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The Application of Next Generation Sequencing to Profile Microbe Related Cheese Quality Defects

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

by

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Agriculture and Food Development Authority

There is no substitute for hard work

- **Thomas Edison**

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Declaration

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

Signed: _____

Daniel O' Sullivan

Date: _____

Abstract

High throughput next generation sequencing, together with advanced molecular methods, has considerably enhanced the field of food microbiology. By overcoming biases associated with culture dependant approaches, it has become possible to achieve novel insights into the nature of food-borne microbial communities. In this thesis, several different sequencing-based approaches were applied with a view to better understanding microbe associated quality defects in cheese. Initially, a literature review provides an overview of microbe-associated cheese quality defects as well as molecular methods for profiling complex microbial communities. Following this, 16S rRNA sequencing revealed temporal and spatial differences in microbial composition due to the time during the production day that specific commercial cheeses were manufactured. A novel Ion PGM sequencing approach, focusing on decarboxylase genes rather than 16S rRNA genes, was then successfully employed to profile the biogenic amine producing cohort of a series of artisanal cheeses. Investigations into the phenomenon of cheese pinking formed the basis of a joint 16S rRNA and whole genome shotgun sequencing approach, leading to the identification of *Thermus* species and, more specifically, the pathway involved in production of lycopene, a red coloured carotenoid. Finally, using a more traditional approach, the effect of addition of a facultatively heterofermentative *Lactobacillus* (*Lactobacillus casei*) to a Swiss-type cheese, in which starter activity was compromised, was investigated from the perspective of its ability to promote gas defects and irregular eye formation. X-ray computed tomography was used to visualise, using a non-destructive method, the consequences of the undesirable gas formation that resulted. Ultimately this thesis has demonstrated that the application of molecular techniques, such as next generation sequencing, can provide a detailed insight into defect-causing microbial populations present and thereby may underpin approaches to optimise the quality and consistency of a wide variety of cheeses.

Glossary of Terms

°C: Degrees Celcius

µg/ml: Microgram per millilitre

µg: Microgram

µl: Microlitre

µM: Micro molar

AGDIc: Agamatine Deaminase Gene Cluster

aguA: Agmatine Deaminase

aguD: Agmatine/Putrescine Transporter

BA: Biogenic Amine

BLAST: Basic Local Alignment Search Tool

Bp: Base Pair

cDNA: Complementary Deoxyribonucleic Acid

CO_2: Carbon Dioxide

DGGE: Denaturing Gradient Gel Electrophoresis

DNA: Deoxyribonucleic Acid

dNTP: Nucleotide Triphosphates containing Deoxyribose

ds: Double Stranded

ED: Early Day

EMA: Ethidium Monoazide

EU: European Union

FASTA: Fast Adaptive Shrinkage Thresh-holding Algorithm

FFA: Free Fatty Acids

FHLb: Facultatively Heterofermentative Lactobacilli

FISH: Fluorescent *In-Situ* Hybridisation

Fla: Flagella related gene cluster

GC: Gas Chromotagraphy

H_2: Dihydrogen

HPLC: High Performance Liquid Chromotography

hsp60: Heat Shock Protein 60

ITS: Internal Transcribed Spacer

Kg: Kilogram

L: Litre

LAB: Lactic Acid Bacteria

LD: Late Day

MEGAN: Metagenome Alignment

mg/l: milligram per litre

Mg: Miligram

NGS: Next Generation Sequencing

Nmol: Nano Mole

NSLAB: Non Starter Lactic Acid Bacteria

nt: Nucleotide

OHLb: Obligately Heterofermentative Lactobacilli

OUT: Operational Transcriptional Unit

PAB: Propionic Acid Bacteria

PCoA: Principal Component Analysis

PCR: Polymerase Chain Reaction

pH: Power of Hydrogen

pheS: Phenyl-tRNA synthase

PMA: Propidium Monoazide

qPCR: Quantitative Polymerase Chain Reaction

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

RDP: Ribosomal Database Project

recA: Recombinase A

RNA: Ribonucleic Acid

rpoB: RNA Polymerase Beta Subunit

rRNA: Ribosomal Ribonucleic Acid

S/M: Salt in Moisture Ratio

SRA: Sequence Read Archive

SSCP: Single Strand Conformation Polymorphism

tdcA: Tyrosine Decarboxylase Gene A

TP: Time Point

T-RFLP: Terminal Restriction Fragment Length Polymorphism

TTGE: Temporal Temperature Gel Electrophoresis

tuf: Elongation Factor Tu

U: Units

V4 Region: Variable Region of the 16S rRNA gene

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Chapter 1

Literature Review: Nucleic acid-based approaches to investigate microbial-related cheese quality defects

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1.0. Abstract

The microbial profile of cheese is a primary determinant of cheese quality. Microorganisms can contribute to aroma and taste defects, form biogenic amines, cause gas and secondary fermentation defects, and can contribute to cheese pinking and mineral deposition issues. These defects may be as a result of seasonality and the variability in the composition of the milk supplied, variations in cheese processing parameters, as well as the nature and number of the non-starter microorganisms which come from the milk or other environmental sources. Such defects can be responsible for production and product recall costs and thus represent a significant economic burden for the dairy industry worldwide. Traditional non-molecular approaches are often considered biased and have inherently slow turnaround times. Molecular techniques can provide early and rapid detection of defects that result from the presence of specific spoilage microbes and, ultimately, assist in enhancing cheese quality and reducing costs. Here we review the DNA-based methods that are available to detect/quantify spoilage bacteria, and relevant metabolic pathways in cheeses and, in the process, highlight how these strategies can be employed to improve cheese quality and reduce the associated economic burden on cheese processors.

1.1. Introduction

There are approximately 1000 varieties of cheeses, corresponding to 9 different cheese families (Cheddar, Dutch, Swiss, Iberian, Italian, Balkan, Middle Eastern, Mould-ripened and Smear-ripened) produced worldwide (1-4). Cheese is one of the most traded dairy products in the world with EU production of more than 8.4 million tonnes in 2011 (www.eurostat.eu). This generates huge revenues for leading cheese exporting economies. The primary ingredients of cheese are milk, rennet and salt. However it is microbial interactions with these major ingredients which allows for the production of the different varieties. These microbial populations are also the least controllable factor in cheese production (5, 6).

Microbial populations in cheese can be split into two distinct groups i.e. starter and non-starter microorganisms. Generally, starter and non-starter populations exhibit an inverse numerical relationship, with starter culture populations dominating during early cheese manufacture but decreasing in number throughout the ripening process to be eventually replaced by the secondary microbiota. The starter microbiota cause rapid acidification via the production of lactic acid and produce enzymes that are important for flavour development during ripening (7). The most commonly used starter cultures are from the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* (8) and are used as either pure or mixed cultures (9). Non-starter/secondary organisms are primarily bacteria but can also include yeasts, moulds and filamentous fungi (5). Secondary, or initially subdominant microbiota, and in particular non-starter lactic acid bacteria (NSLABs), can play a key role in ripening and flavour development, for example propionic acid bacteria and/or smear cultures (including *Brevibacterium linens*). However, they can

also be associated with the occurrence of defects. NSLAB are adventitious bacteria that gain access to cheese *via* the ingredients used and/or the production and ripening environment. They occur as heterogeneous populations with cell densities exceeding 10^6 cfu g^{-1} cheese during the ripening process (10). They primarily consist of facultatively heterofermentative (mesophilic) lactobacilli (FHLb) as well as pediococci, enterococci and leuconostoc (8, 11). FHLb are Gram-positive, non-motile bacteria capable of growth at pH ranging from 5.5 – 6.2, in 4 – 6% salt and temperatures from 2 - 54°C (12). It is the relationship between these non-starter microbes and the physical features of the cheese (salt, pH and moisture) that can lead to specific (un)desirable characteristics (13).

Defects caused by microorganisms that affect the quality of cheese include odour and taste defects, biogenic amine (BA) formation, gas formation and secondary fermentations, mineral deposition and, potentially, cheese pinking. Controlling the strains, and the proportions thereof, is emerging as a key issue to minimise cheese defects (9).

There are a number of strategies which can be employed to facilitate the detection of microorganisms that cause defects. Traditional culture-dependent studies, although relatively inexpensive, suffer biases due to difficulties encountered when culturing many microbes present in the cheese matrix (13). Molecular methods, based on DNA and/or RNA isolation, provide alternative strategies. Some of the molecular approaches which have been quite popular, such as PCR-based denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient electrophoresis (TTGE), single strand conformation polymorphism (SSCP) and terminal–restriction fragment length polymorphism (T-RFLP) techniques, are in turn being replaced by

quantitative real time PCR (qRT-PCR) and next generation sequencing (NGS) technologies (6, 13). These methods are highly accurate and, in the former case, rapid and cost effective. Furthermore, these approaches facilitate the detection of both specific microbial populations and of encoded metabolic pathways as the need arises (14). This paper reviews molecular methods which are currently employed to detect spoilage bacteria in cheese matrices and discusses the potential use of NGS platforms for the cheese industry.

1.2. Defects Associated with Cheese and the Bacteria Responsible

Defects can occur in cheese due to variations in milk quality, milk pre-treatment (pasteurisation), hygiene practices, differences in starter culture activity and acidity profiles, manufacture technology, compositional parameters and ripening temperature/environments. In addition, consumer demand has seen manufacturers endeavour to reduce the salt content of cheese. This in turn has resulted in a noticeable increase in the occurrence of cheese defects due to increased bacterial growth (9). Many defects are cheese-type specific and a selection of defects are presented here that illustrate the influence of microbiota on cheese quality (Figure 1).

