resolution of the ionized molecule based on mass-to-charge (m/z) ratios in an electrostatic field (Croissant, Watson, & Drake, 2011). Every sample analyzed with GCMS produces a total ion chromatogram which then has to be analyzed and interpreted. It is important to have a good-quality chromatogram to facilitate the identification of all volatile compounds inside the sample (nontargeted-analysis). However, the resulting chromatogram may present a complex background which can interfere with the analysis. Specific software is now used to deconvolute the chromatogram to eliminate background (noise). During the chromatographic analysis each peak is identified by comparing the mass spectra to commercial or internally developed libraries in order to identify every peak with a specific compound. It is also possible to calculate the "linear retention index" (LRI), based on retention time in comparison to a set of specific standards to aid sample identification. The LRI of each compound analyzed is compared with data in an internal or/and external database to confirm the identification. In the case of isothermal analysis (constant oven temperature throughout the GC run), the

LRI could be calculated according with Kovats (1965), as shown below:

$$LRI_A = 100N + 100 \frac{\log t_{R(A)} - \log t_{R(N)}}{\log t_{R(N+1)} - \log t_{R(N)}}$$

when a temperature program is applied to the GC analysis (heating the oven at a controlled rate throughout the GC run) the LRI is calculated according with Van den Dool and Kratz (1963) as follows:

$$LRI_A = 100N + 100 \frac{t_{R(A)} - t_{R(N)}}{t_{R(N+1)} - t_{R(N)}}$$

where LRI_A is the linear retention index of compound A, $t_{R(A)}$ is the retention time of compound A, $t_{R(N)}$ and $t_{R(N+1)}$ is the retention time of the alkane with carbon number N and $N+1$, respectively. N is the carbon number of the alkane which elutes earlier than the compound analyzed. For the detection of specific analytes (targeted-analysis) the use of standards, which indicate the compounds to identify, is a more practical method.

The quantification of the spectra is performed by calculating the peak area in the chromatogram for each compound identified. Different software packages are commercially available for deconvolution, identification, and quantification.

The most commonly used MS detectors are quadrupole mass analyzers whereby an electric field is produced by applying a voltage across 4 hyperbolic rods. Each adjacent rod has the opposite charge compared to its nearest counterpart. When DC voltage is applied and the voltage signs are rapidly reversed, varying electrical fields are produced, enabling ions with specific mass-to-charge ratios to pass (Rahman et al., 2015).

MS detector systems can be used in full-scan mode (total ion current, TIC) to identify compounds via their fragmentation profile, comparing the mass spectra to a library which allows the identification of the compounds in relation to the similarity with reference spectra. Alternatively, MS detectors can be used in selected ion storage (SIS) or selected ion monitoring (SIM) mode to detect only a small number of ions and quantify specific compounds with high selectivity and sensitivity (Mariaca & Bosset, 1997).

Recently, triple quadrupole (QqQ) mass spectrometers have been used for confirmatory analysis. This detector is composed by 2 mass filter quadrupoles separated by a collision mass quadrupole (fragmentator). Despite its high efficiency, this type of MS detector is not commonly employed for cheese analysis due to the low mass of volatile compounds in cheese which would be further fragmented with QqQ (Kilcawley, 2017). However, a time-of-flight (TOF) detector has been used in association with GC×GC in Cheddar cheese (Gogus et al., 2006). This detector is based on an ion's charge-to-mass ratio versus time. An electrical field of known strength is used to accelerate ions. Ions of the same charge (equal kinetic energy) are accelerated through this electrical field at the same rate. The velocity of the ion depends on its mass-to-charge ratio. By calculating the length of time it has taken for an ion to reach the detector at a known distance, the mass-to-charge ratio of the ion can be estimated (heavier particles travel at lower speeds) (Rahman et al., 2015). The high cost of this technology is the main reason why TOF MS has not been as widely used as quadrupole MS in cheese flavor research.

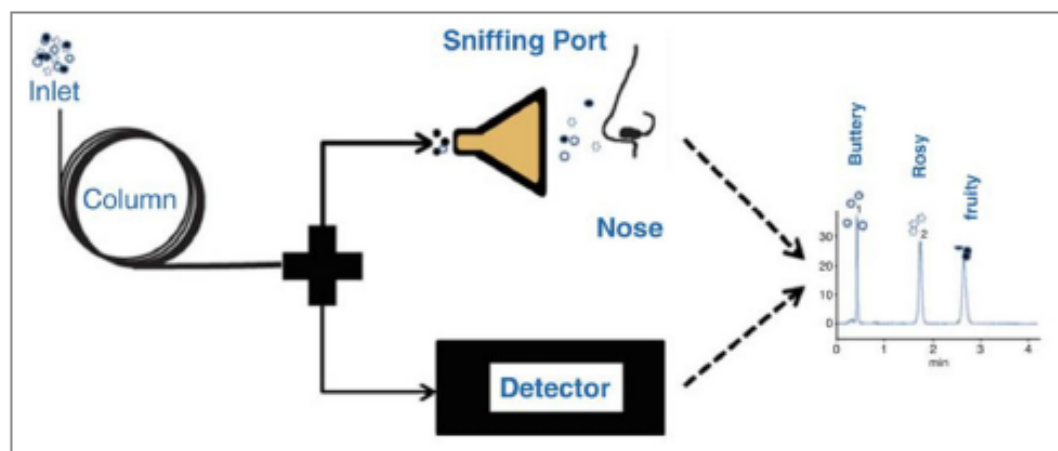


Figure 8—Gas chromatography olfactory system (GC-O), from Kilcawley (2017).

Gas chromatography olfactometry

In this technique the human nose is used as a detector. A sniffing port is connected directly to the end of the GC column and used to perform continuous olfactory analysis by an individual(s). With respect to cheese and food in general, not all volatiles, even if present in high quantities, affect the flavor profile, while others detected in small quantities can be extremely significant (D'Acampora Zellner et al., 2008; Delahunty, Eyres, & Dufour, 2006). Through the GC-O technique it is possible to discriminate between odor-active and nonodor-active compounds and report the duration and the intensity of the corresponding odor activity. Moreover, GC-O enables the association of an odor-active compound with an individual sensory descriptor for aroma characterization.

In GC-O, experienced assessors sniff the effluent of the column to detect the presence of odor-active compounds via a specifically designed odor port (Figure 8). The effluent of the column is split into 2 portions; one goes to the GC-O port and the other one to a detector (usually MS or FID); in this way it is possible to relate the odors sniffed at the GC-O port with the volatile compounds identified by the detector at the same time. Different methodologies varying in complexity are available to interpret data obtained by GC-O. In the "detection frequency" method, the compounds detected more frequently by a panel of assessors are considered the most relevant and the corresponding odor durations are measured. Thus, it is possible to build an aromagram where the breadth of the peaks is related to the odor duration, while the height is related to the number of odor detections. In other methods called "dilution to threshold", such as "aroma extraction dilution analysis" (AEDA) and CharmAnalysis™, the sample is diluted (usually by a factor of 2 to 3) to reach the odor threshold of the compounds in air. In AEDA, it is not necessary to register the odor duration or the intensity, as it only considers the presence of odor-active compounds at different dilutions. Thus, AEDA measures the maximum dilution at which it is still possible to perceive an odor-active compound, and this value is called the "flavor dilution factor". With CharmAnalysis™, the Charm value is calculated for each odor-active compound through an algorithm. This algorithm considers the dilution factor, the number of coincident odor responses detected at a single retention index, and the odor duration (D'Acampora Zellner et al., 2008; Delahunty et al., 2006).

Another method called "direct intensity" is used to classify the intensity of the odor-active compounds using a scale of measurement. The data are elaborated in an aromagram taking into account the intensity and duration of the odors perceived (Delahunty et al., 2006).

GC-O has been widely applied to different varieties of cheese enabling the association between volatile compounds and aroma notes (Fuchsmann, Stern, Brügger, & Breme, 2015; Thomsen et al., 2012; Zabaleta et al., 2016). However, GC-O was also recently used together with GC×GC. The combination of GC-O with GC×GC-TOF considerably increased the identification of odor-active compounds in different food matrices (Cordero, Kiefl, Schieberle, Reichenbach, & Bicchi, 2015).

Volatiles in surface-ripened cheese

The right selection of the techniques and analytical tools for the extraction and separation of volatile compounds is important for the flavor characterization of the various cheese types, including surface-ripened cheese. Surface-ripened cheeses belong to the category of rennet-coagulated cheese, and are a very heterogeneous group. They can be soft (such as Münster or Munster and Reblochon), semi-hard (Tilsit, brick, Port Salut, Livarot, and Limburger) or hard (Gruyère and Comté), with a variable period of ripening (Cogan et al., 2014; Fox et al., 2017b; Mounier et al., 2017).

The sensory properties of surface-ripened cheese are very variable and have been described using a myriad of descriptive terms including oily, rancid (Chaumes), pepper undertones (Appenzeller), and pungent, silage, and sweaty/sour odor with a rancid, mushroom, oily, smoky, silage, bitter, and burnt after-taste (Tête de Moine and Raclette) (Lawlor & Delahunty, 2000; Lawlor et al., 2002). In an in-depth analysis of 152 European cheeses, Koppel and Chambers (2012) defined the general aroma profiles of surface-ripened cheese, soft and semi-hard, as musty, moldy, sour, salty, and buttery.

Even if flavor studies on surface-ripened cheese are limited, different extraction techniques and stationary phases were used for the volatile analysis of several surface-ripened cheeses, thus influencing their volatile profiles (Figure 9), and the volatile compounds most commonly identified were mainly aldehydes, alcohols,

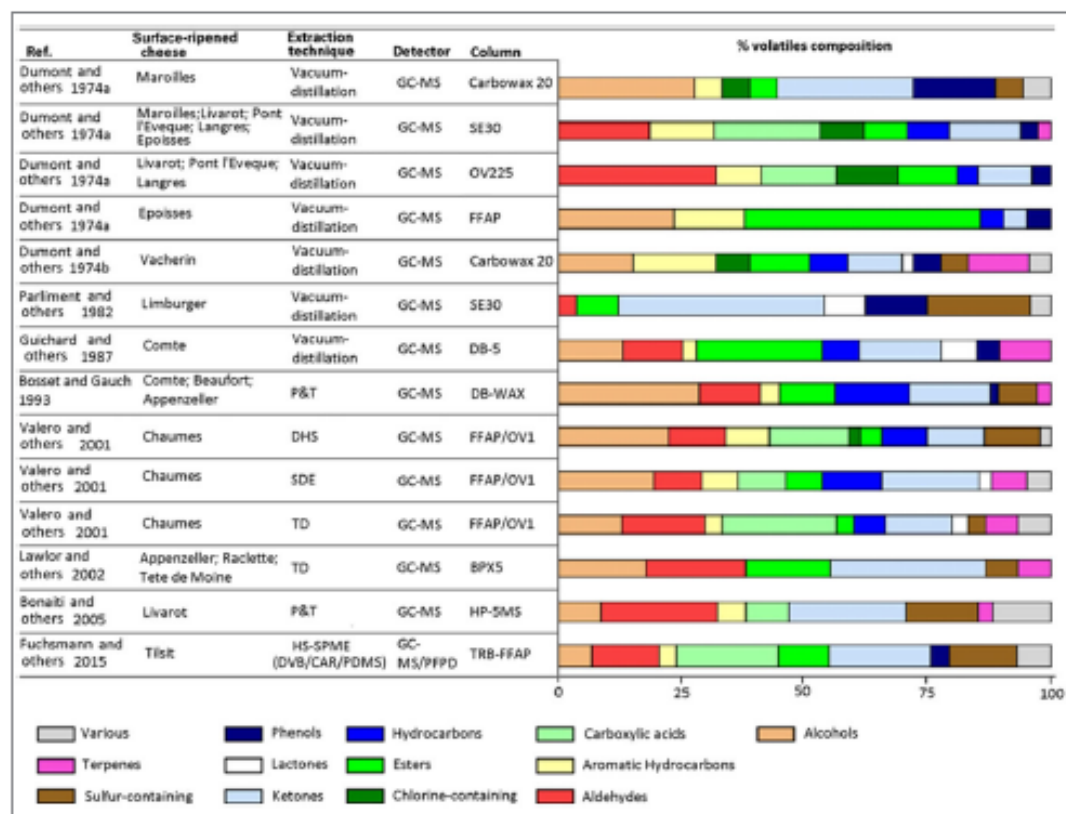


Figure 9—Percentages of volatile compounds detected in different surface-ripened cheese varieties.

carboxylic acids, methyl ketones, ethyl esters, sulfur compounds, and aromatic hydrocarbons (Table 2).

Aldehydes have mainly been identified in some Swiss and French surface-ripened cheeses (Table 2). Hexanal and 3-methylbutanal were the aldehydes most commonly detected. Hexanal is characterized by green, slightly fruity, lemon, and herbal notes, while 3-methylbutanal is related to malty, powerful, cheese, green, and dark-chocolate notes (Curioni & Bosset, 2002; Kilcawley, 2017; Thomsen et al., 2012). Benzaldehyde was also frequently detected, and it was usually associated with bitter almond and sweet cherry flavors (Singh et al., 2003; Smit et al., 2005). In surface-ripened cheese, aldehydes were detected in high percentage using nonpolar/medium-polar stationary phase GC, especially with TD and P&T extractions (Bonaïti, Irlinger, Spinner, & Engel, 2005; Lawlor et al., 2002; Valero et al., 2001) (Figure 9).

Generally in surface-ripened cheese, alcohols are the volatile compounds detected in highest numbers, when compared with other classes of compounds (Table 2). The primary alcohols mostly detected were ethanol (dry dust, alcohol notes), 1-propanol (sweet and wine-like notes), and 3-methylbutanol (fresh cheese, breath-taking, alcoholic, fruity, grainy, and solvent-like notes), while the most common secondary alcohol identified was 2-butanol (sweet, fruity fusel oil, and wine-like) (Kilcawley, 2017). In surface-

ripened cheese, a high percentage of alcohols were identified, using intermediate stationary phase, mainly with vacuum-distillation and P&T extractions (Bosset & Gauch, 1993; Dumont et al., 1974a, 1974b) (Figure 9).

Carboxylic acids, such as butanoic, 2-methylbutanoic, hexanoic, and octanoic acid, have not been frequently identified in surface-ripened cheese (Table 2), because the GC columns used for volatile compound detection are usually nonpolar columns, which are less suitable for polar compounds (Croissant et al., 2011). Moreover, at the pH levels of surface-ripened cheese (pH ~ 6), carboxylic acids are present as nonvolatile salts, thereby reducing their flavor impact (Singh et al., 2003). When identified in surface-ripened cheese types, carboxylic acids (Table 2) had strong and unpleasant odors, described as sweaty, rancid, and cheesy (Kilcawley, 2017). As expected, in surface-ripened cheese the use of medium-polar/polar stationary phases greatly influenced the detection of carboxylic acids, which were extracted with various techniques (TD and HS-SPME) (Fuchsmann et al., 2015; Valero et al., 2001) (Figure 9).

Some methyl ketones, such as 2-butanone, 2-pentanone, 2-nonanone, and 2-heptanone, have commonly been identified in a wide range of surface-ripened cheese varieties (Table 2). Sweet, ether-like, slightly nauseating notes were associated with 2-butanone; orange peel, sweet, and fruity notes, with 2-pentanone;

Table 2—Principal volatile compounds most frequently detected in surface-ripened cheese varieties, and related extraction techniques utilized, namely vacuum-distillation, DHS (dynamic headspace extraction), TD (thermal desorption), HS-SPME (headspace solid-phase microextraction), P&T (purge and trap), and SDE (simultaneous distillation-extraction).