1.2.1 Aroma and Taste Defects

The production of volatile flavour compounds by cheese microbiota is considered a crucial characteristic of cheese quality. However, when certain limits are exceeded, or where an imbalance of flavour compounds occurs, flavour defects are observed (9). Common taste/aroma defects caused by cheese microbiota include bitterness, hydrolytic rancidity and sulphurous defects (15). Bitterness defects, common in Cheddar and Gouda as well as in low salt and low fat cheese, can be as a result of

either excessive proteolysis of caseins or low bacterial peptidase activity among starters (16). Bitter hydrophobic peptides can be liberated from the C-terminal region of β-casein and in α_{s1}-casein and are liberated through the activity of proteinases (9, 15). These enzymes, produced by psychrotrophs such as *Pseudomonas fluorescens* and *P. putrefaciens*, are heat stable and thus unaffected by pasteurisation temperatures consequently allowing for bitter flavours to accumulate (15). Changing the coagulant, using a starter with high peptidase activity and/or manipulating salt content can reduce the occurrence of such bitterness (15).

Hydrolytic rancidity occurs as a result of lipolysis whereby lipids undergo hydrolytic degradation to free fatty acids (FFAs). Levels of FFAs are often used as indicators of lipolysis. Starter cultures, non-starter LAB and moulds/smear organisms all produce lipases that cause lipolysis during ripening (9) and thus have the potential to cause hydrolytic rancidity. Most LAB have a low lipolytic ability and it is the number of bacteria and the time in contact with the cheesefat that leads to the production of significant levels of FFA (17). Propionic acid bacteria (PAB) are considerably more lipolytic than LAB. Moulds such as *Penicillium* spp. are also strong lipolytic agents and are used in mould ripened cheeses such as Brie and Camembert (9, 17).

Volatile agents such as sulphur compounds including (di)/methyl sulphide play a key role in the flavour of many surface ripened and soft cheeses. These compounds give off sulphurous, over ripened and garlic like flavours that contribute to the characteristic flavours associated with surface ripened cheeses such as Brie, Camembert and Limburger. Coryneform bacteria and *B. linens* in particular, are known to be the major producers of sulphur compounds. Flavour thresholds of these

compounds are low (9 – 170 ppb for dimethyl sulphide in Camembert) and thus if these limits are exceeded cheese flavour is adversely affected (18).

Other microbe associated flavour defects include fruity off flavours, harsh and green flavours. Fruity off flavours are as a result of production of ethyl esters by some species of *L. lactis* and *L. lactis* subsp. *cremoris.* This can be controlled by careful selection of starter cultures capable of producing the correct flavour associated with a cheese type as well as standardising storage/handling practices (18, 19). Methyl alcohols/aldehydes produced by certain strains of *L. lactis* are also associated with off flavours (20). Harsh and green flavours are often caused by excessive production of acetaldehyde by some strains of *L. lactis* subsp *cremoris.* This can be controlled through careful starter culture selection, particularly those high in aldehyde reductase, and the inclusion of *Leuconostoc* populations (19). *Leuconostoc* species are known to antagonise detrimental bacteria through formation of organic acids and bacteriocin production (21, 22).

1.2.2. Gas Defects: Split Defects and Secondary Fermentations

Gas defects in cheese can occur for a variety of different reasons. Excess gas production in cheese manifests as cracks, slits, holes and eyes, which while not harmful to the consumer, affects aesthetic properties (23). A variety of microbes can be responsible for gas defects. Gas defects can be subcategorised as either early or late gas. Early gas occurs within 1 – 2 days of manufacture and can affect many cheese varieties. Late gas occurs during later stages of ripening and primarily affects Dutch and Swiss-type cheeses (9, 23-25).

Early Gas Production:

Poor hygiene or the use of unpasteurised milk can result in the presence of coliforms such as *Enterobacter*, *Escherichia*, *Citrobacter* and *Serratia* which are strongly associated with early gas defects. These microbes produce H_2 and/or CO_2 gas aerobically or anaerobically as a by-product of lactose utilisation (23). H_2 is poorly soluble in the aqueous phase of curd and therefore even small quantities can cause serious gas problems. The presence of these gases often also results in development of off-flavours. Coliform levels of approximately 10^7 cfu/g of cheese are sufficient to cause early gas defects (23, 24). Starter bacteria, including sub-species of *L. lactis*, *Streptococcus* and *Leuconostoc,* have also been implicated in undesirable early gas production. Both *Lactococcus* and *Leuconostoc* species are capable of fermenting lactose and citrate to form CO_2. Early gas formation problems often arise when the proportions of these starter bacteria differ from normal allowing one or a group of bacteria to predominate over others (24, 26). Yeasts such as *Kluyveromyces*, *Debaryomyces* and *Candida* are also known to cause gas blowing issues in hard, semi-hard and soft cheeses. Such yeasts are highly resistant to commercial cleaning practices (9, 23, 26).

Late Gas Production:

In many instances this phenomenon is due to the action of PAB which ferment lactose and/or lactate to propionic acid. This gives the characteristic 'nutty' taste and results in the presence of the characteristic 'eyes' associated with Swiss type cheeses (26). In these cases selected strains of PAB are purposely added along with the starter culture to produce different flavour profiles. However, in raw milk cheeses, such as Beaufort, the presence of PAB in milk leads to spontaneous, uncontrolled fermentations (9).

Late gas defects in Swiss and other cheese types can occur within a few weeks of manufacture and up to 4 – 6 months into ripening. There are several factors attributed to irregular late gas production including the presence of butyric acid bacteria (*Clostridium* spp), FHLb, salt tolerant lactobacilli, and the abnormal growth of PAB (9, 23, 26-28). Butyric acid bacteria are anaerobic bacteria that ferment lactate to butyric acid, CO_2 and H_2. These gases are produced when *Clostridium tyrobutyricum* spores germinate during cheese ripening. Other butyric acid bacteria species known to contribute to late gas defects via spore germination include *C. butyricum, C. sporogenes* and *C. beijerinckii* (23). Swiss cheese, and Emmental in particular, is particularly susceptible to spore germination due to the anaerobic environment of cheese as well as higher ripening temperatures (in excess of 20°C). The low salt and acid content also assists in spore germination. Spores often enter milk via fecal contamination of cows udders and are capable of surviving high temperature pasteurisation (23). Good hygiene practices, with respect to both milk and manufacturing equipment, combined with microfiltration or bactofugation of cheese milk reduces the possibility of contamination. Enzymes added to the cheese milk such as lysozyme and the use of bacteriocins such as nisin may also be used in preventing contamination with clostridia spores. Nitrates are also often added for preservation purposes (9, 23).

FHLb, salt tolerant and mesophilic lactobacilli cause gas blowing in Cheddar-type and brine salted cheeses (23). FHLbs such as *Lb. brevis*, and *Lb. casei* ferment residual lactose, galactose and citrate to CO_2 during ripening. This issue is more pronounced in raw milk cheeses due to high levels of NSLAB in comparison to cheese made from pasteurised milk (23). *Lb. brevis* is also present in pasteurised milk but at lower levels

due to pasteurisation and competition by other NSLABs such as *Lb. paracasei* (27). Salt tolerant and mesophilic lactobacilli have been implicated in irregular gas production in both Swiss and Dutch type cheeses. Rapidly growing starter bacteria generally limit the amounts of lactose and galactose present in the cheese and consequently less is available for NSLAB growth (23, 27). When starter populations are affected by bacteriophage attack, incorrect storage conditions and/or elevated salt concentrations, excessive gas formation may result particularly in the presence of FHLb (29). PAB, and *P. freudenreichii* in particular, are responsible for regular eye formation in Swiss-type cheese. However, abnormal growth can lead to late gas defects occurring. Different sub-species of *P. freudenreichii* can have different effects on flavour profile and eye formation. Research has shown that the PAB strains selected, as well as co-cultivation strains, such as *Lb. helveticus* which produces peptides that stimulate activity of PAB particularly during cold room storage, can have a dramatic effect on the occurrence of split defects (28). For example PAB strains with high aspartase activity are associated with excess gas formation. Aspartase is an enzyme responsible for the deamination of aspartate and varies in activity among different strains of PAB. Lactate, in the presence of aspartate, is fermented to acetate, succinate and CO_2 by PAB. Therefore the presence of strains with high aspartase activity causes excess secondary fermentation (27).

1.2.3. BA Formation:

BAs are aliphatic, heterocyclic or aromatic organic nitrogenous compounds with low molecular weight that can be found in a variety of foods including cheese, fish, wine, beer and dry sausage (30-33). They are also naturally present in the body where they function as neurotransmitters and signal transducers (34). BAs can be further sub-

divided into monoamines, such as tyramine, and polyamines, such as putrescine, agmatine and spermidine (31, 32). These amines can exhibit a toxic effect, with reports that histamine concentrations as low as 20 mg kg^{-1} cheese can elicit an adverse reaction in some humans (30, 31). They affect both the vascular and nervous systems (35), with ingestion in susceptible individuals causing a diverse range of symptoms including headache, cardiac palpitations, localised inflammation, nausea, vomiting, and hyper/hypotension (30) (Table 1). BAs have been associated with cases of food poisoning, particularly in fish and cheese, hence the terms scombroid fish poisoning and 'the cheese reaction' have been coined (36). Individuals that are susceptible to adverse reactions following BA ingestion include those prescribed antidepressant drugs classed as monoamine oxidase inhibitor drugs (30, 32, 34, 35, 37) or those with an impaired detoxification system. Furthermore, biogenic amines are also known precursors of carcinogens (36, 38, 39).