Compounds	Surface-ripened cheese	Extraction technique	References
Butanoic acid	Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; TD; HS-SPME	Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
3-Methylbutanoic acid	Limburger; Livarot; Tilsit	Vacuum-distillation; P&T; HS-SPME	Parliment et al., 1982; Bonaiti et al., 2005; Fuchsmann et al., 2015
Hexanoic acid	Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; TD; HS-SPME	Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
Octanoic acid	Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; SDE; TD; HS-SPME	Parliment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
3-Methylbutanal	Comté; Beaufort; Appenzeller; Chaumes; Tilsit	P&T; DHS; TD; HS-SPME	Bosset and Gauch 1993; Valero et al., 2001; Fuchsmann et al., 2015
Hexanal	Comté; Chaumes; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; SDE; TD	Guichard et al., 1987; Valero et al., 2001; Lawlor et al., 2002
Benzaldehyde	Comté; Raclette; Tête de Moine; Livarot; Tilsit	Vacuum-distillation; P&T; TD; HS-SPME	Guichard et al., 1987; Bosset and Gauch 1993; Lawlor et al., 2002; Bonaiti et al., 2005; Fuchsmann et al., 2015
2-Butanone	Vacherin; Maroilles; Livarot; Pont l'Eveque; Comté; Beaufort; Appenzeller; Raclette; Tête de Moine; Tilsit	Vacuum-distillation; P&T; TD; HS-SPME	Groux and others 1974; Dumont et al., 1974a; Bosset and Gauch 1993; Lawlor et al., 2002; Fuchsmann et al., 2015
2-Pentanone	Vacherin; Maroilles; Limburger; Chaumes; Appenzeller; Raclette; Tête de Moine; Livarot	Vacuum-distillation; DHS; TD; P&T	Groux and others 1974; Dumont et al., 1974a; Parliment et al., 1982; Valero et al., 2001; Lawlor et al., 2002; Bonaiti et al., 2005
2-Heptanone	Maroilles; Pont l'Eveque; Langres; Vacherin; Livarot; Limburger; Comté; Beaufort; Appenzeller; Chaumes; Raclette; Tête de Moine	Vacuum-distillation; DHS; SDE; TD; P&T	Groux and others 1974; Dumont et al., 1974a, b; Parliment et al., 1982; Guichard et al., 1987; Bosset and Gauch 1993; Valero et al., 2001; Lawlor et al., 2002; Bonaiti et al., 2005
2-Octanone	Limburger; Comté; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; TD	Parliment et al., 1982; Guichard et al., 1987; Lawlor et al., 2002
2-Nonanone	Maroilles; Pont l'Eveque; Langres; Epoisses; Vacherin; Livarot; Limburger; Comté; Beaufort; Appenzeller; Chaumes; Raclette; Tête de Moine	Vacuum-distillation; DHS; SDE; TD; P&T	Groux and others 1974; Dumont et al., 1974a, b; Parliment et al., 1982; Guichard et al., 1987; Bosset and Gauch 1993; Valero et al., 2001; Lawlor et al., 2002; Bonaiti et al., 2005
2-Undecanone	Maroilles; Epoisses; Vacherin; Limburger; Comté; Livarot	Vacuum-distillation; P&T	Groux and others 1974; Dumont et al., 1974a, b; Parliment et al., 1982; Guichard et al., 1987; Bonaiti et al., 2005
Acetophenone	Maroilles; Livarot; Pont l'Eveque; Langres; Limburger; Comté	Vacuum-distillation	Dumont et al., 1974a; Parliment et al., 1982; Guichard et al., 1987
Ethanol	Vacherin; Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; P&T; TD	Groux and others 1974; Dumont et al., 1974a; Bosset and Gauch 1993; Lawlor et al., 2002
1-Propanol	Maroilles; Pont l'Eveque; Langres; Epoisses; Vacherin; Livarot; Comté; Beaufort; Appenzeller	Vacuum-distillation; P&T	Groux and others 1974; Dumont et al., 1974a, b; Bosset and others 1993
2-Propanol	Pont l'Eveque; Langres; Comté; Appenzeller; Chaumes; Livarot	Vacuum-distillation; TD; P&T	Dumont et al., 1974a; Bosset and Gauch 1993; Valero et al., 2001; Bonaiti et al., 2005
2-Methylpropanol	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Appenzeller	Vacuum-distillation; P&T	Dumont et al., 1974a; Bosset and others 1993
1-Butanol	Pont l'Eveque; Epoisses; Comté; Beaufort; Appenzeller	Vacuum-distillation; P&T	Dumont et al., 1974a; Bosset and others 1993
2-Butanol	Maroilles; Livarot; Pont l'Eveque; Langres; Vacherin; Comté; Beaufort; Appenzeller; Raclette; Tête de Moine	Vacuum-distillation; P&T; TD	Dumont et al., 1974a, b; Bosset and Gauch 1993; Lawlor et al., 2002
2-Methylbutanol	Comté; Beaufort; Appenzeller; Tête de Moine	P&T; TD	Bosset and Gauch 1993; Lawlor et al., 2002
3-Methylbutanol	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Vacherin; Comté; Beaufort; Appenzeller; Chaumes	Vacuum-distillation; P&T; DHS; TD	Dumont et al., 1974a, b; Bosset and Gauch 1993; Valero et al., 2001
2-Pentanol	Vacherin; Maroilles; Pont l'Eveque; Comté; Beaufort; Appenzeller; Chaumes; Raclette	Vacuum-distillation; P&T; DHS; TD	Groux and others 1974; Dumont et al., 1974a; Bosset and Gauch 1993; Valero et al., 2001; Lawlor et al., 2002
Hexanol	Livarot; Pont l'Eveque; Comté; Chaumes	Vacuum-distillation; P&T; TD	Dumont et al., 1974a; Guichard et al., 1987; Bosset and Gauch 1993; Valero et al., 2001
2-Heptanol	Maroilles; Pont l'Eveque; Langres; Vacherin; Comté; Appenzeller; Chaumes	Vacuum-distillation; P&T; DHS; SDE	Groux and others 1974; Dumont et al., 1974a, b; Guichard et al., 1987; Bosset and Gauch 1993; Valero et al., 2001
2-Nonanol	Livarot; Pont l'Eveque; Langres; Vacherin; Comté	Vacuum-distillation	Dumont et al., 1974a, b; Guichard et al., 1987
Phenol	Maroilles; Livarot; Pont l'Eveque; Vacherin; Limburger; Chaumes	Vacuum-distillation; DHS; SDE; TD	Dumont et al., 1974a, b; Parliment et al., 1982; Valero et al., 2001
2-Phenylethanol	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Limburger; Comté; Chaumes	Vacuum-distillation; TD	Dumont et al., 1974a; Parliment et al., 1982; Guichard et al., 1987; Valero et al., 2001
Cresol	Livarot; Pont l'Eveque; Langres; Limburger; Comté	Vacuum-distillation	Dumont et al., 1974a; Parliment et al., 1982; Guichard et al., 1987
Ethyl acetate	Maroilles; Livarot; Pont l'Eveque; Langres; Epoisses; Comté; Beaufort; Appenzeller; Tête de Moine	Vacuum-distillation; P&T; TD	Dumont et al., 1974a; Bosset and Gauch 1993; Lawlor et al., 2002

(Continued)

Table 2—Continued.

Compounds	Surface-ripened cheese	Extraction technique	References
Ethyl butanoate	Langres; Epoisses; Comté; Chaumes; Appenzeller; Raclette; Tête de Moine; Tilsit	Vacuum-distillation; TD; HS-SPME	Dumont et al., 1974a; Guichard et al., 1987; Valero et al., 2001; Lawlor et al., 2002; Fuchsmann et al., 2015
Ethyl hexanoate	Livarot; Pont l'Évêque; Langres; Epoisses; Comté; Beaufort; Chaumes; Tête de Moine; Tilsit	Vacuum-distillation; P&T; TD; HS-SPME	Dumont et al., 1974a; Guichard et al., 1987; Bosset and Gauch 1993; Valero et al., 2001; Lawlor et al., 2002; Fuchsmann et al., 2015
Ethyl octanoate	Livarot; Pont l'Évêque; Langres; Epoisses; Limburger; Chaumes; Tilsit	Vacuum-distillation; SDE; HS-SPME	Dumont et al., 1974a; Parment et al., 1982; Valero et al., 2001; Fuchsmann et al., 2015
Methyl thioacetate	Pont l'Évêque; Epoisses; Limburger; Beaufort; Chaumes	Vacuum-distillation; P&T; DHS	Dumont et al., 1974a; Parment et al., 1982; Bosset and Gauch 1993; Valero et al., 2001
Dimethyl disulfide	Maroilles; Pont l'Évêque; Langres; Epoisses; Vacherin; Livarot; Limburger; Chaumes; Raclette; Tête de Moine	Vacuum-distillation; DHS; SDE; TD; P&T	Dumont et al., 1974a; Parment et al., 1982; Valero et al., 2001; Lawlor et al., 2002; Bonaiti et al., 2005
Dimethyl trisulfide	Maroilles; Pont l'Évêque; Langres; Livarot; Limburger; Chaumes; Tilsit	Vacuum-distillation; DHS; TD; P&T; HS-SPME	Dumont et al., 1974a; Parment et al., 1982; Valero et al., 2001; Lawlor et al., 2002; Bonaiti et al., 2005; Fuchsmann et al., 2015
Indole	Maroilles; Livarot; Pont l'Évêque; Langres; Vacherin; Limburger; Chaumes	Vacuum-distillation; SDE; TD	Dumont et al., 1974a; Parment et al., 1982; Valero et al., 2001
Toluene	Pont l'Évêque; Langres; Epoisses; Vacherin; Livarot; Chaumes	Vacuum-distillation; DHS; SDE; TD; P&T	Dumont et al., 1974a; Valero et al., 2001; Bonaiti et al., 2005
Benzene	Maroilles; Livarot; Pont l'Évêque; Langres; Epoisses; Chaumes	Vacuum-distillation; TD	Dumont et al., 1974b; Valero et al., 2001
Naphthalene	Maroilles; Livarot; Langres; Epoisses; Comté	Vacuum-distillation	Dumont et al., 1974a; Guichard et al., 1987

malty, rotten fruit, hot milk, green, and earthy notes, with 2-nonanone (Kilcawley, 2017). Blue cheese notes were mainly attributed to 2-heptanone (Curioni & Bosset, 2002; Singh et al., 2003). In surface-ripened cheese, a high percentage of ketones were detected, using a nonpolar stationary phase with vacuum-distillation, and TD extractions (Lawlor et al., 2002; Parment et al., 1982) (Figure 9).

Esters have an interesting impact on the development of surface-ripened cheese flavor, due to their sweet, fruity, and floral notes (Niimi et al., 2015; Urbach, 1997). Ethyl acetate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate represent the major esters commonly identified in many varieties of surface-ripened cheese (Table 2). Higher percentages of esters were detected with vacuum-distillation, using both nonpolar or polar stationary phase for the separation (Dumont et al., 1974a; Guichard et al., 1987) (Figure 9).

Another group of volatile compounds widely detected in surface-ripened cheese, and considered one of the key compounds for flavor, are the sulfur compounds (Table 2), especially dimethyltrisulfide and dimethyldisulfide (Curioni & Bosset, 2002; Sablé & Cottenceau, 1999). Dimethyltrisulfide is generally associated with odor notes of vegetable, sulfurous, garlic, putrid, and cabbage-like aromas, while dimethyldisulfide is related to green, sour, and onion notes (Kilcawley, 2017). In surface-ripened cheese, the detection of sulfur compounds was performed effectively using a nonpolar stationary phase, with vacuum-distillation, and P&T extractions (Bonaiti et al., 2005; Parment et al., 1982). However, Fuchsmann et al. (2015) detected high numbers of sulfur compounds using a polar stationary phase, and HS-SPME, with a MS/PTPD detector (Figure 9).

Aromatic hydrocarbons, such as indole and toluene, were identified mainly in soft and semi-soft French surface-ripened cheese (Table 2). Indole is considered one of the compounds responsible for unclean-utensil, rose-like off-flavors, while toluene is associated with nutty, and rancid odors (Delgado, González-Crespo, Cava, & Ramírez, 2011; Yvon & Rijnen, 2001). In surface-ripened cheese, the detection of aromatic hydrocarbons was not particularly affected by the polarity of the stationary phase, but was mainly influenced by the use of the vacuum-distillation extraction, which is considered a reliable technique for the detec-

tion of high-molecular-weight compounds (Dumont et al., 1974a, 1947b) (Figure 9).

Other compounds, often identified in surface-ripened cheese, are terpenes. A range of studies looking at a variety of surface-ripened cheese types such as Vacherin, Comté, Beaufort, and Tête de Moine found that a broad spectrum of terpenes was present (Bosset & Gauch, 1993; Dumont, Roger, Paule, & Adda, 1974b; Guichard et al., 1987). Terpenes, as aromatic hydrocarbons are high-molecular-weight compounds and were predictably extracted in high percentages by vacuum-distillation and SDE (Dumont et al., 1974b; Guichard et al., 1987; Valero et al., 2001) (Figure 9).

Conclusion

Numerous extraction techniques are available for the analysis of cheese volatiles. However, the techniques available all have some limitations. The extraction technique, the nature of the extraction polymeric material, the GC column, and the detector greatly influence the sensitivity and reliability of the detection of specific analytes; evident from studies performed on surface-ripened cheese varieties, where the volatiles detected were predictably based on the characteristics of the extraction techniques and the column stationary phase used. At present, the various analytical approaches have to be considered as complementary techniques in order to get a complete volatile profile. Further work is required to fully elucidate the flavor characteristics of surface-ripened cheese.

Acknowledgment

Funding was provided by the Teagasc Walsh Fellowship program.

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Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese



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ARTICLE INFO

Article history:

Received 12 December 2016

Received in revised form

3 April 2017

Accepted 4 April 2017

Available online 17 April 2017

ABSTRACT

The strains *Staphylococcus saprophyticus* DPC5671 and *Corynebacterium casei* DPC5298 were applied in combination with *Debaryomyces hansenii* DPC6258 to the surface of young Cheddar cheese curd to obtain two different smear-ripened cheeses. A surface microbiota developed over the incubation period, comprising of both yeast and bacteria; pulsed field gel electrophoresis confirmed that the inoculated strains of *S. saprophyticus* DPC5671 or *C. casei* DPC5298 were the dominant bacterial strains on the surface of the cheese at the end of the ripening period. The smear cultures changed the appearance and aroma, which were significantly different from the control cheese. The approach presented in this study represents a method for the development of new cheese varieties with novel aromas within a short ripening time.

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1. Introduction

Smear cheese is a traditional dairy product, which plays an important role in both small and industrial scale dairy production. Smear cheese is characterised by a short ripening time and strong aroma produced by the growth of smear microbiota on the cheese surface. Smear-ripened cheeses are manufactured by inoculating the surface of the cheese curd, dipping, spraying or brushing with a mixture of bacteria and yeasts. The traditional method of production is called "old-young smearing" and consists of washing young curds with the brine from old cheese, to encourage the transfer of the microbiota from the old to the young cheeses (Desmasures, Bora, & Ward, 2015; Fox, Guinee, Cogan, & McSweeney, 2017a).

The microbiota on the surface of the smear cheese is composed of a variety of microorganisms that coexist in symbiotic relationships. Yeasts are normally the first resident microorganisms to establish themselves on the surface of the cheese due to their tolerance to low pH and salt. Yeasts metabolise lactate, producing H₂O and CO₂ and increase the pH (Cholet, Hénaut, Casaregola, &

Bonnarme, 2007; Corsetti, Rossi, & Gobetti, 2001). Moreover, they produce metabolites and growth factors (vitamins and amino acids) which encourage the growth of Gram-positive bacteria, such as *Corynebacterium*, *Staphylococcus* and *Brevibacterium* species (Cogan et al., 2014; Larpin et al., 2011).

The growth of smear microorganisms on the surface of cheese curd modifies the appearance, aroma, proteolysis and lipolysis of the cheese within a relatively short ripening time (McSweeney, 2004). The combined growth of the bacteria and yeasts on the surface of the cheese results in the production of proteolytic and lipolytic enzymes, increasing the amount of free amino acids (FAAs) and free fatty acids (FFAs) (McSweeney & Sousa, 2000; Sousa, Ardô, & McSweeney, 2001). Yeasts and Gram-positive bacteria isolated from smear cheeses have a wide range of proteolytic enzymes that display various peptidase activities, with FAAs increasing within the cheese as a consequence. Additionally, yeasts and Gram-positive bacteria possess esterolytic/lipolytic enzymes capable of catabolising triacylglycerols in cheese, producing FFAs (Curtin, Gobetti, & McSweeney, 2002; Fox, Guinee, Cogan, & McSweeney, 2017b).

The further metabolism of FAAs and FFAs during the ripening produces flavour compounds important for cheese aroma. The catabolism of FAAs, especially of branched chain amino acids, aromatic amino acids and sulphur amino acids, produces mainly aldehydes, alcohols, carboxylic acids and sulphur compounds.

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Moreover, FFAs are involved in reactions leading to the production of flavour compounds such as secondary alcohols, carboxylic acids, esters, lactones and ketones (McSweeney & Sousa, 2000; Singh, Drake, & Cadwallader, 2003; Smit, Smit, & Engels, 2005; Yvon & Rijnen, 2001).

The characteristics of smear-ripened cheese are not strictly controlled inside artisanal smear cheese plants. The resulting product is also affected by the final microbial consortia of the cheese, which is influenced by the individual in-house microbiota of the cheese-making facilities. Microorganisms detected in the environment of artisanal cheese-making plants have also been found on the surface of smear cheeses, indicating a strong relationship between product and the environment in which the cheese is manufactured and ripened (Bokulich & Mills, 2013; Goerges et al., 2008; Mounier et al., 2006a).

In previous studies, smear strains were added to the cheese surface or as adjunct cultures to the milk during manufacture of smear-ripened cheese; however, some of the added strains were not detected at the end of ripening (Feurer, Vallaeys, Corrieu, & Iringer, 2004; Goerges et al., 2008). These commercial smear strains have to compete with the in-house microbiota and do not always successfully establish themselves on the cheese surface (Bokulich & Mills, 2013; Feuer et al., 2004; Goerges et al., 2008). It is likely that the relationship within the smear microbiota promotes the survival of a particular group of microorganisms to the detriment of others.

With the abolition of the milk quotas within the EU in 2015 there is a renewed interest in developing novel cheeses with a range of flavours. There is a progressive increase in global cheese consumption, with an annual production in Ireland of 207,100 tonnes in 2015 (data from Eurostat). Therefore the aim of this work was to develop a novel cheese with diverse aromas and short ripening time using cheese curd made in a traditional Cheddar cheese plant. Ripening time for Cheddar cheese can be from a little as 3 months for mild cheese up to > 9–12 months for mature/extra mature varieties. In this study, the ability of smear bacteria and yeast to grow on the surface of young Cheddar cheese curd was investigated to produce a cheese variety with different flavour and appearance compared with Cheddar cheese within a short time frame of 35 days.

2. Materials and methods

2.1. Preparation of smear suspensions

For the preparation of the *Debaryomyces hansenii* DPC6258 suspension, the strain was streaked onto yeast extract glucose chloramphenicol agar (YGC agar; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated aerobically at 25 °C for 96 h. Using a 5 µL loop, the strain was inoculated into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company) and incubated, shaking at 100 rpm, at 25 °C. When the OD₆₀₀ reached ~1, the cells were centrifuged at 6000 × g at 4 °C for 15 min, washed twice with sterile 0.75% NaCl and the pellet was resuspended in sterile 0.75% NaCl to obtain a suspension of ~10⁸ cfu mL⁻¹.