The bacteria responsible for the production of biogenic amines contain an amino acid decarboxylase which removes the α-carboxyl from a particular amino acid to give the corresponding amine. BAs and corresponding amino acids include: histamine (histidine), tyramine (tyrosine), tryptamine (tryptophan), putrescine (ornithine), cadaverine (lysine) and β-phenylethylamine (phenylalanine) (30). Several species of *Lactobacillus, Clostridium*, *Pseudomonas* as well as *Enterobacteriaceae* display decarboxylase activity (30, 39). While most BAs are produced via decarboxylase activity, amines such as putrescine are produced by LAB, of the genera *Enterococcus* and *Lactobacillus,* through deamination of agmatine by agmatine deaminase (40). In cheese, biogenic amines are generally produced by the non-starter microorganisms (30, 35, 41). Non-starter microbiota capable of BA

formation includes *Lb. bulgaricus*, *Lb. buchneri, Lb. curvatus, Lb. casei, Lb. acidophilus* *Enterobacter*, *Escherichia*, *Citrobacte*r and *Klebsiella*. Certain PAB species have also been implicated in BA formation (30, 35, 36, 38, 42, 43). It is also noteworthy that certain strains of starter microbiota such as *L. lactis* and *Lb. helveticus* are capable of BA formation, although this has become less of an issue due to screening for decarboxylase activity (9).

1.2.4. Mineral Deposition Defects

Mineral deposition, corresponding to calcium lactate crystal (CLC) formation, is a common defect found in Cheddar cheese (44, 45). CLCs appear as white crystals or spots on the external surface of the cheese (44, 46-48). While not harmful, CLC formation is often mistaken for mould by consumers. This results in an increase in complaints to the manufacturer often leading to product recall. CLC formation is influenced by the concentrations of calcium and lactate ions present in the cheese (44, 45, 48). CLCs are formed via the racemisation of L(+)-lactate to the less soluble D(-)-lactate by racemase-positive NSLAB. Agarwal et al found that CLC crystals occurred after 56 days of ripening on cheese inoculated with *Lb. curvatus* but not in *Lb. curvatus* negative cheese (47). Somers et al, provided further evidence to the role of *Lb. curvatus* in CLC formation by demonstrating that *Lb. curvatus* are capable of forming biofilms which survive cleaning practices. These biofilms can then detach from cheese vats and contaminate the cheese matrix (45, 46, 49). Other researchers have shown that many other strains of lactobacillli and pediococci may also be involved in CLC formation (45). Chou et al showed that lactobacilli negative cheese did not suffer from CLC formation. Furthermore, control cheeses and cheeses manufactured with *Lb. helveticus* did not suffer from crystal formation This study

also suggests that accelerated maturation at higher than normal temperatures may accelerate NSLAB growth, and consequently D(-)-lactate formation and CLCs (44). Johnson et al showed that CLCs did not form in cheeses that were gas flushed and vacuum packed. However, controlling populations of racemase positive lactobacilli and concentrations of lactic acid are regarded as more effective methods of controlling CLC formation (48).

1.2.5. Cheese Pinking

Pink discolouration defects can occur either on external surfaces or within the cheese matrix (9, 50). This defect may occur in cheese with or without Annatto. Annatto is a carotenoid food dye comprised mainly of 2 pigments (bixin and norbixin), sourced from the seeds of the Achiote tree. This dye, which gives an orange/red colour to cheese, often suffers from pink discolouration due to photo-oxidation of its pigments, or interactions of the pigments with heat and/or light (51). However, natural non-dyed cheeses can also suffer from pinking. In such cases, thermophilic lactobacilli (particularly *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*) and propionic acid bacteria (*P. shermanii*) have been suggested as potential causes but this remains a matter of much debate (9, 50). Recently, studies using Next Generation Sequencing platforms have provided evidence for the presence of *Thermus*, and more specifically, *T. thermophilus*, in the occurrence of the pinking defect. Further to this, whole genome shotgun metagenomics sequencing has revealed the presence of *Thermus* genes involved in carotenoid biosynthesis in defect cheeses (Quigley *et al*, Unpublished). Pink discolouration is not harmful to consumers but may result in product recall or downgrading (51).

1.3 Detection Methods

Molecular techniques have revolutionised the strategies employed to detect beneficial and detrimental microorganisms in foods. Previously, culture-dependant approaches, which relied on the isolation and cultivation of microbes, were exclusively employed. In these instances, cultured microbes were identified based on their morphology and/or biochemical features (6, 13, 52). Although relatively inexpensive, such approaches are inefficient, time consuming and tedious. Furthermore, many bacterial species cannot be cultured easily, or at all, on standard agar plates. Thus the identification and quantification of bacteria in this way is inherently biased towards those bacteria that grow well in a laboratory setting (13, 53, 54). Selective media such as MRS, MSE, LM17 and KAA are widely used for culturing lactobacilli, leuconostoc, streptococci/lactococci and enterococci, respectively, from cheese (55, 56). These media allow for the selection of the particular species in question only. In the past, BA producing species were detected in cheese by culturing on selective media containing a pH indicator, such as bromocresol purple. A colour change is then noted around decarboxylase producing colonies due to the production of alkaline amines (32, 35, 36, 55). Examples include MRS-decarboxylase broth used by Rea et al for determining production of biogenic amines by enterococci. (56).

As an alternative to traditional culturing, molecular methods provide rapid, reproducible, accurate and non-biased strategies to analyse microbial communities. These techniques allow for specific species identification in foods without the need to culture. Detection of both viable and non-viable bacterial cells, damaged or completely lysed cells is also possible (57). Furthermore, molecular techniques can

be employed to search for particular enzyme-encoding genes such as amino acid decarboxylases. Identifying microbes that cause defects early in the cheese making process enables manufacturers to uncover and remedy potential sources of contamination quickly and thus minimise the risk of a product recall (54, 58).

PCR amplification of a specific target sequence is often the key element with respect to molecular approaches to bacterial identification (6). Frequently the target region within bacterial genomes is the 16S rRNA gene or the 16S/23S spacer region, either using species/genera specific primers or universal primers (13, 59, 60). The 16S rRNA gene is ubiquitous among bacteria, present at high copy number and there is an abundance of species-specific sequence information available in public databases (52, 61, 62). The 16S rRNA gene consists of highly conserved and highly variable regions making it ideal for bacterial typing (13, 52, 53). Amplifying other conserved target genes that contain conserved and variable domains, such as those encoding the RNA polymerase subunit B (*rpoB)*, phenylalanyl-tRNA synthase (*pheS)*, elongation factor Tu (*tuf*)*,* DNA repair gene (*recA)* or heat shock protein (*hsp60)*, or, alternatively, genes that are genera, species and strain specific, can also be very informative. As more and more sequencing information becomes publicly available, this targeted approach is becoming more popular (13, 59, 62).

The first step in amplifying bacterial genes involves extracting high quality DNA or RNA from a food matrix. This is often accomplished using mechanical homogenization in a salt based solution followed by lytic enzyme treatment (lysozyme, mutanolysin, proteinase K). Nucleic acids are then extracted by either phenol chlorophorm or spin column purification systems which use detergents such as guanidine thiocyanate (6, 63-66). RNA isolation is achieved in a similar fashion

with care taken to remove ribonucleases which degrade this single stranded nucleic acid (67). Extracted nucleic acid, or cDNA generated from RNA, then provides the template for PCR amplification using universal, species specific or gene specific primers, depending on the goal of the study, to generate PCR amplicons. Resultant PCR amplicons will vary in size and/or sequence depending on their bacterial origin (59).

There are, however, issues associated with PCR amplification that can affect the accuracy and reproducibility of the detection methods. The quality of the DNA extracted from the cheese source is the first barrier. The cheese matrix contains many PCR inhibitors such as salts, fats and carbohydrates which need to be removed during the extraction procedure (62). The choice of PCR primers also influences the effectiveness of PCR as dominant and sub-dominant bacterial populations may not be amplified in a proportional manner and, furthermore, different species may differ in gene copy numbers (62). Preferential or differential PCR amplification may also lead to the introduction of a biased view of the community present (6, 68). Preferential amplification of certain PCR templates can occur as a result of differences in GC contents and/or primer mismatches at template annealing sites (69). Another issue affecting PCR is the formation of artefacts such as chimeric amplicons which can occur due to heteroduplex formation (6). These issues can be overcome by including co-solvents, hot-starting DNA or by using low numbers of PCR cycles (68). It should also be noted that the amplification of DNA from dead cells may result in false positives. In order to overcome this RNA can be isolated and subsequently used to generate a cDNA template. Inhibitors such as ethidium

bromide monoazide (EMA) or propidium monoazide (PMA) can be used to bind to and inactivate DNA from dead cells (70).

1.3.1. Molecular Approaches to Study Cheese Defects

Molecular techniques have not specifically been used to identify cheese defects but they have been used to profile microbial populations in cheese (Figure 2). Table 2 summarises the techniques used, organisms identified and cheese tested.

1.3.1.1. Conventional and Quantitative Real Time PCR (qRT-PCR)

Conventional PCR assays using genus- or species-specific primers to target 16S rRNA, or other genes commonly used for rapid bacterial detection and as as such there are numerous publications on this topic (71-73). We will provide only two examples. Rossi *et al,* used nested PCR with species-specific primers to amplify propionibacteria from raw milk samples. This approach indicated seasonal variations in propionibacteria in the dairy environment (74). Herman *et al* used a similar approach to detect *C. tyrobutyricum* in hard and semi hard cheeses. (75). Although such assays are useful from a detection perspective, they do not provide a very accurate insight into the quantity of the microbe present. In contrast, qRT-PCR quantifies the number of specific microorganisms or gene copies present in a sample and represents the 'gold standard' in quantifying genes and gene expression (76-78). qPCR is rapid, extremely sensitive and has been applied in food microbiology, genomics, medicine and environmental studies (52, 77).

qRT-PCR differs from conventional PCR by virtue of being performed in real time in the presence of fluorescent reporters, such that the number of newly generated PCR amplicons can be quantified after each amplification cycle. A DNA binding cyanine dye, such as SYBR Green or BOXTO, are added to the reaction mixture and fluoresce

during DNA amplification (79). Probe based PCR is an alternative to fluorescent reporters.