For the preparation of the *Corynebacterium casei* DPC5298 and *Staphylococcus saprophyticus* DPC5671 suspensions, the strains were streaked onto trypticase soy agar (TSA; Becton, Dickinson and Company) and incubated aerobically at 30 °C for 48 h. Using a 5 µL loop, the strains were inoculated into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company) and incubated, shaking at 100 rpm, at 30 °C. When the OD₆₀₀ reached ~1, the cells were centrifuged at 6000 × g at 4 °C for 15 min, washed twice with sterile 0.75% NaCl and the pellets resuspended in sterile 0.75% NaCl to obtain a suspension of ~10⁸ cfu mL⁻¹.

2.2. Smearing of cheese blocks

Cheddar cheese was supplied by a commercial cheese company as 20 kg blocks, <24 h post manufacture. The large cheese block was aseptically cut into smaller blocks (~8 × 6.5 × 30 cm). These blocks were then inoculated by placing them in a saline suspension containing *D. hansenii* DPC6258 (10⁸ cfu mL⁻¹), ensuring an even coating of yeast. Subsequently the blocks of cheese were placed on sterile, plastic coated racks and allowed to drain. Once the excess liquid had completely drained, the cheese pieces were placed inside a sterile plastic bag (Südpack Verpackungen, Ochsenhausen, Germany) on the rack ensuring that the sides of the cheese did not make contact with the plastic bag. Relative humidity % (RH%) was maintained by pouring 100 mL of sterile 0.75% NaCl into the base of the bag and the bag was sealed. The cheese was ripened at 15 °C with a RH% of ~97%. After 5 days of ripening, the blocks of cheese were removed from the bag and dipped in saline suspension containing *C. casei* DPC5298, or *S. saprophyticus* DPC5671 (10⁸ cfu mL⁻¹). The blocks of cheese were placed on the sterile rack and incubated for a further 30 days (for a total ripening period of 35 days), as described above, to produce a smear cheese with *D. hansenii* DPC6258 in combination with *S. saprophyticus* DPC5671 (cheese A) or *C. casei* DPC5298 (cheese B). During the ripening period the surface of the cheese blocks was washed with a sterile sponge soaked in a sterile brine solution (5% NaCl) at day 7, 10 and 15 of ripening to ensure an even growth of the smear microbiota. As a control, blocks of Cheddar cheese were vacuum packed in sterile bags and incubated at 15 °C. These blocks were not smeared with either bacteria or yeasts and were not washed with NaCl solution during the ripening period. However, the control cheese differs from normal Cheddar cheese in that the temperature of ripening was higher (15 °C) than the ripening temperature normally associated with Cheddar cheese (~8 °C). Three replicate cheese trials were performed.

2.3. Sampling cheese

The surface of the cheese A and B was aseptically sampled for enumeration and isolation of bacteria and yeast at 3, 5, 7, 10, 15, 21, 25, 30 and 35 days of ripening.

When analysing the control cheese samples, a composite sample of core and surface was analysed, while for the test cheeses both the surface and core were analysed separately. At day 0 and day 35, samples were taken from the control cheese, cheese A and B for composition, urea-polyacrylamide gel electrophoresis (urea-PAGE), free fatty acid and free amino acid analysis. At day 35, samples were taken from the control cheese, cheese A and B for sensory evaluation and volatile analysis. At days 0, 10, 15, 21, 25, 30, 35 samples were taken from the control cheese, cheese A and B for proteolysis analysis and colorimetric analysis.

2.4. Enumeration of bacteria and yeast from cheese surface

During ripening, ~5 cm² of the surface of the test cheeses were aseptically removed and resuspended in 2% trisodium citrate, serially diluted and plated on TSA 5% NaCl with 50 U mL⁻¹ of nystatin (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) and YGC agar, for the enumeration of smear bacteria and yeasts, respectively. Nystatin was added to TSA to prevent the growth of yeast and moulds. The TSA plates were incubated at 30 °C for 48 h, while YGC plates were incubated at 25 °C for 96 h. Colonies were counted and the results expressed as log cfu g⁻¹ of cheese. Five colonies from the highest countable dilution were re-streaked onto TSA and incubated at 30 °C for 48 h. Isolates were stocked at -80 °C in glycerol for further analysis.

2.5. Pulsed field gel electrophoresis

The cultures isolated from cheese A and B were grown on TSA, incubated aerobically at 30 °C for 24 h and then inoculated in 8 mL of TSB, with shaking at 100 rpm at 30 °C for 24 h. Pulsed field gel electrophoresis (PFGE) was carried out as described by Bannerman, Hancock, Tenover, and Miller (1995) for *S. saprophyticus*, while the method outlined by Brennan et al. (2001) was used for *C. casei*. Before digestion the agarose plugs were cut into small slices (1 by 2 mm), transferred into 100 µL restriction buffer containing 20 U of *Sma*I for *S. saprophyticus*, and 20 U of *Spe*I (all from New England Biolabs, Hitchin, UK) for *C. casei* and incubated overnight at 25 °C or 37 °C, respectively. The gel was run in a CHEF-DR III PFGE apparatus (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK) at 1 V (6 V cm⁻¹) with the pulse ramped from 5 to 40 s at 14 °C for 20 h for *S. saprophyticus* and from 1 to 20 s at 14 °C for 16 h for *C. casei*. Gels were stained with ethidium bromide (0.5 µg mL⁻¹) in water, destained in water and then photographed using a gel imaging system (AlphaImager 2000, Alpha Immotech, San Leonardo, CA, USA).

2.6. Compositional analysis

Cheese samples were analysed for moisture content by oven-drying 3 g of cheese sample at 102 °C for 5 h and for salt content by a potentiometric method on a mixture of 2 g of grated cheese and 60 g of water (IDF, 1988). Total protein was determined on 0.2 g of cheese sample and total fat was determined on 3 g of cheese sample by standard methods (IDF, 1993, 1996). The pH was measured with a standard pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland) on a mixture of 20 g of grated cheese and 12 g of water, as described by British Standards Institution standard (BSI, 1976).

2.7. Determination of colour

The development of the colour during ripening was measured in triplicate on the surface of the cheese at room temperature, using a Minolta Colorimeter CR-300 (Minolta Camera, Osaka, Japan). A white colour tile standard was used to calibrate the instrument and the colour was analysed using L*, a* and b*-values, which describe the colour space. L*-value measures the visual lightness (as values increase from 0 to 100), a*-value measures from the redness to greenness (positive to negative values, respectively) and b*-value from the yellowness to blueness (positive to negative values, respectively).

2.8. Proteolysis

Proteolysis was determined by measuring the levels of the non-casein nitrogen content, soluble at pH 4.6 (pH4.6-SN) (Fenelon & Guinee, 2000) and total nitrogen (TN) on a water soluble extract of a mixture of 60 g of grated cheese and 120 g of water, using the macro-Kjeldahl method (IDF, 1993). The levels of proteolysis were expressed as a percentage of the ratio between non-casein nitrogen content and total nitrogen (% pH4.6-SN/TN).

2.9. Urea-polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was performed according to the method described by Rynne, Beresford, Kelly, and Guinee (2004). The gel system was composed of a separating and stacking gel, using a PROTEANS II xi cell vertical slab gel unit (Bio-Rad Laboratories Ltd). The samples were prepared, maintaining the same concentration of protein (425 g of protein

content L⁻¹ sample buffer) to have a final volume of 1 mL. Ten microlitres of sample solution were loaded into individual wells. The sample buffer (pH 8.7), the sample preparation and the running conditions were as described by Henneberry, Wilkinson, Kilcawley, Kelly, and Guinee (2015). After the run, the gel was removed from the plates and stained overnight in an aqueous solution of Coomassie Blue G250 (0.25% w/v), destained in a destaining solution (acetic acid 10%, methanol 25%), and washed in distilled water. The images were acquired by a gel imaging system (AlphaImager 2000; Alpha Immotech).

2.10. Free amino acid analysis

Individual FAAs were determined on the soluble N extracts as described by McDermott et al. (2016) using a Jeol JLC-500V AA analyser fitted with a Jeol Na+ high performance cation exchange column (Jeol Ltd., Garden City, Herts, UK). The chromatographic analyses were conducted at pH 2.2. Results are expressed as µg mg⁻¹ of cheese.

2.11. Free fatty acid analysis

FFA extraction was performed on 10 g of grated cheese, according to the method described by De Jong and Badings (1990). The FFA extracts were aliquoted into amber glass vials and capped with PTFE/white silicone septa (Agilent Technologies, Little Island, Cork, Ireland). The FFA extracts were derivatised as methyl esters as outlined by Mannion, Purrey, and Kilcawley (2016) using a Sample Prep Workbench (Agilent Technologies). Fatty acid methyl esters extracts were analysed using Varian CP3800 gas chromatograph (Aquilant, Dublin, Ireland) with a CP84000 auto-sampler and flame ionisation detector (GC-FID) and a Varian 1079 injector (Aquilant). For the GC-FID analysis, 0.7 µL were injected into a CP FFAP CB capillary column (30 m × 0.25 mm × 0.32 µm) (Agilent Technologies). Results are expressed as µg mg⁻¹ of cheese.

2.12. Volatiles analysis

After sampling the cheese samples were wrapped in foil, vacuum packed and stored at -20 °C. Before analysis, the samples were defrosted at room temperature and blended with a cheese grater. For the analysis, 4 g of cheese sample were placed in a screw capped SPME vial with a silicone/PTFE septum (Apex Scientific, Maynooth, Ireland). The SPME vials were equilibrated to 40 °C for 10 min with pulsed agitation (5 s on, 2 s off) at 500 rpm. Sample introduction was performed using AOC-5000 injection system (Shimadzu, Albert-Hahn-Str., Duisburg, Germany) and a single 50/30 µm Carboxen TM137/divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre was used for the volatile extraction (Agilent Technologies). The SPME fibre was exposed to the headspace above the samples for 20 min at a depth of 54 mm, then was retracted and injected into the GC inlet and desorbed for 2 min at 250 °C. Injections were made on a Shimadzu 2010 plus with an Agilent DB-5ms column (60 m × 0.25 mm × 0.25 µm) (Agilent Technologies), using a multipurpose injector with a Merlin microseal. The temperature of the column oven was initially held for 30 s at 35 °C, increased to 230 °C at a rate of 6.5 °C min⁻¹, and to 320 °C at a rate of 15 °C min⁻¹, yielding a total GC run time of 415 min. The carrier gas, helium, was at a constant pressure of 1.58 bar, performing a splitless injection. The detector, a Shimadzu TQ8030 MSD triple quadrupole mass spectrometer, was used in single quadrupole mode. The ion source temperature and interface temperature were set at 220 °C and 280 °C, respectively, and the MS mode was electronic ionisation (70 V) with the mass range between 35 and

250 amu. The chromatograms were deconvoluted and the peaks quantified with TargetView (Markes International Ltd, Llantrisant, UK). The compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, and using an internal database with known target and qualifier ions for each compound. Ten microlitres of the standard solution [1-butanol, dimethyldisulphide, butyl acetate, cyclohexane, benzaldehyde at 10 ppm, and 2-phenylethanol at 50 ppm, in methanol/water (1:99)] in a SPME vial were run before and after every series of samples to ensure that both the SPME extraction and MS detection were performing within specification. An autotune of the GCMS was carried out prior to the analysis to ensure optimal GCMS performance. All analyses were performed in triplicate.

2.13. Sensory affective evaluation and ranking descriptive analysis

The blocks of cheese sample (~500 g), vacuum packed and stored at -20°C , were defrosted at 4°C the day before sensory evaluation. The surface of cheese A and B was removed and the blocks were cut into portions for the sensory test. Twenty naïve assessors were recruited in University College Cork, Ireland. Sensory acceptance testing was conducted using these untrained assessors, who were both Cheddar and smear cheese consumers, (age 21–48 years). Assessors used the sensory hedonic descriptors for the control, cheese A and B. Samples underwent monadic presentation to the panel at ambient temperatures ($\sim 21^{\circ}\text{C}$) and coded with a randomly selected 3 digit code. Each assessor was asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like). Ranking descriptive analysis (RDA) was then undertaken using the consensus list of sensory descriptors, which was also measured on a 10 cm line scale. All samples were presented in duplicate.

2.14. Statistical analysis

The statistical analysis for cheese composition, sensorial analysis, FAAs and FFAs were tested with one-way analysis of variance (ANOVA) using Minitab 17 (Minitab Inc., Coventry, UK). A split plot designed with SAS 9.3 (SAS, Dublin, Ireland) was used to determine the singular effect of smear treatments, ripening time and their interaction on the microbiology, pH, % pH 4.6-SN/TN, L^* , a^* and b^* values, measured at several time points during the ripening. The statistical analysis of the volatile compounds was tested with ANOVA-Partial Least Squares Regression (APLSR) using Unscrambler X 10.3, Camo Software, Oslo, Norway). From the results of the APLSR, the individual volatile compounds positively correlated with the samples were tested with one-way analysis of variance (ANOVA) using Minitab 17 (Minitab Inc.), to evaluate the significant differences among the samples for each volatile compound. The level of significance for all analyses was determined at $P < 0.05$.

3. Results

3.1. Growth of the strains and pH development

PFGE analysis established that the inoculated cultures of *S. saprophyticus* DPC5671 and *C. casei* DPC5298 were the dominant bacterial strains isolated at the end of ripening (day 35) (Supplementary data). The total count of yeasts and smear bacteria during ripening is shown in Fig. 1. A significant interactive effect ($P < 0.05$) between ripening time and smear treatments was observed for the growth of the surface microbiota. No significant differences were observed in the growth of yeast and bacteria between cheese A and cheese B.

The variation in pH of the control, and the core and the surface of cheese A and B is shown in Fig. 2. A significant interactive effect ($P < 0.05$) between smear treatments and ripening time was observed for pH. From day 21–35, the pH was significantly higher ($P < 0.05$) at the surface of cheese A compared with its respective core and the control. From day 15–35, the pH was significantly higher ($P < 0.05$) at the surface of cheese B compared with the respective core and the control.

3.2. Cheese composition

The mean composition of the Cheddar cheese before the smearing process was typical of a commercial Cheddar cheese after manufacture, but the smearing treatments influenced the compositional parameters at the end of the ripening (35 days). Compositional data and significant differences ($P < 0.05$) are presented in Table 1.

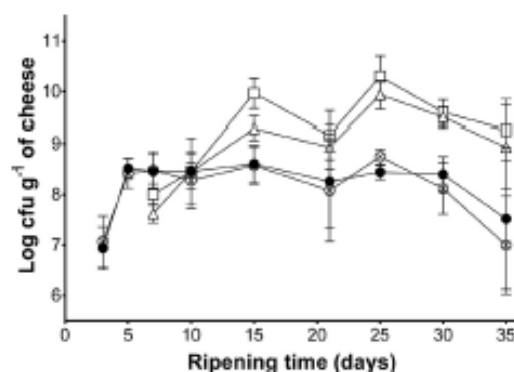


Fig. 1. Enumeration of cfu g^{-1} of the cheese surface of cheese A and cheese B during the ripening time; yeasts total count in cheese A (●) and in cheese B (○), smear bacteria total count in cheese A (▲) and in cheese B (△). Values presented are the means and standard deviations from three replicate trials.

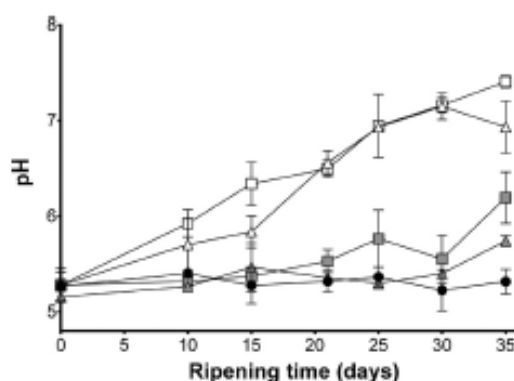


Fig. 2. Change in pH overtime; control cheese (●), surface of cheese A (△), core of cheese A (▲), surface of cheese B (□) and core of cheese B (■). Values presented are the means and standard deviations from three replicate trials.

Table 1

Composition of the control at day 0 and 35 and composition of the surface and core of cheese A and cheese B at day 35.^a

Composition	Control d0	Control d35	Surf A d35	Core A d35	Surf B d35	Core B d35
Moisture (% w/w)	38.80 ± 0.84	38.32 ± 0.62 ^c	39.71 ± 1.20	40.40 ± 0.86	39.61 ± 1.43	41.27 ± 0.39 ^a
Fat (% w/w)	30.45 ± 0.33	30.41 ± 0.27	28.30 ± 0.14 ^b	30.09 ± 0.89	28.01 ± 0.25 ^b	29.80 ± 0.30
Protein (% w/w)	24.87 ± 0.21	25.17 ± 0.11	24.28 ± 0.57	24.5 ± 0.83	24.94 ± 0.32	24.72 ± 0.44
MINFS (% w/w)	55.79 ± 1.44	55.07 ± 1.02 ^c	55.39 ± 1.77	57.89 ± 1.33	55.02 ± 2.13 ^b	58.79 ± 0.39 ^a
FDM (% w/w)	49.77 ± 1.16	49.32 ± 0.80	46.97 ± 1.13 ^b	50.51 ± 1.33	46.41 ± 1.44 ^b	50.74 ± 0.24
S/M (% w/w)	4.02 ± 0.10	4.17 ± 0.26 ^a	3.74 ± 0.20	3.81 ± 0.33	3.58 ± 0.10 ^b	3.88 ± 0.51
Salt (% w/w)	1.56 ± 0.02	1.60 ± 0.09	1.48 ± 0.11	1.55 ± 0.12	1.40 ± 0.08	1.60 ± 0.22

^a The compositional values are for moisture, fat, protein, MINFS (moisture in non-fat substances), FDM (fat in dry matter), S/M (salt in moisture) and salt of control at day 0 (Control d0), for control at day 35 (Control d35), for the surface of cheese A at day 35 (Surf A d35), for the core of cheese A at day 35 (Core A d35), for the surface of cheese B at day 35 (Surf B d35) and for the core of cheese B at day 35 (Core B d35). Values presented are the means ± standard deviations of three replicate trials; values with different superscript letters differ significantly ($P < 0.05$).