Once one or many of the PCR components is depleted (primers, dNTPs, polymerase), a saturation limit is reached and the reaction stops. Fluorescence is then recorded versus the number of cycles needed to reach saturation and from this the cycle threshold (Ct) is calculated (76, 78, 79). There are two methods by which the PCR product can be quantified i.e. absolute or relative quantification. Absolute quantification relies on a comparison between levels of fluorescence of the target amplicon to that of a standard curve of known amounts of the target amplicon. Relative quantification is based on gene expression versus that of a 'housekeeping gene', a gene that is expressed at ubiquitous levels within the cell (80).

A recent review by Postollec *et al* compiled numerous examples in which qRT-PCR has been applied to assess food safety (80). There are also cases in which qRT-PCR has been used to investigate food quality, and examples relevant to cheese are mentioned below. Decarboxylase and agmatine deaminase genes have been targeted by qPCR methods as part of efforts to detect, and ultimately target, biogenic amine producing bacteria (81). Ladero *et al* used a qPCR approach to detect histamine producing strains in 80 French and Spanish commercial cheese samples using *hdc* (histidine decarboxylase) specific primers. This approach allowed for histidine decarboxylase positive strains to be detected and quantified in cheeses long before the BAs could be detected via HPLC (81). Fernandez *et al* also developed a qPCR approach using *hdcA* specific primers for detecting histamine positive, Gram positive bacteria in both milk and cheese. Similarly, the blue cheese Cabrales, which has an inherently high BA content, was analysed by qPCR as well as HPLC.

Quantitative PCR detected *hdcA* positive bacteria in all samples during early ripening whereas histamine was only detected by HPLC on day 7 of ripening, even in the cheeses with the highest amine concentrations. Thus, while HPLC can detect actual levels of histamine (mg/g) in the final product, qPCR can determine if the bacteria responsible for histamine biosynthesis are present and in what numbers (82). In another publication by Ladero *et al*, a qPCR method specific for the LAB tyrosine decarboxylase (*tdcA*) gene was used to detect and quantify tyramine producing bacteria in 57 raw or pasteurised cheese samples. *tdcA*-positive bacteria were found in all cheeses, in varying amounts, but the amine itself was only detected by HPLC in 56% of samples. This study implies that when tyramine producing bacteria exceed 10^4 cfu/g cheese, as revealed by qPCR, tyramine build up becomes a quality/safety issue (83). Further studies have also targeted putrescine decarboxylase genes. Strains of *Enterococcus, Lactococcus* and *Lactobacillus* are all implicated in putrescine formation due to the presence of the agmatine deaminase gene cluster (AGDIc). A multiplex qPCR approach to detect and quantify the intergenic spacer region between *aguD* and *aguA* of the AGDIc was proposed by Ladero *et al*. In this study 29 cheese samples made from raw and pasteurised milk were analysed for putrescine producers. Results determined that producers corresponding to the three genera were present in all except 3 cheeses. A direct correlation was also observed between cheeses with the highest numbers of putrescine producers and cheeses with the highest levels of putrescine present, as determined by HPLC. As with other qRT-PCR approaches, this method has the potential to facilitate the early detection of putrescine producers and/or levels of the deaminase gene in raw materials with a view to controlling putrescine levels in the final product (40).

Late blowing issues caused by *C. tyrobutyricum* are a common problem in hard and semi-hard cheeses. As few as 50 spores per litre of milk is enough to cause late blowing effects and thus detection methods must be highly sensitive. Lopez-Enriquez *et al*, targeted the flagellin gene (*fla*) of *C. tyrobutyricum* to successfully detect spores in inoculated raw milk samples. Enzymatic treatment of samples prior to analyses allowed for detection of as few as 25 spores per 25 ml of raw milk (84). Falentin *et al*, 2010 also performed studies using both qPCR and RT-PCR, the latter being employed to reflect RNA levels and thus metabolically active cells, to quantify levels of growth of *P. freudenreichii* and *L. paracasei* at different ripening stages of Emmental cheese. Monitoring these bacteria over time allows for greater understanding of LAB and PAB behaviour in a complex cheese matrix and the roles they play in the occurrence of cheese defects (65). Both *Staphylococcus aureus* and *Listeria monocytogenes* have also successfully been quantified in bovine milk and raw milk cheeses, respectively, using a qRT-PCR approach (85, 86).

Ultimately, it is conceivable that qPCR could be applied to any cheese defect associated with bacteria provided that there is sufficient genome sequencing data available to design target specific primers. Some issues may arise due to detection of dead cells but this can be overcome using inhibitors such as EMA and PMA. qPCR can therefore become a key tool in detecting and quantifying microorganisms known to contribute to cheese defects. Early detection prior to observation of a defect in the final product will enhance cheese quality and decrease overall costs.

<u>1.3.1.2. Denaturing gradient gel electrophoresis (DGGE) and temporal temperature gel electrophoresis (TTGE)</u>

DGGE/TTGE methods allow for separation of PCR amplicons based on differing sequences. These are among the most commonly used methods to assess complexity of microbial communities in food products (64, 87) but are more typically employed for scientific rather than industrial applications. DGGE uses denaturing (urea and formamide-containing) acrylamide gels. As amplicons migrate through the gel matrix, the denaturing agents cause the amplicons to denature partially at melting domains within the sequence. Amplicons are separated due to differences in melting domains as a direct result of sequence differences (6, 64, 88, 89). DGGE is usually performed at a constant temperature between 55°C and 65° C (64, 88). TTGE separates amplicons in the absence of denaturing chemicals and uses temperature variation over time to denature and separate DNA (6). The addition of a GC clamp, a 30 – 40bp GC rich region, added to the PCR primers ensures that amplicons do not completely degrade (53, 64, 89, 90). This approach yields banding patterns which reflect the complexity of microbial populations.

There are many examples where these technologies have been applied for identifying microbes in cheese, although, in the majority of cases, the detection of microorganisms responsible for cheese defects has not been a priority. Cocolin *et al* optimized a protocol for using PCR-DGGE for directly detecting *Clostridium* species responsible for late blowing in cheese. Results obtained showed there was a strong correlation between DGGE and conventional plating techniques. This method has an estimated sensitivity of 10^4 cfu/g cheese making it ideal for detecting spoiled samples (91). PCR-DGGE has also been used for investigating microbial biodiversity in artisanal and protected designation of origin (PDO) cheeses. Randazzo *et al*, 2002 and 2006, applied this method to Ragusano and Pecorino Siciliano cheeses,

respectively. In the former case, *Lactobacillus* specific 16s rRNA primers were used to profile microbial communities. The biodiversity of cheeses provided from three different farmers was assessed and revealed the changes in microbial populations during the production process i.e. from raw milk, curd and 15 to 30 day old cheeses (92). The dynamics of the PDO cheese Pecorino Siciliano made from raw milk, raw milk plus starter culture and pasteurised milk was investigated using a combined PCR-DGGE and culturing approach. Similar microbial profiles were observed in all three cheese samples, however a predominance of wild *L. lactis* and *S. bovis* species in the raw milk cheese is likely responsible for the unique flavour associated with this cheese (93). The microbial composition of the Spanish artisanal cheese Casín, thought to be among the oldest traditional cheeses in Spain, was also investigated using both DGGE and standard culturing methods. Although the aim was to attempt to identify LAB to replace or complement those currently used, the results demonstrate the success of the technique for microbial detection. Interestingly, *S. thermophilus*, a species not previously isolated from traditional Spanish cheeses, was identified by PCR-DGGE but not by culturing methods. Added to this high numbers of coliforms, indicating poor hygienic practices, were identified in the initial stages of production but not in the final product sampled at day 30 (94). Many other studies are available in which microbial populations in artisanal cheeses have been analysed using PCR-DGGE. These include, Fontina (Giannino *et al*, 2009), Robiola di Roccaverno (Bonetta *et al*, 2008), Cabrales (Flores *et al*, 2006), Oscypek (Alegria *et al*, 2012), Fossa (Barbieri *et al*, 2012) and other raw milk cheeses (Quigley *et al*, 2011) (52, 87, 94-98).

Ogier *et al* applied a TTGE approach to investigate the microbiota of model miniature cheeses. This 16S approach was able to differentiate between dominant species such as *L. delbueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. delbrueckii* subsp. *lactis* within a cheese matrix. However, it failed to identify minor species that were present at concentrations below 1% making it unsuitable for the detection of many potential pathogens (99). A similar study by Abriouel *et al* profiled the biodiversity of the Spanish farmhouse cheese Alberquilla using PCR-TTGE. The 16S rRNA gene was amplified with results showing the presence of LABs such as *L. paracasei*, *L. brevis* and *L. acidophilus* as well as less desirable species such as *E. coli* and enterococci (100).

It is noteworthy that TT/DGGE techniques can suffer from reproducibility-related issues due to variable staining, primer dimer formation and the loss of bands corresponding to less abundant strains in a community (62). Similar migration patterns of amplicons with similar melting domains but different sequences also pose a problem. Sekiguchi et al found that a single DGGE band contained several different sequences (101). In addition, prior knowledge of the primer sequence is required for identifying a specific species or genus (13, 59).

1.3.1.3. Single Strand Conformation Polymorphism (SSCP)

SSCP allows for separation of DNA amplicons of similar size based on differences in the conformation of folded single strand DNA in a non-denaturing gel (62). Single strand nucleotide sequences fold into tertiary structures, depending on intramolecular interactions, under non-denaturing conditions and are then separated based on movement through an acrylamide gel (6, 63). This method was used by Takahashi *et al* to study histidine decarboxylase (*hdc*) genes in Gram-

negative bacteria associated with Scombroid poisoning. Bands produced by SSCP were identified by comparison with reference strains and were successfully matched in 8 out of 10 fish samples (102). With respect to cheese, SSCP has not been extensively employed to assess defect-causing populations. Duthoit *et al* used PCR-SSCP combined with microbial clone library sequencing (i.e. amplicons are cloned into vectors, and ultimately host cells, to facilitate DNA sequencing) to profile community dynamics of the raw milk Salers cheese during production. Universal and high GC primers were used to amplify regions of the 16S rRNA gene. Members of the LAB family including *L. lactis, S. thermophilus, L. plantarum* and *E. faecium* were identified (63). SSCP has also been used to determine if certain cheese microbes can inhibit growth of *Listeria monocytogenes* by comparing communities in affected and unaffected cheeses. Saubusse *et al*, demonstrated that on day 8, cheese samples with the lowest counts of *L. monocytogenes* contained *Enterococcus faecium, Enterococcus saccharominimus, Chryseobacterium* spp, and *Corynebacterium flavescens, Lactococcus garvieae* and *Lactococcus lactis,* respectively. Further studies revealed that *L. monocytogenes* inhibition occurred where *L. lactis*, *L. garvieae* and to a lesser extent *C. flavescens* and *E. saccharominimus* were present. This could be as a result of competitive inhibition or an indication of bacteriocin production (103).

1.3.1.4. Fluorescence *In Situ* Hybridisation (FISH)

FISH is based on hybridising regions of a target bacterial genome to a taxon specific DNA probe labelled with a fluorescent dye. These regions can then be detected using fluorescence microscopy or flow cytometry (62). FISH requires prior knowledge of the microbial populations present in a sample (59). Ercolini *et al*, used FISH to detect *L. lactis, Lactobacillus plantarum* and *Leuconostoc mesenteroides* in Stilton cheese.

This approach was successfully used to identify microbes resident in different locations within the cheese matrix. *L. mesenteroides* colonies were found to be distributed throughout the cheese while *L. plantarum* was only found beneath the crust of the cheese. Lactococci were found in the core and veins (104). Bunthof *et al*, employed FISH and flow cytometry to study the viability of LABs using probes labelled with different dyes to discriminate between live and dead cells. The dyes were selected based on their spectroscopic properties to stain DNA. Carboxyfluorescein diacetate (cFDA), a non-fluorescent precursor which is converted to a fluorescent product by cellular enzymes, was used as a live cell stain. Impermeant exclusion dyes propidium iodide (PI) and cyanine dye TOTO-1 were attached to probes and used to stain dead cells. In experiments performed on bile salt stressed cultures of *L. lactis*, *L. helveticus* and *L. mesenteroides* both TOTO-1 and cFDA proved to be accurate indicators of live and dead cells in comparison to plate counts (105).

1.3.1.5. Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Terminal Restriction Fragment Length Polymorphism (T-RFLP)

ARDRA, also known as restriction fragment length polymorphism (RFLP), involves restriction enzyme digestion of multiple PCR amplicons (62). As restriction enzymes digest DNA at specific cleavage sites, differences in amplicon sequences may result in the absence or presence of cleavage sites. Gel electrophoresis of digested amplicons allows for comparative analyses. PCR products can be labelled, at the 5' and/or 3' ends, with a fluorescent dye and are then identified based on differences in multiple restriction enzyme sites (59, 62). This method was used to study the microbial dynamics of the smear ripened Tilsit cheese by Rademaker *et al*, using conserved

bacterial primers and two restriction enzymes (*Hae*III and *Cfo*I) (106). T-RFLP has been used for bacterial profiling in many dairy products however, these methods suffer from a lack of resolution and thus have been of limited use in complex food matrices (62).

1.3.1.6. Ribosomal Intergenic Spacer Analysis (RISA)

RISA focuses on the 16S/23S ribosomal spacer region. The spacer region between these two genes represents a good target for bacterial identification due to heterogeneity in nucleotide length and sequence (62). RISA has been automated (automated RISA or ARISA) using fluorescently labelled PCR primers where a laser is used to detect fluorescent amplicons, (62). This method was used by Cardinale *et al*, to profile bacterial communities in goat's milk using universal primer sets. Results showed that the primer set employed is very effective for evaluating bacterial profiles in complex communities as it yields a wide range of spacer sizes (134 to 1387 bp), produces reproducible profiles and amplifies bacteria at DNA template concentrations from 280 to 0.14 ng/µl (107).

1.3.1.7. Denaturing High Performance Liquid Chromotography (DHPLC)

DHPLC is a relatively new technique that has been employed to study microbial populations in the intestine and in environmental samples. This method involves the separation of PCR amplicons via an automated ion-pairing HPLC system (6). Ercolini *et al* used this technique in conjunction with DGGE to study natural whey cultures in Caciocavallo Silano cheese. PCR fragments generated after amplification of a region of 16S rRNA gene were separated by DHPLC on a C18 reverse phase column. Peaks generated by DHPLC were collected and sequenced. DHPLC generated the same results as DGGE, under the same conditions (108). Major advantages of this system

include that it is fully automated and avoids gel preparation. However, problems with fragment co-migration or the presence of many copies of a DNA fragment may again result in inaccurate representation of microbial diversity (108).

1.3.1.8. DNA Microarrays

DNA microarray technology, originally developed for gene expression analysis, has recently been adapted for profiling microbial communities (109, 110). This approach is of particular interest because of its high density and high throughput capacity. DNA microarray technology is based on the hybridisation of fluorescently labelled target sequences to immobilised complementary sequences (oligonucleotides or small single strand PCR amplicons). The detector sequences are covalently attached to a solid support, either nylon or nitrocellulose membrane (low density macroarrays) or a glass slide (high density microarrays) (62, 110, 111). Detector oligonucleotides are adapted to have nearly identical melting temperatures by including amine salts and/or by manipulating their lengths. The length of oligonucleotide probes are of key importance. Short probes of 20 – 25 nucleotides in length are preferred for microbial ecology studies and require PCR amplification of marker genes. Longer probes (50 – 70 nucleotides) yield better sensitivity and are therefore generally used for transcriptome studies. Long probes also do not require PCR amplification thus avoiding potential PCR bias issues (111). Target sequences, which are fluorescently labelled, then hybridise with complementary detector oligonucleotides to produce a detectable signal (6, 62).

There are three classes of microarrays, functional gene arrays (FGAs), community genome arrays (CGAs) and phylogenetic oligonucleotide arrays (POAs) (62, 109). FGAs are used to monitor the activity of genes that encode functional enzymes in

microbial populations (112). CGAs consist of whole genomic DNA, isolated from pure cultures, which are used as a probe for profiling microbes in complex communities. (112-114). CGA relies on fluorescence based detection on a non-porous surface and is of particular use for bacterial identification at the species and strain level (109). A genome probing microarray (GPM) was used by Bae *et al* to monitor community dynamics of LABs in the Korean fermented food Kimchi (114). The method employed could potentially be applied to a cheese matrix. The major disadvantage of CGAs is that only cultivable microbes in a community can be analysed because genomic DNA from pure isolates are required as probes (109). POAs employ rRNA or other highly conserved sequences as phylogenetic probes. This approach allows for the analysis of both highly variable and highly conserved regions of bacterial DNA and can facilitate species level resolution. A 16S rRNA targeting microarray was used by Treimo *et al* to quantify both *L. lactis* ssp. *lactis* as well as several species of propionibacteria in a liquid cheese model after 0 h, 24 h, 48 h, 7 days and 5 weeks. DNA from the propionibacteria was shown to increase from 48 h up to 7 days, albeit at a slower growth rate than was observed in corresponding broth samples (115). POAs were also used by Kostic *et al* to identify pathogenic bacteria in a predominantly non-pathogenic community. Rather than using 16S rRNA, *gyrB* (encoding the B subunit of bacterial gyrase) was used as a phylogenetic marker in that instance.

There are some issues arising when attempting to apply DNA microarrays to analyse environmental or food samples. These fall into 3 main categories. Firstly, the diversity between the target and probe sequences, particularly in environmental samples, may affect hybridisation particularly if probes are sourced from pure

cultures. Secondly, while recoverable DNA is not an issue when dealing with pure cultures, the amounts of DNA retrieved from environmental samples may be below accurate detection limits. Finally, the presence of hybridisation inhibitors in cheese may also be an issue (112).

1.4. New Detection Methods: Next Generation Sequencing (NGS)

Next generation, also known as massively parallel or high throughput, sequencing technologies represent a dramatic improvement over the traditional Sanger DNA sequencing method when it comes to investigating microbial communities (116). High throughput screening can be applied to specific target genes, such as the 16S rRNA gene, as well as to (meta)genomic and (meta)transcriptomic applications (117). Sequencing of the 16S rRNA gene allows one to determine the relative proportions of different microbial populations within complex communities. In situations where there is a need to differentiate between species that are very closely related, and thus have highly conserved 16S rRNA genes, metagenomic sequencing, i.e. the analysis of the total genetic content of a particular community, is an alternative (14, 117). Whole genome sequencing of harmful (cheese defect bacteria) and beneficial bacteria (117) is also facilitated. Once entire genomes have been sequenced, comparisons can be made better to understand the relationships between microbes within a cheese matrix (117). The majority of NGS platforms currently employed are supplied by three companies i.e. Roche 454 (GS-FLX, GS-FLX+, GS Junior), Illumina (GA, GA II, HISEQ, MISEQ), Applied Biosystems (ABI SOLiD). The data output for each of the above is summarised in Table 3 (14, 117, 118). Less common systems include the Helicos Heliscope, Pacific Biosciences SMRT, Life Technologies Ion Torrent PGM and Oxford NanoPore Technologies (14, 119). NGS instruments share certain

similarities such as the removal of the need for bacterial cloning. Sequences are typically amplified on a glass slide or within microbeads to produce sufficient signal for detection. NGS systems are also capable of sequencing DNA from both ends of single fragments or fragments which are many kbp apart. This process is termed paired end sequencing (14, 119).