3.3. Proteolysis

The proteolysis was particularly high at the surface of cheese A and B, where the smear treatments significantly ($P < 0.05$) influenced the level of % pH 4.6-SN/TN (Supplementary data). Urea-PAGE (Fig. 3) confirmed the high level of proteolysis on the surface of cheese A and B. α_{S1} -Casein was partially degraded to α_{S1} -CN (f102–199) and α_{S1} -CN (f24–199) in the control cheese and in the core of cheese A and B. However there was evidence of further breakdown products on the surface samples of the test cheeses. Similar β -casein degradation patterns were observed for the control and core of test cheeses, while samples from the surface of the test cheeses showed that the β -casein was almost totally degraded producing β -CN (106–209) and β -CN (108–209) with higher intensity (Fig. 3).

3.4. Free amino acids and free fatty acids

Significant differences ($P < 0.05$) on the total amount of FAAs were observed between the control and the surface of cheese A and B at the end of ripening on day 35 (Fig. 4). In the cheese A and B,

some individual FAAs were significantly higher ($P < 0.05$) on the surface compared with their respective cores or the control. No significant difference was determined between the surface samples of cheese A and B with respect to the total FAA content.

Significant differences ($P < 0.05$) in the total amount of FFAs were observed between the control and the surface of cheese A and B at day 35 (Fig. 5). In the cheese A and B, all individual FFAs

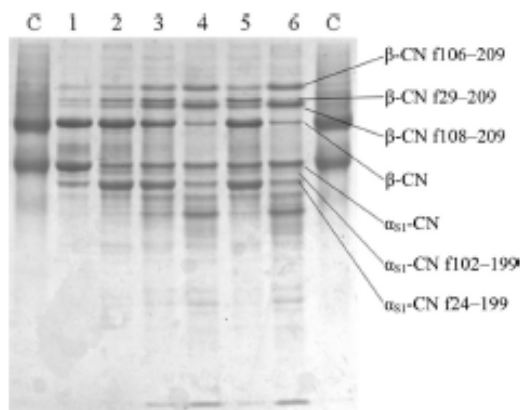


Fig. 3. Urea-PAGE electrophoretogram of sodium caseinate (lane C), control cheese at day 0 (lane 1), control cheese at day 35 (lane 2), core of cheese B at day 35 (lane 3), surface of cheese B at day 35 (lane 4), core of cheese A at day 35 (lane 5) and surface of cheese A at day 35 (lane 6). The urea-PAGE was performed on the basis of fixed weight protein for all three replicate trials.

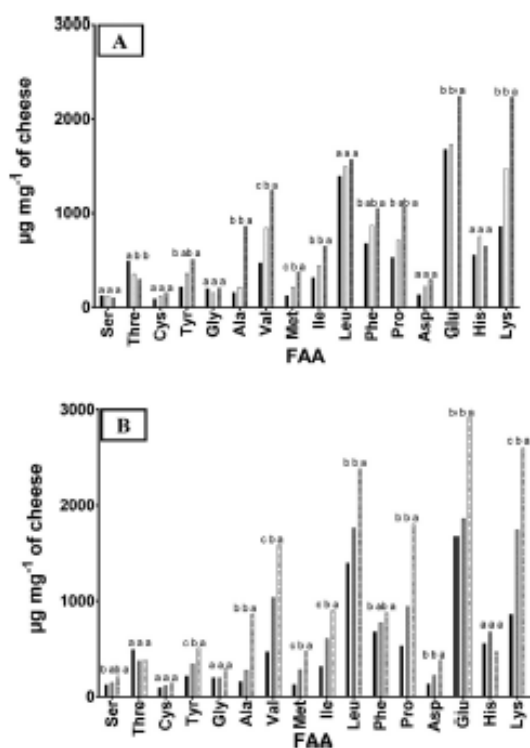


Fig. 4. Individual free amino acids content of (A) the control (■), core cheese A (□), surface cheese A (▨), and (B) the control (■), core cheese B (□), and surface cheese B (▨) at day 35. The values presented are the means of the three replicate trials; values within an amino acid group with different letters are significantly different ($P < 0.05$).

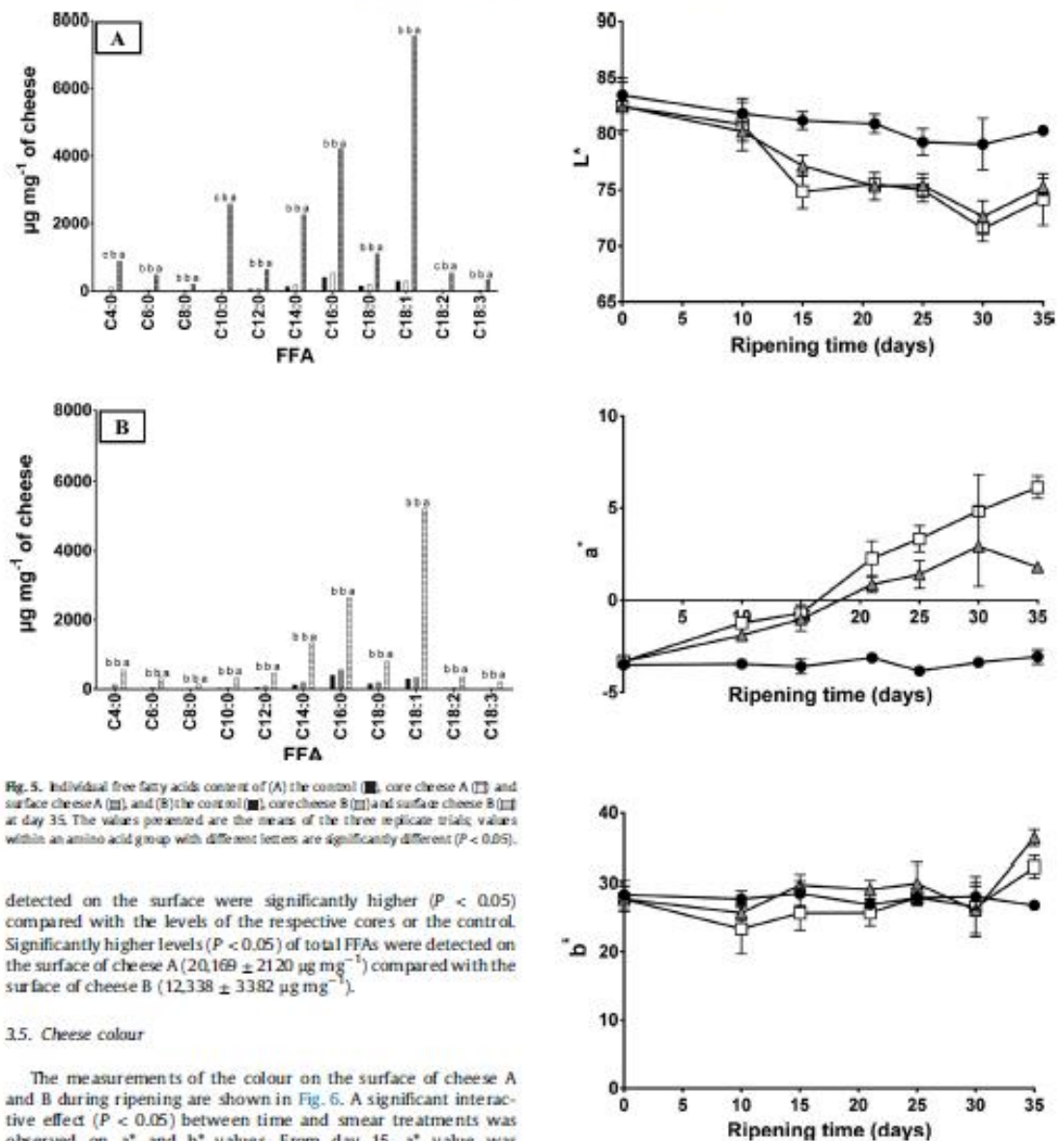


Fig. 5. Individual free fatty acids content of (A) the control (■), core cheese A (□) and surface cheese A (▨), and (B) the control (■), core cheese B (□) and surface cheese B (▨) at day 35. The values presented are the means of the three replicate trials; values within an amino acid group with different letters are significantly different ($P < 0.05$).

detected on the surface were significantly higher ($P < 0.05$) compared with the levels of the respective cores or the control. Significantly higher levels ($P < 0.05$) of total FFAs were detected on the surface of cheese A ($20,169 \pm 21.20 \mu\text{g mg}^{-1}$) compared with the surface of cheese B ($12,338 \pm 3382 \mu\text{g mg}^{-1}$).

3.5. Cheese colour

The measurements of the colour on the surface of cheese A and B during ripening are shown in Fig. 6. A significant interactive effect ($P < 0.05$) between time and smear treatments was observed on a^* and b^* values. From day 15, a^* value was significantly higher ($P < 0.05$) compared with the control for the cheese A and B. At the end of the ripening (day 35), a^* value was significantly higher ($P < 0.05$) for cheese B compared with cheese A, resulting a redder colour on the surface of cheese B.

3.6. Volatile compounds and sensory analysis

The analysis of variance enabled the selection of 40 volatile compounds that were significantly different ($P < 0.05$) and

Fig. 6. Colour development on the surface of the cheeses during the ripening. The colour values (L^* , a^* , b^*) of the control cheese (●), of cheese A (▲) and of cheese B (□). Values presented are the means and standard deviations from three replicate trials.

positively correlated with the samples (Table 2). In total, 22 volatile compounds (7 acids, 4 alcohols, 5 esters, 4 sulphur compounds, 1 ketone and 1 aromatic hydrocarbon) were significantly associated ($P < 0.05$) with the surface of cheese A. Eight compounds (2 acids, 2 aldehydes, 1 alcohol 1 ester, 1 pyrazine and 1 sulphur compound)

Table 2

Volatile compounds detected with SPME-GCMS in cheese A, cheese B and control, and relative aroma notes.^a

Volatile compound	CAS number	Aroma note
Aldehydes		
3-Methyl-1-butanol ^b	590-86-3	Malty, powerful, cheese, green, dark chocolate (Kilcawley, 2017)
2-Methyl-1-butanol ^b	96-17-3	Malty, dark chocolate, almond, cocoa (Qian, Subbank, & Wang, 2006; Singh et al., 2008; Urbach, 1993)
Alcohols		
Ethanol ^a	64-17-5	Dry, dust, alcohol (Kilcawley, 2017)
2-Butanol ^a	78-92-2	Sweet, fruity, fusel oil, wine-like (Kilcawley, 2017)
3-Methyl-1-butanol ^a	123-51-3	Fresh cheese, breath-taking, alcoholic, fruity, grainy (Kilcawley, 2017)
2-Methyl-1-butanol ^a	137-32-6	Malty, wine, onion (Kilcawley, 2017)
2,3-Butanediol ^a	513-85-9	Fruity (Singh et al., 2003)
2-Heptanol ^{a,b}	543-49-7	Fruity, earthy, green, sweetish, dry (Kilcawley, 2017)
2-Ethyl-1-hexanol ^{a,c}	104-76-7	Animal, cardboard (Thomson et al., 2012)
Phenylethyl-alcohol ^a	60-12-8	Unclean, rose, violet-like, honey, floral (Kilcawley, 2017)
Ketones		
2,3-Butanedione ^c	431-03-8	Buttery, strong (Kubicková & Grouch, 1997; Singh et al., 2003)
2-Pentanone ^a	107-87-9	Orange peel, sweet, fruity (Kilcawley, 2017)
3-Methyl-2-pentanone ^a	565-61-7	Minty-camphoraceous, sharp (Barron et al., 2005)
2-Hexanone ^a	591-78-6	Floral, fruity (Qian et al., 2006)
8-Nonen-2-one ^a	5009-32-5	Animal, stinky (Poveda, Sánchez-Palomo, Pérez-Collado, & Cabezas, 2008; Varming, Andersen, Petersen, & Ardl, 2013)
2-Nonanone ^a	821-55-6	Malty, rotten fruit, hot milk, green, earthy (Kilcawley, 2017)
2-Decanone ^a	693-54-9	Fruity, musty (Qian et al., 2006; Varming, et al., 2013)
Acids		
Acetic acid ^a	64-19-7	Vinegar, peppers, green, fruity, floral (Kilcawley, 2017)
2-Methyl-propanoic acid ^a	79-31-2	Rancid butter, sweaty, sweet, apple-like (Curioni & Bosset, 2002)
Butanoic acid ^a	107-92-6	Sweaty, butter, cheese, strong, acid (Kilcawley, 2017)
3-Methyl-butyric acid ^b	503-74-2	Cheesy, sweaty, socks, rancid, rotten fruit (Kilcawley, 2017)
2-Methyl-butyric acid ^a	116-53-0	Fruity, waxy, sweaty (Singh et al., 2003)
Pentanoic acid ^a	109-52-4	Rain, wood, vegetable, spicy, nutty, grain, Swiss cheese, stable, sweaty, sheep (Curioni & Bosset, 2002)
Heptanoic acid ^a	111-14-8	Soapy, fatty, goat, rancid (Curioni & Bosset, 2002)
Octanoic acid ^b	124-07-2	Cheesy, rancid, pungent, sweat (Kilcawley, 2017)
n-Decanoic acid ^a	334-48-5	Stale, butter, sour, fruity, pungent (Kilcawley, 2017)
Esters		
Ethyl acetate ^a	141-78-6	Solvent, pineapple, fruity (Kilcawley, 2017)
Ethyl propionate ^a	105-37-3	Pineapple, solvent, fruity (Barron et al., 2005; Qian et al., 2006)
3-methylbutyl acetate ^c	123-92-2	Fruity, banana, candy, sweet (Barron et al., 2005; Curioni & Bosset, 2002; Qian et al., 2006)
Methyl hexanoate ^a	106-70-7	Pineapple, fruity (Qian et al., 2006; Varming et al., 2013)
Ethyl hexanoate ^b	123-66-0	Pineapple, sweet, fruity, banana (Kilcawley, 2017)
Ethyl octanoate ^a	106-32-1	Pear, a pisco, sweet, fruity, banana, pineapple (Kilcawley, 2017)
Isopentyl hexanoate ^a	2198-61-0	Sweet, fruity (Gárbuz, Rouzeff, & Rouzeff, 2006)
Sulphur compounds		
Methionol ^a	505-10-2	Orange (Capino et al., 2004)
Methanethiol ^{a,b}	74-93-1	Rotten cabbage, cheese, vegetative, sulphur (Kilcawley, 2017)
Dimethylsulphide ^a	624-92-0	Green, sour, onion (Kilcawley, 2017)
Dimethyltrisulphide ^a	3658-80-8	Vegetable-like, sulphurous, garlic, putrid, cabbage-like (Kilcawley, 2017)
Aromatic hydrocarbons		
Benzaldehyde ^c	100-52-7	Bitter almond, sweet cherry (Singh et al., 2008; Smit et al., 2005)
Benzene carboxaldehyde ^a	122-78-1	Honey-like, rose, violet-like, hyacinth, green (Kubicková & Grouch, 1997; Qian et al., 2006; Singh et al., 2003; Smit et al., 2005; Varming et al., 2013)
Pyrazines		
3-Ethyl-2,5-dimethyl-pyrazine ^b	13,360-66-1	Roasted, baked (Qian & Reinhardt, 2002)

^a Superscript letters denote that volatile compounds are significantly different ($P < 0.05$) and positively correlated to: (°) the surface of cheese A; (°) the surface of cheese B; (°) the core of cheese A; (°) the core of cheese B; (°) the control cheese.

were significantly associated with the surface of cheese B. Two compounds (1 ketone and 1 hydrocarbon) were significantly associated ($P < 0.05$) with the core of cheese A. Two compounds (1 alcohol and 1 ketone) were significantly associated ($P < 0.05$) with the core of cheese B. Nine compounds (4 alcohols, 4 ketones and 1 ester) were significantly associated ($P < 0.05$) with the control cheese.

Using hedonic sensory analysis (Fig. 7) the control cheese scored significantly higher ($P < 0.05$) for "Liking of Aroma" compared with the cheese A and B. Cheese A scored significantly higher ($P < 0.05$) for "Liking of Aroma" compared with cheese B. The control and cheese A scored significantly higher ($P < 0.05$)

for "Liking of Flavour" compared with cheese B. As seen in Fig. 8, the control cheese, as expected, scored significantly higher ($P < 0.05$) for "Cheddar flavour" compared with cheese A, which scored significantly higher ($P < 0.05$) for "Cheddar flavour" compared with cheese B. Cheese A and B scored significantly higher ($P < 0.05$) for "Pungent flavour" and "Mould cheese flavour" compared with the control cheese. Cheese B scored significantly higher ($P < 0.05$) for descriptors "Sweaty/Sour Aroma", "Pungent Aroma" and "Off-Aroma" compared with the control cheese. The control scored significantly higher ($P < 0.05$) for "Crumbly texture" compared with cheese A and B.

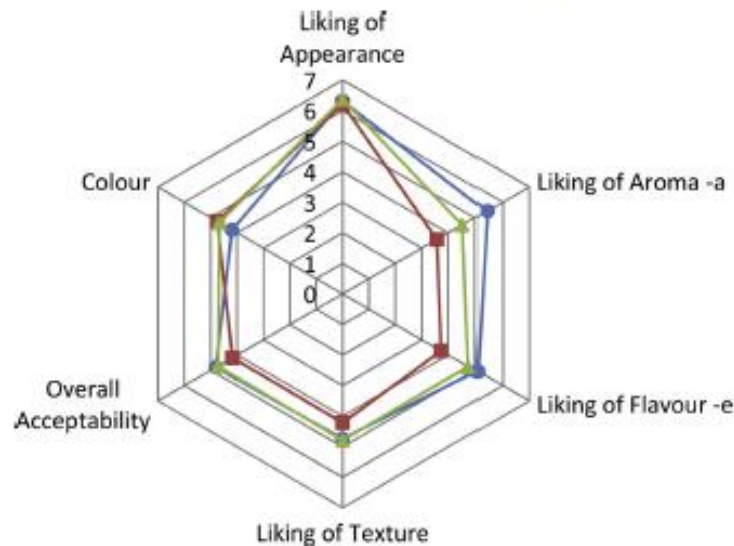


Fig. 7. Sensory affective (hedonic) analysis performed in duplicate on cheese A (Δ), cheese B ($\blacksquare$) and control ($\bullet$) at 35 days of ripening. Letters after an attribute descriptor denote: (a) control significantly higher ($P < 0.05$) than cheese A and B; (e) cheese A and control significantly higher ($P < 0.05$) than cheese B.

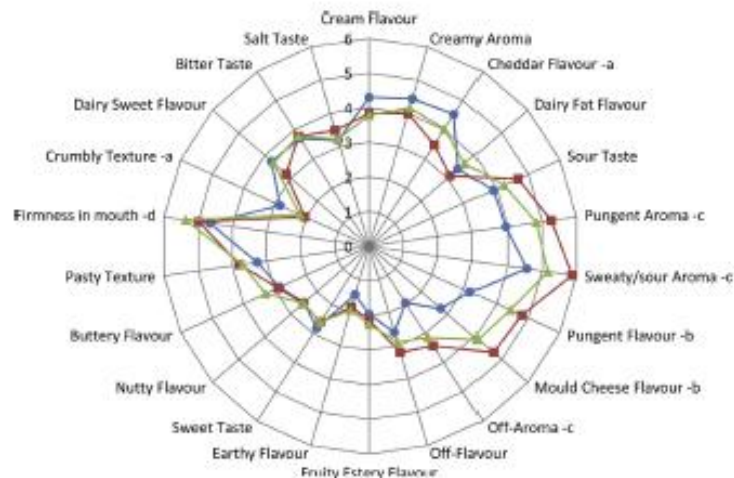


Fig. 8. Ranking descriptive analysis performed in duplicate on cheese A (Δ), cheese B ($\blacksquare$) and control ($\bullet$) at 35 days of ripening. Letters after an attribute descriptor denote: (a) control significantly higher ($P < 0.05$) than cheese A and B; (b) cheese A and B significantly higher ($P < 0.05$) than control; (c) cheese B significantly higher ($P < 0.05$) than cheese A and control; (d) cheese A significantly higher ($P < 0.05$) than cheese B and control.

4. Discussion

C. casei and *S. saprophyticus*, bacteria commonly isolated from smear-ripened cheeses (e.g., Limburger, Reblochon, Livarot, Tilsit, Gubbeen) (Cogan et al., 2014; Larpin et al., 2011), do not belong to the traditional microbiota of Cheddar cheese, although in this study both strains established themselves on the surface of young Cheddar cheese curd and they were the dominant population on the cheese surface throughout the ripening.

C. casei DPC5298 or *S. saprophyticus* DPC5671 in combination with *D. hansenii* DPC6258 developed a coloured layer on the cheese surface after 15 days of ripening. Unlike studies with *Brevibacterium linum* that showed that colour development was influenced by the yeast strain used (Leclercq-Perlat, Corrieu, & Spindler, 2004a), in this study the type of colour developed was dependent on the bacteria used. The combination *D. hansenii* DPC6258 with *C. casei* DPC5298 on cheese B developed a redder colour compared with the combination of *D. hansenii* DPC6258 with *S. saprophyticus* DPC5671

on cheese A. Similar results were shown by Mounier et al. (2006b) who reported in a cheese model an increase of the colour after the 15th day of ripening at 14 °C and higher a^* value for the combination *D. hansenii* with *C. casei*, compared with *D. hansenii* combined with *S. saprophyticus*, resulting in higher development of red colour.

The development of the typical aroma and flavour in cheese A and B is associated with the lipolytic and proteolytic processes of the yeast and bacterial component of the smear consortium. These processes were slower in the control cheese, which did not develop the same levels of proteolysis, FAAs and FFAs in the short ripening time (35 days).

During cheese ripening, a gradual decomposition of caseins into small peptides and FAAs occurs. FAAs are considered precursors of flavour compounds during the development of cheese flavour. It is likely that the smear treatments increased the proteolysis of β -casein by plasmin (more active at alkaline pH) on the surface of the test cheeses (Fig. 3), increasing the pH levels. At the end of ripening the dominant FAAs detected in high amount on the surface of the test cheeses were valine, leucine, proline, glutamate and lysine (Fig. 4), due to their relative concentration in casein and the peptidase activity of the smear consortium, especially *D. hansenii* DPC6258. Analysis of cell free supernatants of the cultures used in this study confirmed that *D. hansenii* DPC6258 had high peptidase activities (PepX and PepN), while activities were low for *S. saprophyticus* DPC5671 and *C. casei* DPC5298 (Supplementary data). Similar results have been previously reported for other *D. hansenii*, *S. saprophyticus* and *C. casei* strains (Bintsis, Vafopoulou-Mastrogiannaki, Litopoulou-Tzanetaki, & Robinson, 2003; Casaburi, Villani, Toldrá & Sanz, 2006; Curtin et al., 2002).

The hydrolysis of triglycerides is the main biochemical transformation of fat during cheese ripening, which leads to the production of FFAs. Individual FFAs contribute to the cheese aroma with their specific flavours and especially with their metabolites. At the end of ripening high amounts of FFAs were detected on the surface of cheese A and B. The levels of all individual FFAs detected were higher on the surface of cheese A compared with cheese B, especially for C10:0 (Fig. 5). It has been previously reported that the lipolysis by *D. hansenii* is weak, while studies on Gram-positive bacteria showed good activity on substrates with different glyceride chain lengths (Bintsis, Vafopoulou-Mastrogiannaki, Litopoulou-Tzanetaki, & Robinson, 2003; Cardoso et al., 2015; van den Tempel & Jakobsen, 2000). Experimental work showed that *S. saprophyticus* DPC5671 had greater lipolytic activity on tributyrin than either *C. casei* DPC5298 or *D. hansenii* DPC6258 (Supplementary data). These results are in agreement with what was previously reported by Talon and Montel (1997), who detected lipolytic activity on tributyrin in a range of staphylococcus strains, including *S. saprophyticus*. The higher lipolytic activity of *S. saprophyticus* DPC5671 compared with *C. casei* DPC5298 may explain the higher amount of FFAs in cheese A.

Numerous volatile compounds were significantly ($P < 0.05$) associated to cheese A and B, especially those particularly characterised by strong aroma notes, such as some specific carboxylic acids, alcohols, esters, ketones and sulphur compounds, suggesting that the smear treatments have modified the aroma profile of Cheddar cheese curd in only 35 days of ripening (Table 2).

The metabolism of FAAs in cheese A and B in this study was responsible for the development of specific branched alcohols and branched chain acids detected (3-methyl-1-butanol, phenylethyl-alcohol, 3-methyl-butanoic acid). *D. hansenii* has been identified as a possible producer of alcohols in previous studies (Arfi, Spinnler, Tache, & Bonnamy, 2002; Gori, Sørensen,

Petersen, Jespersen, & Arneborg, 2012; Leclercq-Perlat, Corrieu, & Spinnler, 2004b), while the production of carboxylic acids has been previously attributed to both yeasts and smear bacteria (*Geotrichum candidum* and *B. linens*) (Jollivet, Bézenger, Vayssier, & Belin, 1992; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994).

The high amount of esters detected in cheese A (ethyl acetate, ethyl octanoate, methyl hexanoate, 3-methylbutyl acetate and isopentyl hexanoate) is likely related to the high FFA content and the presence of alcohols in cheese A, considering they originate from the esterification or alcoholysis of alcohols with carboxylic acids. While information on the biosynthesis of esters by corynebacteria is sparse, numerous studies reported ester production by staphylococci isolated from fermented foods, including *S. saprophyticus* strains (Talon, Chastagnac, Vergnais, Montel, & Berdague, 1998). The formation of esters in cheese A, not detected in high amount in cheese B, is likely due to the metabolic activity of *S. saprophyticus* DPC5671 rather than by *D. hansenii* DPC6258.

Other products of FFA metabolism such as ketones and alcohols were detected in all cheeses. However 2-pentanone, 2-hexanone, 2-nonanone and 2-decanone were particularly associated with the control cheese and not with cheese A and B, suggesting an involvement of LAB rather than the smear cultures. It is known that methylketones result from the β -oxidation of FFA, by lipolytic enzymes due to autolysis of the LAB during ripening (Collins, McSweeney, & Wilkinson, 2003), although it is also postulated that methylketones can be produced by the heating of milk or directly from esterification of β -keto acids (Alewijn, 2006; Forss, 1979).

Sensory analysis showed different results between cheese A, cheese B and the control cheese. The sensory panel was not influenced by the colour of the cheese as the surface was removed before sensory analysis. "Mould Cheese Flavour" and "Pungent Flavour" are descriptors associated with smear-ripened cheeses and they can be correlated with a range of volatiles with strong aroma notes (Table 2) detected on the surface and core of both cheese A and B, namely butanoic, octanoic acid (originated from lipolysis of lipids), 2-heptanol (from reduction of ketones), 2-methyl-propanoic, 3-methyl-butanoic, 2-methyl-butanoic, pentanoic acid, 3-methyl-butanol, 2-methyl-butanol, phenylethyl-alcohol, 3-methyl-butanol (from metabolism of branched chain amino acids or possibly phenylalanine for phenylethyl-alcohol), methanethiol, dimethyldisulphide and dimethyldisulphide (from metabolism of sulphur amino acids). As expected these descriptors were significantly associated ($P < 0.05$) with cheese A and/or B, suggesting that the activities of yeast and Gram-positive bacteria on the cheese conferred a typical smear cheese flavour, not perceived in the control. The descriptors significantly associated ($P < 0.05$) with cheese B, such as "Sweaty/Sour Aroma", "Pungent Aroma" and "Off-Aroma", are considered as "undean" and off-odorants and were associated with some compounds detected in abundance on the surface and core of cheese B, such as 3-methyl-butanoic acid, octanoic acid, methanethiol and particularly 8-nonen-2-one (originated from β -oxidation of fatty acids) which was identified only in cheese B.

Sensory analysis showed the smear cultures on the cheese surface affected the cheese ripening giving strong and intense aroma and flavour to cheese A and B, while the control cheese was characterised by mild aroma and flavour. In a short ripening time of 35 days, the smear treatments induced the development of different aroma profiles.

Overall, the bacterial strains in conjunction with *D. hansenii* may have the potential to modify cheese colour and produce novel cheeses with diverse aromas using a Cheddar cheese curd.

5. Conclusion

The cheese-making method described in this paper gives a new approach for the production of novel smear cheeses starting from a Cheddar cheese curd. Both the yeast and bacterial cultures were able to establish themselves on the surface of the cheese and become the dominant microbiota on the cheese surface, producing a cheese variety with acceptable appearance and novel flavour and aroma profiles. The method proposed could be used as a model to produce novel cheese types with a range of flavours and aromas through the growth of combinations of yeast and bacterial cultures on the surface using cheese curd produced on a traditional Cheddar cheese plant.

Acknowledgements

The authors wish to thank to Sarah Henneberry, David Mannion and Hope Faulkner for their assistance in the analysis. Andrea Bertuzzi is currently in a receipt of Teagasc Walsh Fellowship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.idairyj.2017.04.001>.

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Genome Sequence of *Staphylococcus saprophyticus* DPC5671, a Strain Isolated from Cheddar Cheese

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ABSTRACT The draft genome sequence of *Staphylococcus saprophyticus* DPC5671, isolated from cheddar cheese, was determined. *S. saprophyticus* is a common Gram-positive bacterium detected on the surface of smear-ripened cheese and other fermented foods.

Staphylococci are Gram-positive catalase-positive bacteria with halotolerance that enables their growth in salted fermented food. Strains belonging to the *Staphylococcus* genus are commonly identified in soft cheese varieties made from cow, ewe, or goat milk, and together with *Brevibacterium*, *Corynebacterium*, and *Microbacterium*, *Staphylococcus* is considered the most important genus making up the microbiota of the cheese surface (1). *S. saprophyticus* is a species frequently detected on the surface of smear-ripened cheese and other fermented foods (2–4). Here, we present the draft genome sequence of *S. saprophyticus* DPC5671, which will allow a full safety assessment and further analysis on its role in cheese ripening.

The draft genome of *S. saprophyticus* DPC5671 was sequenced using paired-end 454 pyrosequencing to a coverage of 23×. Sequencing took place at the Teagasc 454 sequencing facility on a genome sequencer FLX platform (Roche Diagnostics, West Sussex, United Kingdom), according to the manufacturer's protocols. This was followed by initial assembly into 24 contigs using the Newbler program (Roche Life Science). The software Prodigal (5) was used to predict open reading frames within the draft genome, and the RAST annotation server (6) was used to determine complementary gene calling and automated annotation. The draft genome was manually analyzed using the ARTEMIS genome browser (7), and comparative analysis with the genome of *S. saprophyticus* ATCC 15305 (8) was performed using the Artemis Comparison Tool (ACT) (9). The PHAST (PHAge Search Tool) Web server (10) was used to determine the presence of putative phage within the genome. The Comprehensive Antibiotic Resistance Database (CARD) software (11) was used to determine the presence of genes potentially involved in antibiotic resistance, and the presence of known staphylococcal virulence factors was analyzed using the BlastP Web server (12).

The draft genome of *S. saprophyticus* DPC5671 includes 2,676,318 bp, with an average G+C content of 33.1%. It consists of a single circular chromosome and does not appear to harbor any plasmids. Within the draft genome, there are 2,647 coding regions predicted, in addition to four rRNA and 59 tRNA genes. The genome sequence also includes two putative novel phages of ~43.6 kb and ~42.9 kb within the chromosome. Overall, the genome of *S. saprophyticus* DPC5671 shows high similarity to *S. saprophyticus* ATCC 15305 in genome size, G+C content, and gene synteny (8).

S. saprophyticus DPC5671 is coagulase negative, nonhemolytic, and does not appear to produce any toxins associated with *Staphylococcus aureus*. Genomic analysis re-

Received 25 February 2017 Accepted 7 March 2017 Published 20 April 2017

Citation Bertuzzi AS, Guinane CM, Crispie F, Kilcawley KN, McSweeney PLH, Rea MC. 2017. Genome sequence of *Staphylococcus saprophyticus* DPC5671, a strain isolated from cheddar cheese. *Genome Announc* 5:e00193-17. <https://doi.org/10.1128/genomeA.00193-17>.

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vealed no obvious transferable antibiotic resistance loci by the methods used in this study. Previous studies showed an involvement of *S. saprophyticus* in urinary tract infections, showing a specific adhesin, *uafA*, to be associated with adherence to the eukaryotic cell in the urinary tract (8). The *S. saprophyticus* DPC5671 genome was found to have a predicted coding sequence (CDS) for adhesin, with similarities examined with the BlastP Web server to *uafA* in *S. saprophyticus* ATCC 15305 with 39% of query cover and 97% of protein identity.

The availability of the genome sequence of DPC5671 will allow its role in flavor development in cheese ripening to be studied.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number MUX100000000. The version described in this paper is version MUX100000000.

ACKNOWLEDGMENT

Andrea Bertuzzi is currently in receipt of a Teagasc Walsh Fellowship.

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Omics-Based Insights into Flavor Development and Microbial Succession within Surface-Ripened Cheese

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ABSTRACT In this study, a young Cheddar curd was used to produce two types of surface-ripened cheese, using two commercial smear-culture mixes of yeasts and bacteria. Whole-metagenome shotgun sequencing was used to screen the microbial population within the smear-culture mixes and on the cheese surface, with comparisons of microorganisms at both the species and the strain level. The use of two smear mixes resulted in the development of distinct microbiotas on the surfaces of the two test cheeses. In one case, most of the species inoculated on the cheese established themselves successfully on the surface during ripening, while in the other, some of the species inoculated were not detected during ripening and the most dominant bacterial species, *Glutamicibacter arilattensis*, was not a constituent of the culture mix. Generally, yeast species, such as *Debaryomyces hansenii* and *Geotrichum candidum*, were dominant during the first stage of ripening but were overtaken by bacterial species, such as *Brevibacterium linens* and *G. arilattensis*, in the later stages. Using correlation analysis, it was possible to associate individual microorganisms with volatile compounds detected by gas chromatography-mass spectrometry in the cheese surface. Specifically, *D. hansenii* correlated with the production of alcohols and carboxylic acids, *G. arilattensis* with alcohols, carboxylic acids and ketones, and *B. linens* and *G. candidum* with sulfur compounds. In addition, metagenomic sequencing was used to analyze the metabolic potential of the microbial populations on the surfaces of the test cheeses, revealing a high relative abundance of metagenomic clusters associated with the modification of color, variation of pH, and flavor development.

IMPORTANCE Fermented foods, in particular, surface-ripened cheese, represent a model to explain the metabolic interactions which regulate microbial succession in complex environments. This study explains the role of individual species in a heterogeneous microbial environment, i.e., the exterior of surface-ripened cheese. Through whole-metagenome shotgun sequencing, it was possible to investigate the metabolic potential of the resident microorganisms and show how variations in the microbial populations influence important aspects of cheese ripening, especially flavor development. Overall, in addition to providing fundamental insights, this research has considerable industrial relevance relating to the production of fermented food with specific qualities.