1.4.1. Roche 454 FLX Pyrosequencer

The Roche 454 pyrosequencing based technology was first released in 2005 (116) and relies on the generation of a library of DNA fragments which are hybridised to beads. These beads carry oligonucleotide sequences that complement adaptor sequences ligated to the DNA fragments of interest (119). The bead/fragment complex is then amplified using emulsion PCR in an aqueous microreactor (120). After emulsion PCR, amplification fragments are sequenced in a picotiter plate. Within the picotiter plate, a sequencing-by-synthesis approach is used to measure the release of pyrophosphate (PPi). The response to the incorporation of a complementary nucleotide is then measure by a Charge-Coupled Device (CCD) (116). The use of this and other NGS based technologies allows for the study of microbial populations in many environments including foods, and is of particular use in examining spatial and/or temporal variability of a specific microbial community as well as examining microbial co-existence (120, 121). Indeed, this technology has been used by Quigley *et al* to investigate the sub-dominant bacteria in artisanal cheeses. More specifically, 116,000 16S rRNA amplicon reads, corresponding to 62 different cheese types, were sequenced to reveal the presence of 5 bacterial phyla including *Firmicutes*, *Bacteriodetes*, *Proteobacteria*, *Acintobacteria* and the fungal phylum *Ascomycota*. Indeed, several genera not previously associated with cheese,

including *Faecalibacterium*, *Prevotella* and *Helcococcus* were detected and, for the first time, the presence of *Arthrobacter* and *Brachybacterium* in goats' milk cheese was noted. The detection of populations not previously associated with cheese shows the benefits of using high throughput screening to investigate these microbial populations (122). Masoud *et al* also used this technology to profile the microbial communities present in Danish raw milk and cheeses at different stages of ripening. This study showed that the microbial diversity of Danish raw milk cheeses declined during ripening. This is due to the impact of the cooking temperature and acidification that occur prior to and during the ripening process. Further studies into the effects of cooking temperature, acidification and starter culture addition on the growth of pathogenic bacteria including *E. coli, Listeria innocua* and *S. aureus,* in 4 inoculated cheeses, was investigated using both NGS and qPCR. Results showed that *E. coli* numbers increased until day 7 of ripening and then decreased thereafter. Adjunct starters *Brevibacteria linens* and *Microbacterium lacticum* also did not affect growth of the pathogenic strains during ripening (123). Roche-based pyrosequencing was also used by Alegria *et al* to investigate the microbial biodiversity within the traditional Polish cheese Oscypek. Four bacterial phyla were identified i.e. *Firmicutes*, *Actinobacteria*, *Bacteriodetes* and *Proteobacteria*. This was also the first observation of *Bifidobacteriaceae* present in cheese as sub-dominant populations belonging to both *Bifidobacteriaceae* and *Moraxellaceae* were identified using pyrosequencing (97). In a further study, 16S rRNA amplicon sequencing was used by O'Sullivan et al, to profile the microbial community dynamics of brine salted Continental-type cheese produced early and late in the production day. Interestingly, the genera *Thermus*, *Pseudoalteromonas* and *Bifidobacterium*, not routinely

associated with a Continental-type cheese produced from pasteurised milk were identified (124).

454 pyrosequencing has also been used to sequence the genomes of many dairy associated bacteria. This would allow for determining particular species which contain a specific gene cluster, such as biogenic amine gene clusters. Examples of cheese associated microbes sequenced include, *Lactobacillus cypricasei* KCTC 13900 (125), *Corynebacterium casei* UCMA 3821 (126), *Streptococcus macedonicus* ACA-DC 198 (127) and *Corynebacterium variabile* DSM 44702 (128) among many others.

1.4.2. Illumina/Solexa Genome Analyzer

The Illumina Genome Analyzer was commercially released in 2006 (129) and has since been updated in the form of the HiSeq and MiSeq platforms. For these instruments single stranded DNA fragments are attached to a flow cell, a solid, multi-channel single molecule array (119). DNA fragments are attached to the flow cell via an adaptor molecule and form bridges by hybridising to complementary adaptors. The bridge is then used as the template for generation of complementary strands through bridge amplification (116). After amplification, the flow cell contains upwards of 40 million clusters, where each cluster contains clones of the template DNA fragment (116). This system also uses sequencing by synthesis approach except that all four nucleotides are added together with a DNA polymerase rather than individually as in the 454 system. The DNA polymerase incorporates fluorescently labelled reversible terminator sequences to growing nucleotide chains. Each terminator sequence is labelled with a different fluorophore to differentiate between the different nucleotide bases. Therefore each cluster is sequenced by the colour associated with the nucleotide added (116, 119, 129). It has recently been

established that Illumina based 16S rRNA sequencing is a valid alternative to other 16S based sequencing approaches (130). Recently, whole genome shotgun sequencing using the Illumina HiSeq 2000 platform, provided an in-depth profile of not only bacterial and fungal populations, but also revealed functional diversity of populations present in cheese rind communities (131).

1.4.3. ABI SOLiD

The Applied Biosystems SOLiD sequencer was released in 2007 and relies on sequencing by ligation rather than by synthesis (116). Sequencing libraries are generated by emulsion PCR, similarly to pyrosequencing, and then sequenced on a glass surface by repeating rounds of hybridisation and ligation with 8-mer fluorescent oligonucleotides. The 8-mer oligonucleotides contain fluorescent markers that identify a two base combination which is termed di-nucleotide encoding (129). The 2 base encoding method allows for an accuracy of 99.94%. The library preparation however is time consuming (116). To date this system has not been used to investigate cheese microbiology.

1.4.4. Ion Torrent Personal Genome Machine (PGM)

The Ion Torrent PGM, commercialised in 2010, is similar to 454 pyrosequencing as it relies on an emulsion PCR and sequencing by synthesises approach (132). Ion Torrent technologies do not, however, depend on optical scanning instead using highly sensitive pH probes to detect hydrogen liberated during the incorporation of nucleotides (133). This allows for faster run times and reduced costs (132). The use of various chip sizes (314, 316 and 318) also allows for flexibility with respect to read length, bp yield and consequently cost (134). To date, Ion PGM sequencing has primarily been used for studies on environmental, faecal and oral microbiomes

(135). More recently however, O'Sullivan *et al,* used the PGM platform to screen a range of different cheese varieties for the presence of microbial populations capable of producing biogenic amines. This study identified common amines producers such as *Lb. curvatus*, *Lb. brevis*, *Enterococcus faecalis* and *E. faecium* as well as species commonly used as cheese starters (*Lb. delbrueckii* and *S. thermophilus*) (136).

It is anticipated that these and new sequencing technologies, such as clonal library independent third generation sequencing platforms (Oxford Nanopore, Helicos Heliscope Sequencer) (133), will be widely employed to provide a detailed insight into cheese-associated microbial populations in the future.

1.5. Conclusions and Future Perspectives

Traditional culture-based approaches to detect bacteria in cheeses are being replaced by culture-independent molecular methods. Researchers are shifting from a polyphasic approach which relies on both culture dependent and independent techniques to PCR based culture independent methods only. This is due to the rapid ability of PCR to detect viable, non-viable, damaged/permeabilised and non-cultivable microbes. Molecular methods, therefore, allow for more effective studies of dominant and sub-dominant populations in complex matrices such as cheese, promoting a greater understanding of microbial community structure and activity. The relationships between different microbes as well as the different pathways involved in creating many of the varieties of cheese are now better understood than ever before (13, 52, 62).

While the advent of PCR has revolutionised the way in which microbes are detected in food products, it is important to note that there is no 'one size fits all' PCR-based approach. Thus, selecting the correct method/s for sample analysis is as important as

the technique itself. Techniques such as PCR-TTGE/DGGE and SSCP provide some insight to microbes present in a food sample and have thus predominantly been used for population based studies. Conventional PCR or qPCR are more frequently employed when targeting specific taxa or genes. DNA microarrays can also be employed in a number of situations, depending on which genes are present on the array. qPCR based approaches are already available to detect and quantify decarboxylase gene expression in fermented foods. In the case of decarboxylase genes, sequence variability has led to the development of multiplex PCR assays to facilitate the simultaneous detection of the major enzyme groups (40, 82, 137, 138). Notably, current BA detection is often through HPLC, with a detection limit of 0.1 mg/kg. However this does not assist in pre-empting product recall issues. Thus quantifying the levels of certain decarboxylase genes present via qPCR or DNA microarrays, at various stages of production, could potentially prevent contaminated products entering the market and consequently reduce product recall costs.

Finally, next generation sequencing represents the most recent advance with respect to the evolution of microbial ecology. NGS will significantly enhance our understanding of the genomes and transcriptomes of food microbes and provide greater insight into structural community interactions and metabolic activity. Further reductions in labour time and costs will make NGS even more attractive for food quality and safety studies (52).

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1.7. References

1. **Sandine WE, Elliker PR.** 1970. MICROBIALLY INDUCED FLAVORS AND FERMENTED FOODS - FLAVOR IN FERMENTED DAIRY PRODUCTS. Journal of Agricultural and Food Chemistry **18:**557-&.
2. **Fox PF, McSweeney PLH.** 2004. Cheese: An Overview, p 1-18. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed), Cheese: Chemistry, Physics and Microbiology, vol Volume 1. Academic Press.
3. **Anonymous.** 1987. Cheese: chemistry, physics and microbiology. Volume 2. Major cheese groups. Elsevier Applied Science Publishers Ltd., Barking, Essex.
4. **Fox PF, McSweeney PLH, Cogan TM, Guinee TP.** 2004. Cheese: Chemistry, Physics and Microbiology: Major Cheese Groups, vol 2. Academic Press.
5. **Fox PF.** 2000. Fundamentals of cheese science, *on* Aspen Pub. http://encompass.library.cornell.edu/cgi-bin/checkIP.cgi?access=gateway_standard%26url=http://www.knovel.com/web/portal/browse/display?_EXT_KNOVEL_DISPLAY_bookid=1040. Accessed
6. **Jany JL, Barbier G.** 2008. Culture-independent methods for identifying microbial communities in cheese. Food Microbiol **25:**839-848.
7. **Leroy F, De Vuyst L.** 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology **15:**67-78.
8. **Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM.** 2001. Recent advances in cheese microbiology. International Dairy Journal **11:**259-274.

9. **McSweeney PLH.** 2007. Cheese problems solved. Woodhead Publishing Ltd, Cambridge.

10. **Swearingen PA, O'Sullivan DJ, Warthesen JJ.** 2001. Isolation, characterization, and influence of native, nonstarter lactic acid bacteria on Cheddar cheese quality. Journal of dairy science **84:**50-59.

11. **Beresford T, Williams A.** 2004. The Microbiology of Cheese Ripening, p 287-317. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed), Cheese: Chemistry, Physics and Microbiology, vol Volume 1. Academic Press.

12. **Lynch CM, McSweeney PLH, Fox PF, Cogan TM, Drinan FD.** 1996. Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. International Dairy Journal **6:**851-867.

13. **Ndoye B, Rasolofo EA, LaPointe G, Roy D.** 2011. A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. Dairy Science & Technology **91:**495-524.

14. **Mardis ER.** 2011. A decade's perspective on DNA sequencing technology. Nature **470:**198-203.

15. **Lemieux L, Simard RE.** 1991. Bitter Flavor in Dairy-Products .1. A Review of the Factors Likely to Influence Its Development, Mainly in Cheese Manufacture. Lait **71:**599-636.

16. **Sousa MJ, Ardö Y, McSweeney PLH.** 2001. Advances in the study of proteolysis during cheese ripening. International Dairy Journal **11:**327-345.

17. **Collins YF, McSweeney PLH, Wilkinson MG.** 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. International Dairy Journal **13:**841-866.

18. **Sable S, Cottenceau G.** 1999. Current knowledge of soft cheeses flavor and related compounds. Journal of Agricultural and Food Chemistry **47:**4825-4836.

19. **Sandine WE, Daly C, Elliker PR, Vedamuthu ER.** 1972. Causes and Control of Culture-Related Flavor Defects in Cultured Dairy Products. Journal of Dairy Science **55:**1031-1039.

20. **Morales P, Fernandez-Garcia E, Gaya P, Nunez M.** 2003. Formation of volatile compounds by wild Lactococcus lactis strains isolated from raw ewes' milk cheese. International Dairy Journal **13:**201-209.

21. **Stiles ME.** 1994. Bacteriocins Produced by Leuconostoc Species. Journal of Dairy Science **77:**2718-2724.

22. **Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C.** 1991. Detection and activity of a bacteriocin produced by Leuconostoc mesenteroides. Applied and environmental microbiology **57:**3450-3455.

23. **Sheehan JJ.** 2011. Cheese | Avoidance of Gas Blowing, p 661-666. *In* Editor-in-Chief: John WF (ed), Encyclopedia of Dairy Sciences (Second Edition) doi:10.1016/b978-0-12-374407-4.00079-0. Academic Press, San Diego.

24. **Mullan WMA.** 2000. Causes and control of early gas production in cheddar cheese. International Journal of Dairy Technology **53:**63-68.

25. **Jimeno J, Lazaro MJ, Sollberger H.** 1995. Antagonistic interactions between propionic acid bacteria and non-starter lactic acid bacteria. Lait **75:**401-413.

26. **Sheehan JJ, Wilkinson MG, McSweeney PLH.** 2008. Influence of processing and ripening parameters on starter, non-starter and propionic acid

bacteria and on the ripening characteristics of semi-hard cheeses. International Dairy Journal **18:**905-917.

27. **Daly DFM, McSweeney PLH, Sheehan JJ.** 2010. Split defect and secondary fermentation in Swiss-type cheeses - A review. Dairy Science & Technology **90:**3-26.

28. **White SR, Broadbent JR, Oberg CJ, McMahon DJ.** 2003. Effect of Lactobacillus helveticus and Propionibacterium freudenrichii ssp. shermanii Combinations on Propensity for Split Defect in Swiss Cheese. Journal of Dairy Science **86:**719-727.

29. **O'Sullivan DJ, McSweeney PLH, Cotter PD, Giblin L, Sheehan JJ.** Compromised Lactobacillus helveticus starter activity in the presence of facultative heterofermentative Lactobacillus casei DPC6987 results in atypical eye formation in Swiss-type cheese. Journal of Dairy Science **99:**2625-2640.

30. **Shalaby AR.** 1996. Significance of biogenic amines to food safety and human health. Food Research International **29:**675-690.

31. **Novella-Rodriguez S, Veciana-Nogues MT, Izquierdo-Pulido M, Vidal-Carou MC.** 2003. Distribution of biogenic amines and polyamines in cheese. Journal of Food Science **68:**750-755.

32. **Bover-Cid S, Holzapfel WH.** 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. International Journal of Food Microbiology **53:**33-41.

33. **Önal A.** 2007. A review: Current analytical methods for the determination of biogenic amines in foods. Food Chemistry **103:**1475-1486.

34. **Santos MHS.** 1996. Biogenic amines: their importance in foods. International Journal of Food Microbiology **29:**213-231.

35. **Spano G, Russo P, Lonvaud-Funel A, Lucas P, Alexandre H, Grandvalet C, Coton E, Coton M, Barnavon L, Bach B, Rattray F, Bunte A, Magni C, Ladero V, Alvarez M, Fernández M, Lopez P, de Palencia PF, Corbi A, Trip H.** 2010. Biogenic amines in fermented foods. European Journal of Clinical Nutrition **64:**S95-S100.

36. **Joosten HM, Northolt MD.** 1989. Detection, growth, and amine-producing capacity of lactobacilli in cheese. Applied and environmental microbiology **55:**2356-2359.

37. **Innocente N, Biasutti M, Padovese M, Moret S.** 2007. Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. Food Chemistry **101:**1285-1289.

38. **Joosten HMLJ.** 1988. The Biogenic-Amine Contents of Dutch Cheese and Their Toxicological Significance. Netherlands Milk and Dairy Journal **42:**25-42.

39. **Giraffa G.** 2003. Functionality of enterococci in dairy products. International Journal of Food Microbiology **88:**215-222.

40. **Ladero V, Cañedo E, Pérez M, Martín MC, Fernández M, Alvarez MA.** 2012. Multiplex qPCR for the detection and quantification of putrescine-producing lactic acid bacteria in dairy products. Food Control **27:**307-313.

41. **Stratton JE, Hutkins RW, Taylor SL.** 1991. Biogenic amines in cheese and other fermented foods: a review. Journal of Food Protection **54:**460-470.

42. **Marino M, Maifreni M, Moret S, Rondinini G.** 2000. The capacity of Enterobacteriaceae species to produce biogenic amines in cheese. Letters in applied microbiology **31:**169-173.

43. **Sumner SS, Roche F, Taylor SL.** 1990. Factors Controlling Histamine Production in Swiss Cheese Inoculated with Lactobacillus buchneri. Journal of Dairy Science **73:**3050-3058.

44. **Chou YE, Edwards CG, Luedecke LO, Bates MP, Clark S.** 2003. Nonstarter Lactic Acid Bacteria and Aging Temperature Affect Calcium Lactate Crystallization in Cheddar Cheese. Journal of Dairy Science **86:**2516-2524.

45. **Swearingen PA, Adams DE, Lensmire TL.** 2004. Factors Affecting Calcium Lactate and Liquid Expulsion Defects in Cheddar Cheese. Journal of Dairy Science **87:**574-582.

46. **Agarwal S, Costello M, Clark S.** 2005. Gas-Flushed Packaging Contributes to Calcium Lactate Crystals in Cheddar Cheese. Journal of Dairy Science **88:**3773-3783.

47. **Agarwal S, Sharma K, Swanson BG, Yüksel GÜ, Clark S.** 2006. Nonstarter Lactic Acid Bacteria Biofilms and Calcium Lactate Crystals in Cheddar Cheese. Journal of Dairy Science **89:**1452-1466.

48. **Johnson ME, Riesterer BA, Chen C, Tricomi B, Olson NF.** 1990. Effect of Packaging and Storage Conditions on Calcium Lactate Crystallization on the Surface of Cheddar Cheese. Journal of Dairy Science **73:**3033-3041.

49. **Somers EB, Johnson ME, Wong ACL.** 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. Journal of dairy science **84:**1926-1936.

50. **Shannon EL, Olson NF, von Elbe JH.** 1969. Effect of Lactic Starter Culture on Pink Discoloration and Oxidation-Reduction Potential in Italian Cheese. Journal of Dairy Science **52:**1557-1561.

51. **Shumaker EK, Wendorff WL.** 1998. Factors Affecting Pink Discoloration in Annatto-colored Pasteurized Process Cheese. Journal of Food Science **63:**828-831.

52. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2011. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. International Journal of Food Microbiology **150:**81-94.

53. **Temmerman R, Huys G, Swings J.** 2004. Identification of lactic acid bacteria: culture-dependent and culture-independent methods. Trends in Food Science & Technology **15:**348-359.

54. **Delbes C, Ali-Mandjee L, Montel MC.** 2007. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. Applied and environmental microbiology **73:**1882-1891.

55. **Landete JM, De Las Rivas B, Marcobal A, Munoz R.** 2008. Updated molecular knowledge about histamine biosynthesis by bacteria. Critical Reviews in Food Science and Nutrition **48:**697-714.

56. **Rea MC, Franz CMAP, Holzapfel WH, Cogan TM.** 2004. Development of enterococci and production of tyramine during the manufacture and ripening of Cheddar cheese. Irish Journal of Agricultural and Food Research **43:**247-258.

57. **Sheehan A, O'Cuinn G, FitzGerald RJ, Wilkinson MG.** 2009. Distribution of microbial flora, intracellular enzymes and compositional indices throughout a 12 kg Cheddar cheese block during ripening. International Dairy Journal **19:**321-329.

58. **Carraro L, Maifreni M, Bartolomeoli I, Martino ME, Novelli E, Frigo F, Marino M, Cardazzo B.** 2011. Comparison of culture-dependent and -independent methods for bacterial community monitoring during Montasio cheese manufacturing. Research in Microbiology **162:**231-239.