KEYWORDS dairy science, flavor, microbiology

Recent studies involving both metabolomic and metagenomic analyses have begun to address the relationship between the microbiota and biochemical pathways during the fermentation process (1–4). It is clear that in fermented food, the metabolic

Received 8 November 2017 Accepted 4 January 2018 Published 30 January 2018

Citation Bertuzzi AS, Walsh AM, Sheehan JJ, Cotter PD, Crispie F, McSweeney PLH, Kilcawley KN, Rea MC. 2018. Omics-based insights into flavor development and microbial succession within surface-ripened cheese. mSystems 3:e00211-17. <https://doi.org/10.1128/mSystems.00211-17>.

Editor Rachel J. Dutton, University of California, San Diego

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Interactions which regulate the composition of the microbial population influence the taste, shelf life, and safety of the subsequent product (5). The ability to manipulate the microbiota of fermented food represents an important avenue for the food industry for developing new food products with precise characteristics.

Surface-ripened cheese (e.g., Münster, Tilsit, Livarot, Limburger, and Comté) is characterized by the growth of a heterogeneous microbiota on the cheese surface, with the consequent development of a strong flavor. The flavor and the appearance of these types of cheese are related to the metabolic activities of bacteria and yeasts, which comprise the smear consortium. Generally, the cheese is brined or surface salted, which also influences the growth of surface microbiota. In some traditional procedures, young cheese is smeared by transferring the smear from older cheese to a younger curd (old-young technique) (6, 7). However, today, commercial mixtures of smear bacteria and yeasts are more commonly used to produce a more standardized product.

So far, metagenomic sequencing represents a valid method to investigate the microbial population on the exterior of surface-ripened cheese (4, 8–10). In studies of complex microbial communities in fermented foods, such as kefir, the information gained through whole-metagenome shotgun sequencing allowed the variations of the microbial population and also the metabolic pathways involved in the fermentation process to be monitored (1).

The aim of the current study was to investigate, at both the species and the strain level, the succession of the microbial populations present on the rind of a surface-ripened cheese produced with young Cheddar cheese curd as a base, using two different commercial smear-culture mixes. Studies were performed over the course of 30 days of ripening to correlate volatile analysis with data generated through whole-metagenome shotgun sequencing in order to understand how microbial composition relates to flavor development. Moreover, metagenomic analysis allowed for the screening of metagenomic clusters during cheese ripening, showing the involvement of the surface microbiota in a variety of biochemical processes.

RESULTS

Microbial compositions of the smear-culture mixes. Two smear-culture mixes, D4 and S5, were used for the cheese trials and contained, as outlined in the supplier specification sheet, *Brevibacterium linens*, *Debaryomyces hansenii*, *Cyberlindnera jadinii*, and *Brevibacterium casei* (for D4) or *Staphylococcus xylosum*, *B. linens*, *D. hansenii*, *Geotrichum candidum*, and *Glutamicibacter arilaitensis* (previously classified as *Arthrobacter arilaitensis*) (for S5). Using metagenomic analysis, performed with Kaiju (11), the relative abundances of the individual species within the mixes were determined (Fig. 1). Overall, Kaiju was able to assign $81.7\% \pm 1.5\%$ of reads from the starter mix samples to the species level. The proportion of assigned reads for each starter mixture sample is presented in Fig. S1 in the supplemental material. *B. casei* (60.83%) and *C. jadinii* (15%) were the most abundant bacterial and yeast species in D4, while *B. linens* and *D. hansenii* were minor components in the smear-culture mix, with relative abundances of 5.25% and 1.92%, respectively (Fig. 1; Table S1). In the S5 mix, *G. arilaitensis* (64.25%), *D. hansenii* (14.56%), and *G. candidum* (11.83%) were the most abundant bacteria and yeasts; *S. xylosum* (0.59%) and *B. linens* (3.52%) were present at lower relative abundances. Other species, not specified by the suppliers, were identified at low relative abundances in the smear-culture mixes D4 and S5 and are reported in Table S1.

Microbial compositions of the cheese surfaces. Two test cheeses, D4 and S5, were prepared by smearing young Cheddar cheese curd with the two aforementioned commercial smear-culture mixes and ripened for 30 days at 15°C. Kaiju was used to determine the bacterial and yeast compositions of the cheese surfaces at days 0, 18, 24, and 30 for both the control cheese (unsmearied and ripened under vacuum) and the two test cheeses (11). Overall, Kaiju was able to assign $57.5\% \pm 8.3\%$ of reads from the cheese samples to the species level. The proportions of assigned reads for each cheese sample are presented in Fig. S2. Compositional data of the cheese surface were analyzed by a one-way analysis of variance (ANOVA), designed with SAS 9.3, to

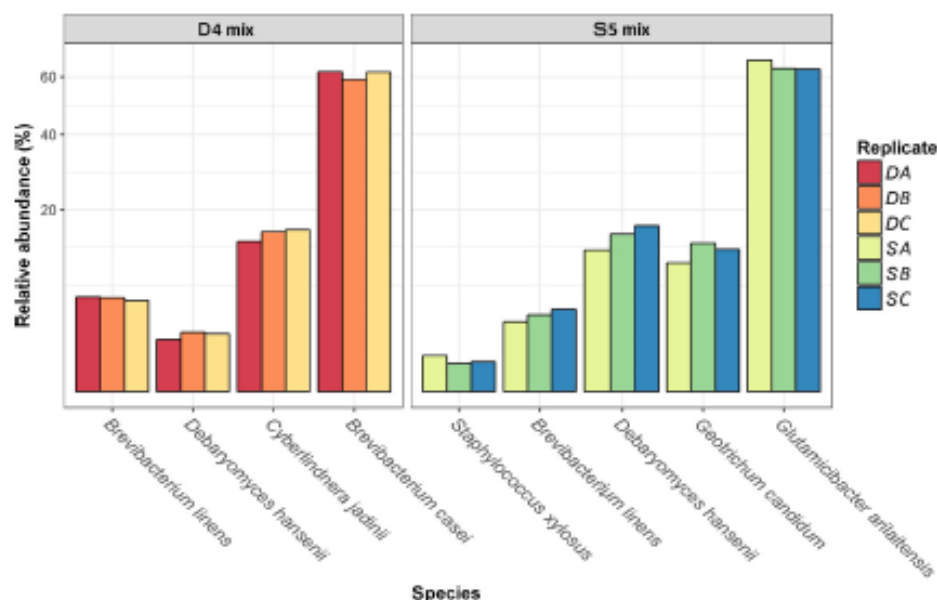


FIG 1 Relative abundances of the species (percentages) which were indicated as being present by the supplier within the smear-culture mixes D4 and S5 (results are from replicates of three analyses [DA, DB, DC and SA, SB, SC]).

determine the significant differences in the proportions of the individual species present over time (12). The metagenomic sequences of the bacteria used as starter cultures in the Cheddar cheese curd (*Lactococcus lactis* and *Streptococcus thermophilus*) and as smearing cultures (*B. linens*, *S. xyloso*, and *G. arilaitensis*) were compared at the strain level, using PanPhlAn, to determine the presence/absence of the inoculated bacterial strains on the cheeses throughout ripening (13).

As expected, lactic acid bacteria dominated the surfaces of all samples at day 0, and their relative abundances on the surface of the control cheese did not significantly change throughout the 30 days of ripening (Fig. 2). *L. lactis* and *S. thermophilus* were identified in all samples analyzed (D4, S5, and control) (Fig. 3). *L. lactis* was the dominant species in the control, constituting 75.85% of the initial population at day 0 and decreasing to 65.99% at day 30. *S. thermophilus* increased from 19.65% at day 0 to 28.21% at day 30, while the relative abundance of *Lactobacillus (Lb.) helveticus* was low throughout the ripening period (2.12% at day 0 and 2.72% at day 30) (Table S2). However, over the course of 30 days of ripening, the smearing processes clearly influenced the microbial populations of the cheese surfaces of both test cheeses, D4 and S5, causing a significant reduction in the relative abundances of *Lb. helveticus* ($P < 0.03$) and *L. lactis* ($P < 0.0001$). From day 0 to day 18, the population on the surface of D4 changed from predominately lactic acid bacteria to *Debaryomyces hansenii* and *Glutamicibacter arilaitensis* (Fig. 2). Subsequently, over the course of ripening, the relative abundance of *D. hansenii* significantly decreased ($P < 0.0001$) from 34.12% at day 18 to 4.14% at day 30 (Table S2). In parallel, the relative abundance of *G. arilaitensis* significantly increased ($P < 0.0001$) from 30.9% at day 18 to become the dominant population on the cheese surface (73.75%) at day 30 (Table S2). Using PanPhlAn, it was determined that the strain of *G. arilaitensis* detected on the cheese surface of D4 was different from the *G. arilaitensis* strain used in the smear-culture mix inoculated onto the surface of S5, confirming that the growth of this strain on D4 did not result from cross contamination of the two cheeses during inoculation or ripening (Fig. S3 and S4).

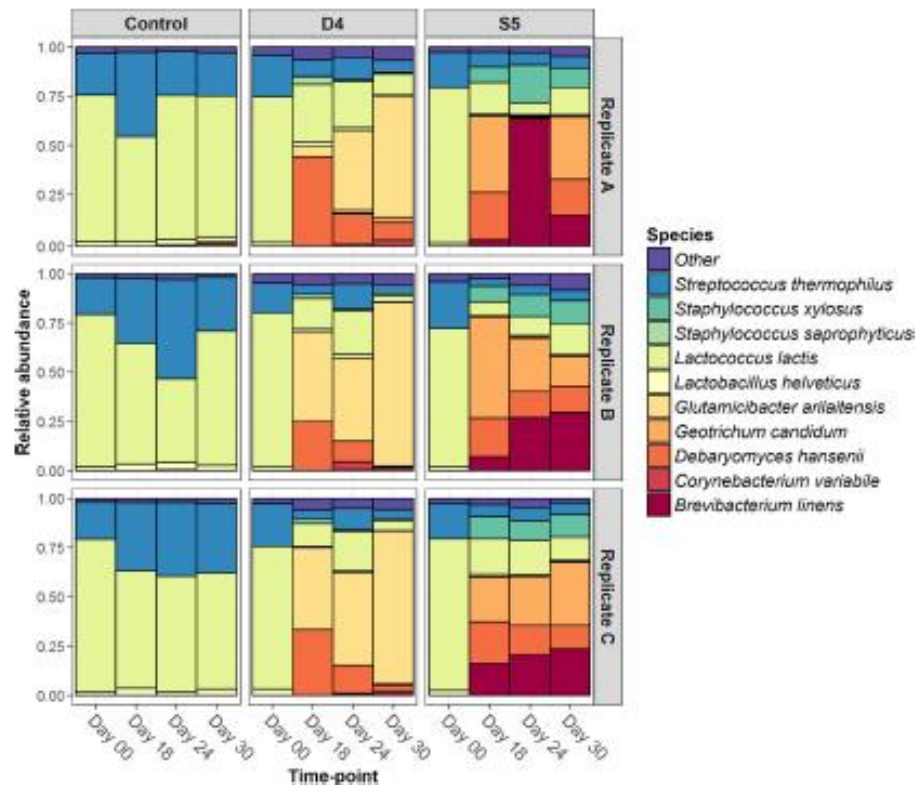


FIG 2 Relative abundances at the species level of the microbiotas on the surfaces of the control, D4, and S5 cheeses at days 0, 18, 24, and 30. Data shown are from the three replicate trials (A, B, and C).

However, the *G. arilaitensis* strain detected on D4 did appear to be more closely related to the strain on the control cheeses (Fig. S4). The secondary microbial population (individually between 1% and 3% of the population) of the D4 surface was composed of species not included in the initial smear-culture mix and included *Arthrobacter* sp., *Corynebacterium variabile*, *Debaryomyces fabryi*, *G. candidum*, *Staphylococcus equorum*, and *Staphylococcus saprophyticus* (Table S2). In addition, some species present in the initial smear-culture mix (*C. jadinii* and *B. casei*) were not detected during ripening, while the inoculated *B. linens* strain was detected at only at a very low relative abundance on the cheese surface of D4 throughout ripening (Table S2).

By comparison, the microbiota was more diverse in cheese S5 (Fig. 2; Table S2). On the cheese surface of S5, the relative abundances of the lactobacilli decreased, while that of *B. linens* increased significantly ($P < 0.004$) from day 18 to day 24, reaching 37.05% before decreasing, but not significantly, to 22.84% at day 30 (Table S2). The strain detected was confirmed by PanPhlan to be that inoculated within the S5 mix (Fig. S3 and S4). The yeasts *D. hansenii* and *G. candidum* (components of the S5 mix) were the most abundant populations on the cheese surface at day 18, comprising 21.2% and 37.54% of the microbiota, respectively, but their relative abundances significantly decreased ($P < 0.04$) by day 24 to 9.57% and 17.6%, respectively, without showing further significant reductions at day 30 (Table S2). *S. xylosum*, did not correspond to the strain present in the S5 mix (Fig. S3 and S4) and was detected at 9.08% at day 18 but did not change significantly throughout the ripening period (Table S2).

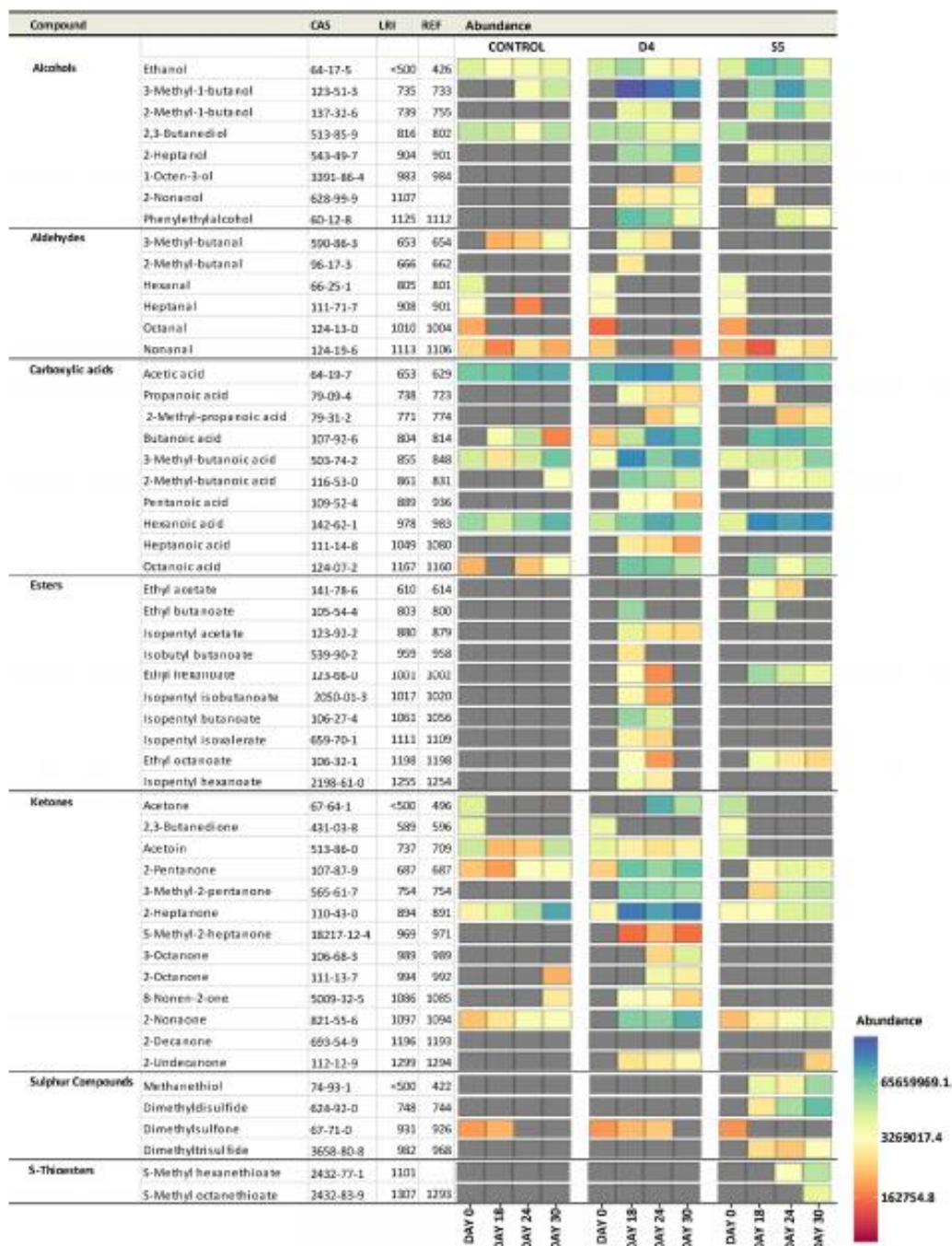


FIG 3 Volatile compounds detected in cheese by GC-MS and faceted heat map showing the variation of volatile compounds between the cheeses at days 0, 18, 24, and 30. The gray tiles indicate when the volatile compounds were not detected. The linear retention index (LRI) was calculated and compared with the reference linear retention index (REF) to confirm the identification. Values are the means of results from three replicates.

In addition, a secondary microbial population, comprising *D. fabryi* (detected in the S5 mix) (Table S1) and *Psychrobacter* sp. (not detected in the S5 mix) (Table S1), developed at low relative abundance (1 to 2%) on the surface of cheese S5 (Table S2) over the course of the ripening period. However, some inoculated species were either not detected (*S. equorum*) at any stage throughout ripening or detected at a very low relative abundance (*G. arilaitensis*, ~0.44%) on the cheese surface during ripening (Table S2).

Volatile compounds present on the cheese surface. Headspace solid-phase microextraction (HS-SPME) gas chromatography-mass spectrometry (GC-MS) was used to analyze the development of volatile compounds at days 0, 18, 24, and 30 of ripening for both the control and test cheeses. In total, 53 volatile compounds that potentially contributed to the flavor development were detected on the cheese surfaces. These compounds are predicted to arise from a variety of substrates and consisted of 8 alcohols, 6 aldehydes, 10 carboxylic acids, 10 esters, 13 ketones, 2 S-thioesters, and 4 sulfur compounds (i.e., a total of 53 compounds) (Fig. 3). As expected, given the microbial diversity on the surface, there was a greater variety and intensity of volatile compounds detected than on the control cheese, on which only 23 of the aforementioned 53 compounds were detected (Fig. 3). In all cheeses, the levels of all volatile compounds detected increased throughout the ripening period, apart from those of 2,3-butanediol, hexanal, heptanal, octanal, nonanal, 2,3-butanedione, and dimethylsulfone (Fig. 3).

Correlations between microbial taxa and volatile compounds. The correlation analysis between the relative abundances of microbial species and the abundances of volatile compounds detected on the cheese surface was performed using the Spearman correlation test, as described previously by Walsh et al. (1). From the results of the metagenomic analysis (performed with Kallu) and the volatile analysis, it was possible to associate both yeasts and bacteria, at the species level, with specific volatile compounds. Figure 4 demonstrates the degrees of correlation between the volatile compounds and the organisms detected.

There was a strong correlation between *B. linens* and *G. candidum* and sulfur compounds and 2-methyl-1-butanol. *S. xylophilus* was correlated with sulfur compounds, 2-methyl-1-butanol, and some ethyl esters; *Corynebacterium variabile* was correlated with ketones. *D. hansenii* was correlated with acids and alcohols, *G. arilaitensis* was correlated with ketones, alcohols, and acids, and *S. saprophyticus* was correlated with ketones, esters, acids, and alcohols (Fig. 4; Table 1).

Gene content of cheese surface microbiota. Using SUPER-FOCUS (<https://edwards.sdsu.edu/SUPERFOCUS>) (14), whole-metagenome shotgun sequencing was used to characterize the functional potential of the whole microbial community on the cheese surfaces at different stages of ripening. Overall, SUPER-FOCUS was able to assign 62.5% ± 10.9% of reads from the cheese samples to a function. The proportions of assigned reads for each cheese sample are presented in Fig. S2. The functional clusters analyzed were initially organized into three different levels, in relation to the specificity of the metabolic pathways. Pathway data were analyzed to determine the significant differences of the individual metabolic clusters by ANOVA, using SAS 9.3 (12), with the selection of 16 specific functional clusters with relative abundances significantly higher ($P < 0.05$) on the cheese surfaces of S5 and D4 than on that of the control (Fig. 5).

Color and pH variation. pH and color analyses were performed on the three cheese types, and the resultant data were examined using a split-plot test, designed with SAS 9.3 (12). A significant interactive effect ($P < 0.0001$) between smear treatments and ripening time was observed for pH. At days 18, 24, and 30, the pH was significantly higher ($P < 0.0001$) on the surfaces of S5 and D4 than on that of the control. In addition, the pH was significantly higher ($P < 0.0001$) on the surface of S5 than on that of D4 from day 18 onwards (Fig. S5).

A significant interactive effect ($P < 0.0001$) between time and smear treatments was observed for L^* , a^* , and b^* values. The L^* value measures the visual lightness (as values

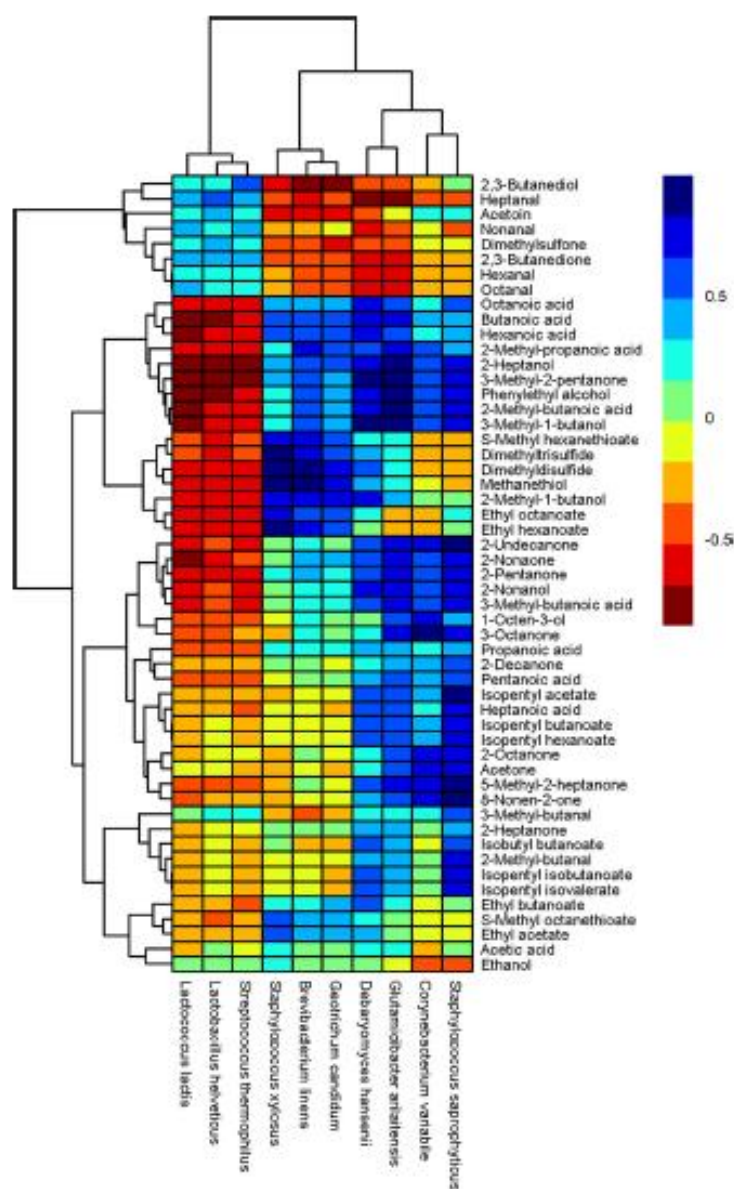


FIG 4 Hierarchically clustered map showing the correlation between the relative abundances of the microbial species and the levels of volatile compounds detected on the cheese surface. Clustering was performed by using the *hclust* function in R. The color of each tile of the heat map indicates the level of correlation for a given species-compound combination, as indicated by the color key.

increase from 0 to 100), the a^* value measures the redness to greenness (positive to negative values, respectively), and the b^* value measures the yellowness to blueness (positive to negative values, respectively). At days 18, 24, and 30, the a^* value was significantly higher ($P < 0.0001$) for the surfaces of S5 and D4 than for the surface of

TABLE 1 List of strong positive correlations^a between the levels of volatile compounds and the relative abundances of species on the cheese surface

Correlation species and compound	Potential precursor	R value
<i>Debaryomyces hansenii</i>		
2-Methyl butanoic acid	Isoleucine	0.81
3-Methyl-1-butanol	Leucine	0.85
Octanoic acid	Lipolysis	0.76
Hexanoic acid	Lipolysis	0.81
2-Heptanol	2-Heptanone (fatty acid oxidation)	0.8
<i>Glutamicibacter arilaitensis</i>		
2-Methyl butanoic acid	Isoleucine	0.9
3-Methyl-1-butanol	Leucine	0.86
3-Methyl butanoic acid	Leucine	0.77
Phenylethyl alcohol	Phenylalanine	0.83
3-Methyl-2-pentanone	Fatty acid oxidation	0.89
2-Undecanone	Fatty acid oxidation	0.82
5-Methyl-2-heptanone	Fatty acid oxidation	0.78
2-Pentanone	Fatty acid oxidation	0.77
2-Nonaone	Fatty acid oxidation	0.76
2-Heptanol	2-Heptanone (fatty acid oxidation)	0.86
<i>Geotrichum candidum</i>		
2-Methyl-1-butanol	Isoleucine	0.76
Methanethiol	Methionine	0.76
Dimethyldisulfide	Methanethiol	0.79
<i>Brevibacterium linens</i>		
2-Methyl-1-butanol	Isoleucine	0.81
Methanethiol	Methionine	0.82
Dimethyldisulfide	Methanethiol	0.85
Dimethyltrisulfide	Methanethiol	0.77
<i>Staphylococcus xylosum</i>		
2-Methyl-1-butanol	Isoleucine	0.77
Methanethiol	Methionine	0.84
Dimethyldisulfide	Methanethiol	0.95
Dimethyltrisulfide	Methanethiol	0.86
Methylthio hexanoate	Methanethiol + hexanoic acid	0.78
Ethyl hexanoate	Ethanol + hexanoic acid	0.85
Ethyl octanoate	Ethanol + octanoic acid	0.77
<i>Staphylococcus saprophyticus</i>		
2-Methyl-butanoic acid	Isoleucine	0.76
3-Methyl-1-butanol	Leucine	0.77
Heptanoic acid	Lipolysis	0.76
5-Methyl-2-heptanone	Fatty acid oxidation	0.98
2-Undecanone	Fatty acid oxidation	0.88
8-Nonen-2-one	Fatty acid oxidation	0.87
3-Methyl-2-pentanone	Fatty acid oxidation	0.77
2-Nonanol	2-Nonaone (fatty acid oxidation)	0.78
Isopentyl acetate	3-Methyl-1-butanol + acetic acid	0.87
Isopentyl butanoate	3-Methyl-1-butanol + butanoic acid	0.8
Isopentyl hexanoate	3-Methyl-1-butanol + hexanoic acid	0.8
<i>Corynebacterium variabile</i>		
3-Octanone	Fatty acid oxidation	0.99
2-Octanone	Fatty acid oxidation	0.78
5-Methyl-2-heptanone	Fatty acid oxidation	0.77

^aCorrelations for which the *P* value was <0.001 (corrected for multiple comparisons using the Bonferroni method) and the *R* value was >0.75.

the control. At day 30, the α^* value was also significantly higher ($P < 0.02$) on the surface of D4 than on that of S5 (Fig. S6).

FAA and FFA analyses. Free amino acid (FAA) and free fatty acid (FFA) analyses were performed on the three cheese types, and the experimental results were examined by one-way ANOVA, using SAS 9.3 (12). The concentrations of total FAAs on the

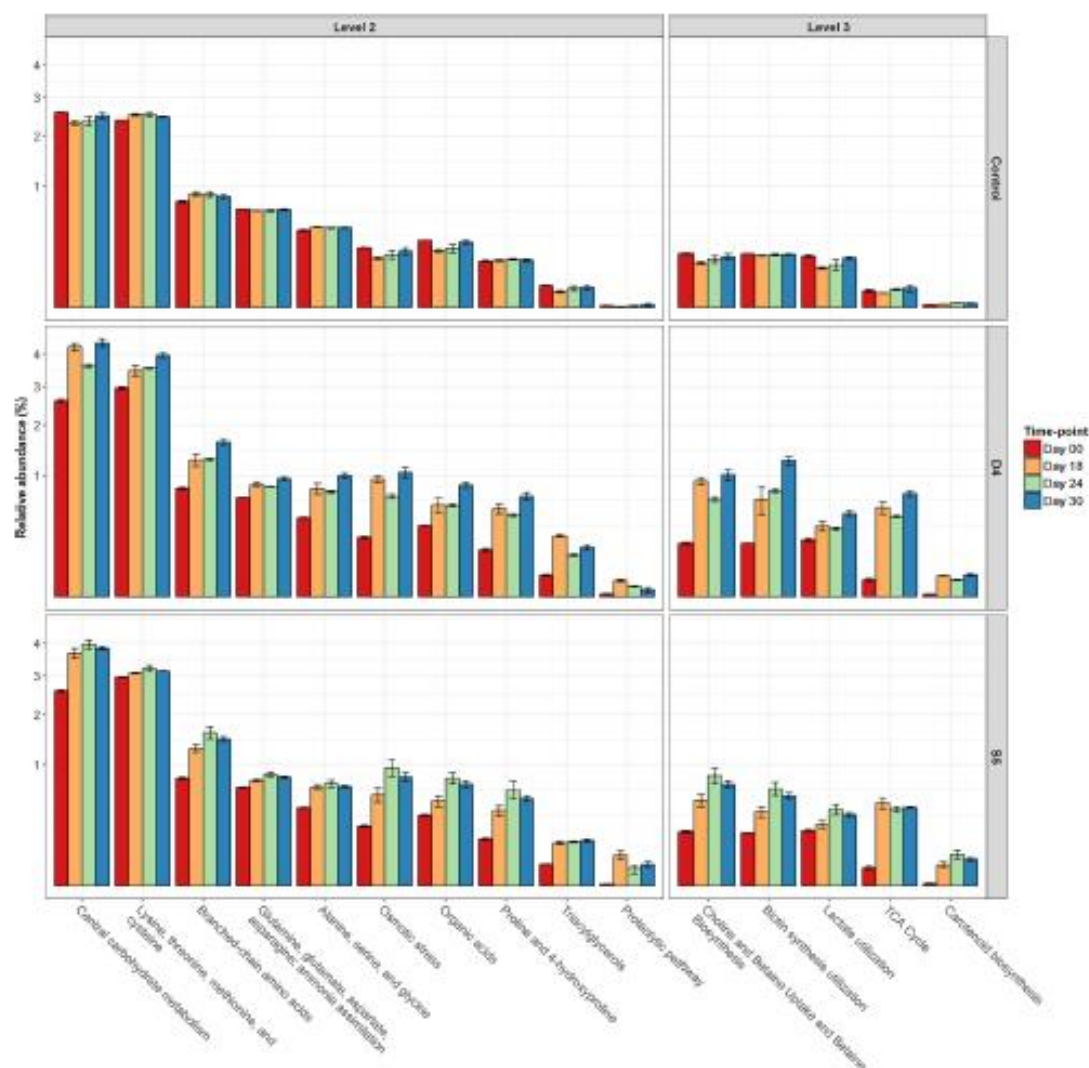


FIG 5 Averages and standard errors from the three replicate trials of the relative abundances of significantly different ($P < 0.05$) metagenomic clusters detected with SUPER-FOCUS at days 0 (red), 18 (orange), 24 (green), and 30 (blue) for the surfaces of the control, D4, and S5 cheeses.

surfaces of S5 ($15,158 \pm 1,683 \mu\text{g} \cdot \text{mg}^{-1}$) and D4 ($11,914 \pm 1,769 \mu\text{g} \cdot \text{mg}^{-1}$) were significantly higher ($P < 0.05$) than those on the control surface ($6,605 \pm 819 \mu\text{g} \cdot \text{mg}^{-1}$). In addition, the concentrations of some individual FAAs, such as tyrosine, proline, and histidine, were significantly higher ($P < 0.05$) on the surface of S5 than on the surfaces of D4 and the control (Fig. S7).

The concentrations of total FFAs on the surfaces of S5 ($22,069 \pm 3,875 \mu\text{g} \cdot \text{mg}^{-1}$) and D4 ($26,562 \pm 2,606 \mu\text{g} \cdot \text{mg}^{-1}$) were significantly higher ($P < 0.05$) than on the surface of the control ($1,336 \pm 70 \mu\text{g} \cdot \text{mg}^{-1}$). The concentrations of some individual FFAs, such as $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, and $C_{16:0}$, were significantly higher ($P < 0.05$) on the surface of D4 than on that of S5 or the control (Fig. S7).

DISCUSSION

In this study, the use of whole-metagenome shotgun sequencing facilitated the study, at the species and the strain level, of microbial succession among smear microorganisms (both bacteria and yeasts) on cheese surfaces and facilitated the analysis of the metabolic potential of the whole microbial community at different stages of ripening. Volatile flavor compounds were analyzed over time, using HS-SPME GC-MS, and correlated with the microbial species that developed during ripening.

Cheddar cheese curd <24 h postmanufacture was inoculated with two different smear-culture mixes and incubated at 15°C for 30 days. Unsmear Cheddar cheese curd, vacuum packed to prevent the growth of spoilage molds on the cheese surface, was used as a control. This model was chosen to investigate microbial succession and flavor development, as it had been shown in a previous study that yeasts and bacteria establish themselves satisfactorily on the surface of young Cheddar cheese curd, producing cheese with modified flavor and appearance (15).

On the cheese surfaces of S5 and D4, a very heterogeneous microbial consortium developed during ripening, triggering an array of biochemical processes. Yeasts are considered to be responsible for the deacidification of the cheese surface (observed on S5 and D4) (Fig. 5S) by the degradation of lactate (to CO₂ and H₂O) (16, 17), as well as for the formation of alkaline metabolites (from metabolism of FAAs) (18) and the secretion of growth factors (vitamins and amino acids) that support the growth of bacteria (17, 19). As expected, in parallel with the growth of the yeasts, the relative abundances of the metagenomic clusters related to lactate utilization and the biosynthesis and uptake of biotin were greater for the cheese surfaces of D4 and S5 than for that of the control (Fig. 5). During ripening, the surfaces of D4 and S5 were washed with a 5% salt solution, causing hyperosmotic stress on the microbial population of the cheese surface (20). This correlated with higher relative abundances of the metagenomic clusters related to osmotic-stress resistance and the metabolism of choline and betaine (osmoprotectants) (21) for the washed cheeses than for the unwashed control (Fig. 5).

The development of a red/orange color on the surface is an important characteristic of many smear-ripened cheeses. This color development is usually derived through the metabolism of carotenoids (22, 23), and correspondingly higher relative abundances of metagenomic clusters involved in carotenoid biosynthesis were observed on the surfaces of the cheeses S5 and D4 than on that of the control (Fig. 5).

Surface-ripened cheeses are also characterized by a strong flavor, which is driven by the biochemical metabolism of the microbial consortium that develops on the cheese surface over time. These are associated with proteolytic and lipolytic pathways, driving the increase in the levels of FAAs and FFAs. These pathways, together with lactose and citrate metabolism, are considered to be responsible for the main precursors of flavor compounds in cheese. In the current study, the relative abundances of the metagenomic clusters associated with the proteolytic pathway and the metabolism of triacylglycerols were higher for D4 and S5 than for the control, which was consistent with FAA- and FFA-related data (Fig. 57). During ripening, the relative abundances of metagenomic clusters directly related to the formation of volatile compounds, such as carbohydrates, organic acids (including FFAs), and FAAs (except aromatic amino acids), and of clusters indirectly related to the formation of volatile compounds, such as those used in the tricarboxylic acid (TCA) cycle (important for α -ketoglutarate production), were significantly higher ($P < 0.05$) for the surfaces of both the D4 and S5 cheeses than for that of the control cheese (Fig. 5). Correspondingly, numerous volatile compounds (alcohols, aldehydes, carboxylic acids, ketones, sulfur compounds, esters, and S-thioesters) (Fig. 3) were produced on the surfaces of cheeses S5 and/or D4, conferring an intense flavor to them.

During ripening, on the cheese surfaces of S5 and D4, a microbial succession involving various inoculated, and indeed some noninoculated, microorganisms was apparent. Consistently with other studies, specific smear strains, added as adjunct

cultures to the milk or to the exterior of surface-ripened cheese during manufacture, have not been detected at the end of ripening (24–28). In this study, the species detected on the cheese surface by metagenomic analysis did not fully correspond with the components of the smear-culture mixes. Different contaminant populations developed on the surfaces of both test cheeses, especially on that of D4, probably due to the different interactions and competition between the cultures of the two mixes (Fig. 2; Table S2).

D. hansenii was part of the inoculum used for both S5's and D4's surface. *D. hansenii* is a component of the surface microbiota of many surface-ripened cheeses and is very tolerant to high-salt and low-pH conditions (16, 29). Presumably due to these characteristics, *D. hansenii* was present at a high relative abundance in both test cheeses, mainly in the early stage of ripening (at day 18), and then decreased gradually in the later stages (days 24 and 30) (Table S2). Volatile compounds significantly ($P < 0.001$) associated with *D. hansenii* were mainly alcohols and carboxylic acids (Fig. 4; Table 1). The biosynthesis of branched-chain alcohols and carboxylic acids from FAA metabolism and the biosynthesis of medium-to-long carboxylic acids from FFA metabolism are processes attributed mainly to yeast and mold metabolism, including that of *D. hansenii* (30–35).

On cheese D4, the relative reduction of *D. hansenii* with time corresponded to an increase in the number of Gram-positive bacteria. *G. arilaitensis*, a component of S5's mix, did not grow on the cheese surface of S5 and, though it was not inoculated as part of the culture mix, was the dominant bacterium on the surface of D4 (Fig. 2; Table S2). Through the use of PanPhlAn, which uses metagenomic data to achieve strain-level microbial profiling resolution, we have demonstrated that the *G. arilaitensis* strain present on D4 was not the same strain as inoculated onto S5 (Fig. S3 and S4). The inability of the inoculated *G. arilaitensis* strain to grow on the S5 cheese is most likely due to the different interactions within the microbiota on the cheese surface. Other studies on the microbial composition of the surface of Limburger cheese observed that *G. arilaitensis* behaved in a similar manner, showing high relative abundance when it was coinoculated only with *D. hansenii* but showing low relative abundance when combined with both *D. hansenii* and *G. candidum* (17). That *G. arilaitensis* contributes to cheese flavor has been shown previously in model cheese media (36) (producing alcohols and especially ketones) and in the current study, where it was significantly ($P < 0.001$) associated with 3-methyl-1-butanol and phenylethyl alcohol, branched carboxylic acids (from FAA metabolism), 2-heptanol, and ketones (from FFA metabolism) (Fig. 4; Table 1). In addition, a genomic study showed numerous genes encoding protein degradation and fatty acid oxidation in *G. arilaitensis* (37).

On the cheese surface of S5, *G. candidum* was coinoculated with *D. hansenii* and established itself to become the most abundant yeast population by day 18. The successful cohabitation of *G. candidum* and *D. hansenii* may be explained by the fact that they do not compete for energy sources in the same way in cheese. *D. hansenii* uses lactate or the limited amount of lactose present in the cheese postmanufacture (0.8 to 1%), while *G. candidum* preferentially uses only lactate (21, 38). During ripening, sulfur compounds were significantly ($P < 0.001$) associated with *G. candidum* (Fig. 4; Table 1), which is in agreement with other studies which have shown that *G. candidum* is able to catabolize methionine in a one-step degradation, with the biosynthesis of sulfur compounds (34, 39, 40).

The production of sulfur compounds is an important characteristic of many surface-ripened cheese, and *B. linens* is considered one of the main species responsible for the development of the strong flavor of many surface-ripened cheeses through the biosynthesis of sulfur compounds derived from methanethiol. In this study, *B. linens* was present at relatively low abundances in the original culture mixes (5.26% and 3.53% for D4 and S5, respectively) (Table S1). However, although it was detected at a very low relative abundance on the cheese surface of D4, it was one of the most dominant bacteria detected on S5 (37.05% at day 24) (Table S2). While this may be due to interstrain differences, it is most likely due to the different interactions within the microbiotas of

S5 and D4. Studies have shown that *B. linens* does not always establish itself on the cheese surface during ripening, even if it is present in the initial culture mix (25–27, 41, 42). However, in previous studies, *G. candidum* has been shown to stimulate the growth of *B. linens* in coculture (43), suggesting the hypothesis that in S5, *G. candidum*, present at high relative abundance, might have produced growth factors that supported the growth of *B. linens* but that in D4, it was out-competed by *G. arilantensis*, which established itself very quickly on the surface of S5 and made up 75% of the microbiota at the end of ripening. *B. linens* was significantly ($P < 0.001$) associated with methanethiol and its derivatives (dimethyldisulfide and dimethyltrisulfide) (Fig. 4; Table 1), which likely originated from the one-step degradation of methionine (30, 36, 44, 45).

Other species, while present at lower relative abundances on the cheese surfaces of S5 and D4, were also responsible for the biosynthesis of some volatile compounds. A strain of *S. xyloso* different from the one within the smear-culture mix of S5 (Fig. S4) was detected during ripening only at 10.83 to 13.36% of its relative abundance on the cheese surface of S5 (Table S2). This is most likely due to competition for nutrients within the microbiota, as suggested by Mounier et al. (38). Members of the genus *Staphylococcus* can establish themselves on surface-ripened cheese in the early stages of ripening but are regularly overtaken by other bacteria at the later stages (26, 46, 47).

In this study, specific species detected in low relative abundances in S5, such as *S. xyloso* (9.08 to 13.36%), and in D4, such as *S. saprophyticus* (1.06 to 2.69%) and *C. variable* (2.04 to 2.08%) (Table S2), were significantly ($P < 0.001$) associated with a range of flavor compounds important in surface-ripened cheese (Fig. 4; Table 1), and interestingly, while *S. xyloso* has previously been shown to produce sulfur compounds only in fermented meat (48, 49), in this study, it was correlated with specific sulfur compounds in cheese. These data suggest that some smear bacteria, though present at relatively low abundances in cheese, are likely contributors to the release of FFAs and to their degradation due to their esterase activity and, hence, that they contribute to the aroma and flavor in the final cheese product (50, 51).

In the study reported here, whole-metagenome shotgun sequencing was employed as a novel method for the analysis of a fermented product with a complex microbiota. Metagenomic analysis was an efficient tool to understand the variations of the microbial population of the cheese surface over time and the related metabolic potential. Moreover, the association between the volatile compounds and the species represents a novel system for studying flavor development in cheese. In conclusion, the approach used in this study enabled us to determine the microbial succession during ripening and also to begin to unravel the contributions of the various components of the surface microbiota when present within a complex microbial environment. The method proposed in this study can be adopted in industry to control the microbiotas of fermented food, resulting in the production of food products with specific flavor characteristics.

MATERIALS AND METHODS

Smearing of cheese blocks. A block of commercial Cheddar cheese <24 h after manufacture was aseptically cut into smaller blocks (~8 by 6.5 by 30 cm) and washed with smearing solutions, as described in our previous study (15). Two commercial smear-culture mixes comprising *G. candidum*, *D. hansenii*, *B. linens*, *G. arilantensis*, and *S. xyloso* (S5 mix) (Sacco, Cadore, Italy) and *D. hansenii*, *C. casei*, and *B. linens* (D4 mix) (DuPont Danisco, Beaminster, Dorset, United Kingdom) were used to inoculate the surfaces of the cheese curds. The blocks of cheese were washed with the smearing solutions and placed in sterile racks inside sterile plastic bags (Südpack Verpackungen, Ochsenhausen, Germany), as previously described (15). The cheese was ripened for 30 days at 15°C, with a relative humidity of ~97%. At days 7, 10, and 15 of ripening, the cheese blocks were brushed with a sterile sponge that had been soaked in a sterile brine solution (5% NaCl) to uniformly spread the smear microbiota on the cheese surface. As a control, unsmear cheese blocks were vacuum packed in sterile bags and incubated at 15°C, as with the test cheeses.

Sampling cheese. Three replicate cheese trials were performed at different times during Cheddar cheese making season. All data presented are the results of the analysis performed on samples taken from the cheese surface (at a depth of ~0.5 cm). All analyses were performed in triplicate.

pH measurement. The pH level was measured on days 0, 18, 24, and 30 using a standard pH meter (MP220; Mettler-Toledo, Schwerzenbach, Switzerland) (52). The data were analyzed by one-way analysis of variance (ANOVA) using SAS 9.3 (12).

Determination of color. At days 0, 18, 24, and 30 of ripening, the color was measured on the cheese surface at room temperature, using a Minolta CR-300 colorimeter (Minolta Camera, Osaka, Japan). The instrument was calibrated on white tile, and the color of the cheese surface was measured using L^* , a^* , and b^* values. The L^* value measures the visual lightness (as values increase from 0 to 100), the a^* value measures the redness to greenness (positive to negative values, respectively), and the b^* value measures the yellowness to blueness (positive to negative values, respectively).

Total DNA extraction from the cheese surface. The total DNA was extracted from the smear culture mixes and the cheese samples using the PowerSoil DNA isolation kit as described in the manufacturer's protocol (Cambio, Cambridge, United Kingdom). For the DNA extraction from the cheese surface, at days 0, 18, 24, and 30, a pretreatment step was included as follows. Samples were removed from different parts of the cheese block and pooled to give a representative sample of 5 g. The cheese was placed in a stomacher bag with 50 ml of 2% trisodium citrate and homogenized using a masticator mixer (JUL SA, Barcelona, Spain) for 5 min.

Fifteen milliliters of the smear-culture mix, or the cheese solution, was placed into sterile Falcon tubes and centrifuged for 30 min at $4,500 \times g$. After centrifugation, the supernatant was discarded and the pellet was placed in a 2-ml Eppendorf tube. The pellet was washed several times with sterile phosphate-buffered saline (PBS) by centrifuging it at $14,500 \times g$ for 1 min, until the supernatant was completely clear. The pellet was then added to PowerBead tubes (Cambio, Cambridge, United Kingdom) provided with the kit as described in the protocol and homogenized by shaking on the TissueLyser II (Qiagen, West Sussex, United Kingdom) at 20 Hz for 10 min. The DNA was then purified according to the protocol of the standard PowerSoil DNA isolation kit (Cambio, Cambridge, United Kingdom).

Total DNA was initially qualified and quantified by gel electrophoresis and the NanoDrop 1000 (Bio-Sciences, Dublin, Ireland) before more-accurate quantification with the Qubit high-sensitivity DNA assay (Bio-Sciences, Dublin, Ireland).

Whole-metagenome shotgun sequencing. Whole-metagenome shotgun libraries were prepared in accordance with the Nextera XT DNA library preparation guide from Illumina (53). Libraries for the starter mixture samples were sequenced on the Illumina MiSeq with a 2 $\times$ 300-bp cycle v3 kit. Libraries for the cheese samples were sequenced on the Illumina NextSeq 500 with a v2 NextSeq 500/550 high-output reagent kit (300 cycles). All sequencing was done in the Teagasc sequencing facility in accordance with standard Illumina sequencing protocols.

Bioinformatic analysis. Raw whole-metagenome shotgun sequencing reads were processed on the basis of quality and quantity using a combination of Picard tools (<https://github.com/broadinstitute/picard>) and SAMtools (54). Processing of raw sequence data produced a total of $3,214,480 \pm 841,719$ filtered reads for samples sequenced on the MiSeq and $10,210,475 \pm 12,478,696$ filtered reads for samples sequenced on the NextSeq. The metagenomic binning tool Kaiju (11) was used to determine the species-level microbial compositions of samples. The NCBI nonredundant protein database (55) was used with Kaiju. PanPhlan (13) was used for strain-level analysis of species of interest. PanPhlan works by aligning sequencing reads against a species pangenome database, built from reference genomes, to identify the gene families present in strains from metagenomic samples. The reference genomes included for each pangenome database are outlined in Table S3. SUPER-FOCUS (14) was used to characterize the microbial metabolic potential of samples. SUPER-FOCUS measures the abundances of subsystems, or groups of proteins with shared functionality, by aligning sequencing reads against a reduced SEED (56) database.

Free amino acid analysis. FAA analysis was performed at the end of the ripening (day 30) on the soluble N extracts using a JEOL JLC-500V AA analyzer fitted with a JEOL Na⁺ high-performance cation-exchange column (JEOL, Garden City, Herts, United Kingdom) (57). The chromatographic analyses were conducted at pH 2.2. Results are expressed as micrograms per milligram of cheese.

Free fatty acid analysis. FFA extractions were performed at the end of the ripening (day 30) according to the method outlined by De Jong and Badings (58). The FFA extracts were derivatized as methyl esters as described by Mannion et al. (59). Fatty acid methyl ester extracts were analyzed using a Varian CP3800 gas chromatograph (Aquilant, Dublin, Ireland) with a CP84000 autosampler and flame ionization detector and a Varian, Inc., 1079 Injector (Aquilant, Dublin, Ireland). Results are expressed as micrograms per milligram of cheese.

Volatile analysis. The volatile compounds were analyzed at days 0, 18, 24, and 30. The surface of the cheese was removed, wrapped in foil, and stored vacuum packed at -20°C until analysis. Before analysis, the samples were defrosted and grated, and 4 g of the cheese surface was used. Analysis was carried out as outlined by Bertuzzi et al. (15).

Statistical analysis. Statistical analysis was done with SAS 9.3 (12) and R-3.2.2 (60). The R packages ggplot2 and phatmap were used for data visualization. The vegan package was used to calculate the Bray-Curtis dissimilarity between samples, while the Hmisc package was used for correlation analysis.

Accession number(s). Sequencing reads have been deposited in the European Nucleotide Archive under the project accession number PRJEB15423.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSystems.00211-17>.

FIG S1, TIF file, 0.1 MB.

FIG S2, TIF file, 0.5 MB.

FIG S3, TIF file, 2.1 MB.

FIG S4, TIF file, 0.2 MB.
 FIG S5, TIF file, 0.04 MB.
 FIG S6, TIF file, 0.1 MB.
 FIG S7, TIF file, 0.1 MB.
 TABLE S1, DOCX file, 0.02 MB.
 TABLE S2, DOCX file, 0.02 MB.
 TABLE S3, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

Andrea Bertuzzi is currently in receipt of a Teagasc Walsh fellowship. Aaron Walsh is funded by the APC Microbiome Institute, via a Science Foundation Ireland grant (SFI/12/RC/2273). Research in the Cotter laboratory is also funded by the Science Foundation Ireland through grant 11/PI/1137.

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First I would like to say thank you to Dr Mary Rea and Dr Kieran Kilcawley for their time, the support, the guidance and suggestions over the course of the PhD, and thanks to Prof Paul Mcsweeney for his supervision. Thanks to the other minor contributors, Dr Caitriona Guinane, Dr Diarmuid Sheehan, Dr Fiona Crispie, Dr Maurice O’Sullivan, and Dr Paul Cotter, who have preciously improved my research.

I would like to mention all colleagues who taught, helped me, and contributed to my PhD research, especially Aaron Walsh, David Mannion, Fergus Collins, Hope Faulkner, Lisa Henihan, Sarah Henneberry, Dr Brian Healy, Dr Cian Hickey, Dr Deirdre Kennedy, Dr Ewelina Stefanovic, and Dr Michelle O’Donnell.

I would like to say thank you to the numerous Italian friends that I have met on the road in these four years, Andrea Badellino, Eleonora Bonvissuto, Giulio Visentin, Mattia Boiani, Vincenzo Pennone, Dr Annalisa Segat, Dr Felicia Ciocia, Dr Giuseppe Mazzola, Dr Leonardo Mancini, and Dr Luigi Scarano.

Thanks to Aoife who have supported me for the last years when the time was tough.

Thanks to all the members of Bertuzzi family, Pierfelice, Tullia, Germana, Cecilia, and Pietro who have encouraged me to work hard to get something important for my life. I am sure my grandparents would be proud of me. I have to say thank you to my brother in law Alberto and my crazy nephews Cristiano and Jacopo, who came to visit me in the delightful mice house in Fermoy.

I have to apologize with my new nephews Diego, Cesare and Silvia, who were born when I had already moved here in Ireland... I hope in the future to catch up the time lost with you!

“Questo viaggio di mille miglia è iniziato con un passo”