59. **Randazzo CL, Caggia C, Neviani E.** 2009. Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. Journal of microbiological methods **78:**1-9.

60. **Clarridge JE, 3rd.** 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical microbiology reviews **17:**840-862, table of contents.

61. **Scheu PM, Berghof K, Stahl U.** 1998. Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction. Food Microbiology **15:**13-31.

62. **Justé A, Thomma BPHJ, Lievens B.** 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiology **25:**745-761.

63. **Duthoit F, Godon JJ, Montel MC.** 2003. Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. Applied and environmental microbiology **69:**3840-3848.

64. **Ercolini D.** 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods **56:**297-314.

65. **Falentin H, Postollec F, Parayre S, Henaff N, Le Bivic P, Richoux R, Thierry A, Sohier D.** 2010. Specific metabolic activity of ripening bacteria

quantified by real-time reverse transcription PCR throughout Emmental cheese manufacture. International Journal of Food Microbiology **144:**10-19.

66. **Parayre S, Falentin H, Madec M-N, Sivieri K, Le Dizes A-S, Sohier D, Lortal S.** 2007. Easy DNA extraction method and optimisation of PCR-Temporal Temperature Gel Electrophoresis to identify the predominant high and low GC-content bacteria from dairy products. Journal of Microbiological Methods **69:**431-441.

67. **Ulve VM, Monnet C, Valence F, Fauquant J, Falentin H, Lortal S.** 2008. RNA extraction from cheese for analysis of in situ gene expression of Lactococcus lactis. Journal of Applied Microbiology **105:**1327-1333.

68. **Reysenbach AL, Giver LJ, Wickham GS, Pace NR.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. Applied and environmental microbiology **58:**3417-3418.

69. **Walsh PS, Erlich HA, Higuchi R.** 1992. Preferential PCR amplification of alleles: mechanisms and solutions. PCR methods and applications **1:**241-250.

70. **Nocker A, Camper AK.** 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiology Letters **291:**137-142.

71. **Lantz P-G, Abu Al-Soud W, Knutsson R, Hahn-Hägerdal B, Rådström P.** 2000. Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples, p 87-130, Biotechnology Annual Review, vol Volume 5. Elsevier.

72. **Hill WE, Wachsmuth K.** 1996. The polymerase chain reaction: Applications for the detection of foodborne pathogens. Critical Reviews in Food Science and Nutrition **36:**123-173.

73. **Malorny B, Tassios PT, Rådström P, Cook N, Wagner M, Hoorfar J.** 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. International Journal of Food Microbiology **83:**39-48.

74. **Rossi F, Torriani S, Dellaglio F.** 1999. Genus- and Species-Specific PCR-Based Detection of Dairy Propionibacteria in Environmental Samples by Using Primers Targeted to the Genes Encoding 16S rRNA. Applied and environmental microbiology **65:**4241-4244.

75. **Herman L, DeBlock J, VanRenterghem R.** 1997. Isolation and detection of Clostridium tyrobutyricum cells in semi-soft and hard cheeses using the polymerase chain reaction. Journal of Dairy Research **64:**311-314.

76. **Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D.** 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiology **28:**848-861.

77. **Bustin SA, Benes V, Nolan T, Pfaffl MW.** 2005. Quantitative real-time RT-PCR - a perspective. Journal of Molecular Endocrinology **34:**597-601.

78. **Bustin SA.** 2005. Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences. Expert Review of Molecular Diagnostics **5:**493-498.

79. **Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N.** 2006. The real-time polymerase chain reaction. Molecular Aspects of Medicine **27:**95-125.

80. **Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D.** 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food microbiology **28:**848-861.

81. **Ladero V, Linares DM, Fernández M, Alvarez MA.** 2008. Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: Relation with histamine content. Food Research International **41:**1015-1019.

82. **Fernández M, del Río B, Linares DM, Martín MC, Alvarez MA.** 2006. Real-Time Polymerase Chain Reaction for Quantitative Detection of Histamine-Producing Bacteria: Use in Cheese Production. Journal of Dairy Science **89:**3763-3769.

83. **Ladero V, Martínez N, Martín MC, Fernández M, Alvarez MA.** 2010. qPCR for quantitative detection of tyramine-producing bacteria in dairy products. Food Research International **43:**289-295.

84. **Lopez-Enriquez L, Rodriguez-Lazaro D, Hernandez M.** 2007. Quantitative detection of Clostridium tyrobutyricum in milk by real-time PCR. Applied and environmental microbiology **73:**3747-3751.

85. **Graber HU, Casey MG, Naskova J, Steiner A, Schaeren W.** 2007. Development of a Highly Sensitive and Specific Assay to Detect Staphylococcus aureus in Bovine Mastitic Milk. Journal of Dairy Science **90:**4661-4669.

86. **Hagi T, Kobayashi M, Nomura M.** 2010. Molecular-based analysis of changes in indigenous milk microflora during the grazing period. Bioscience, biotechnology, and biochemistry **74:**484-487.

87. **Bonetta S, Bonetta S, Carraro E, Rantsiou K, Cocolin L.** 2008. Microbiological characterisation of Robiola di Roccaverano cheese using PCR–DGGE. Food Microbiology **25:**786-792.

88. **Ercolini D, Moschetti G, Blaiotta G, Coppola S.** 2001. The Potential of a Polyphasic PCR-DGGEApproach in Evaluating Microbial Diversity of Natural Whey Cultures for Water-Buffalo Mozzarella Cheese Production: Bias of Culture-Dependent and Culture-Independent Analyses. Systematic and Applied Microbiology **24:**610-617.

89. **Cocolin L, Diez A, Urso R, Rantsiou K, Comi G, Bergmaier I, Beimfohr C.** 2007. Optimization of conditions for profiling bacterial populations in food by culture-independent methods. International Journal of Food Microbiology **120:**100-109.

90. **Ogier J-C, Serror P.** 2008. Safety assessment of dairy microorganisms: The Enterococcus genus. International Journal of Food Microbiology **126:**291-301.

91. **Cocolin L, Innocente N, Biasutti M, Comi G.** 2004. The late blowing in cheese: a new molecular approach based on PCR and DGGE to study the microbia1 ecology of the alteration process. International Journal of Food Microbiology **90:**83-91.

92. **Randazzo CL, Torriani S, Akkermans AD, de Vos WM, Vaughan EE.** 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied and environmental microbiology **68:**1882-1892.

93. **Randazzo CL, Vaughan EE, Caggia C.** 2006. Artisanal and experimental Pecorino Siciliano cheese: Microbial dynamics during manufacture assessed by culturing and PCR–DGGE analyses. International Journal of Food Microbiology **109:**1-8.

94. **Alegría Á, Álvarez-Martín P, Sacristán N, Fernández E, Delgado S, Mayo B.** 2009. Diversity and evolution of the microbial populations during

manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk. International Journal of Food Microbiology **136:**44-51.

95. **Barbieri E, Schiavano GF, De Santi M, Vallorani L, Casadei L, Guescini M, Gioacchini AM, Rinaldi L, Stocchi V, Brandi G.** 2012. Bacterial diversity of traditional Fossa (pit) cheese and its ripening environment. International Dairy Journal **23:**62-67.

96. **Giannino ML, Marzotto M, Dellaglio F, Feligini M.** 2009. Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. International Journal of Food Microbiology **130:**188-195.

97. **Alegria A, Szczesny P, Mayo B, Bardowski J, Kowalczyka M.** 2012. Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. Applied and environmental microbiology **78:**1890-1898.

98. **Florez AB, Mayo B.** 2006. Microbial diversity and succession during the manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by PCR-DGGE. International Journal of Food Microbiology **110:**165-171.

99. **Ogier JC, Son O, Gruss A, Tailliez P, Delacroix-Buchet A.** 2002. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Applied and environmental microbiology **68:**3691-3701.

100. **Abriouel H, Martín-Platero A, Maqueda M, Valdivia E, Martínez-Bueno M.** 2008. Biodiversity of the microbial community in a Spanish farmhouse

cheese as revealed by culture-dependent and culture-independent methods. International Journal of Food Microbiology **127:**200-208.

101. **Sekiguchi H, Tomioka N, Nakahara T, Uchiyama H.** 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnology Letters **23:**1205-1208.

102. **Takahashi H, Kimura B, Yoshikawa M, Fujii T.** 2003. Cloning and sequencing of the histidine decarboxylase genes of gram-negative, histamine-producing bacteria and their application in detection and identification of these organisms in fish. Applied and environmental microbiology **69:**2568-2579.

103. **Saubusse M, Millet L, Delbes C, Callon C, Montel MC.** 2007. Application of Single Strand Conformation Polymorphism - PCR method for distinguishing cheese bacterial communities that inhibit Listeria monocytogenes. International Journal of Food Microbiology **116:**126-135.

104. **Ercolini D, Hill PJ, Dodd CER.** 2003. Bacterial community structure and location in Stilton cheese. Applied and environmental microbiology **69:**3540-3548.

105. **Bunthof CJ, Bloemen K, Breeuwer P, Rombouts FM, Abee T.** 2001. Flow cytometric assessment of viability of lactic acid bacteria. Applied and environmental microbiology **67:**2326-2335.

106. **Rademaker JLW, Peinhopf M, Rijnen L, Bockelmann W, Noordman WH.** 2005. The surface microflora dynamics of bacterial smear-ripened Tilsit cheese determined by T-RFLP DNA population fingerprint analysis. International Dairy Journal **15:**785-794.

107. **Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D.** 2004. Comparison of

different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Applied and environmental microbiology **70:**6147-6156.

108. **Ercolini D, Frisso G, Mauriello G, Salvatore F, Coppola S.** 2008. Microbial diversity in Natural Whey Cultures used for the production of Caciocavallo Silano PDO cheese. International Journal of Food Microbiology **124:**164-170.

109. **Zhou J.** 2003. Microarrays for bacterial detection and microbial community analysis. Current Opinion in Microbiology **6:**288-294.

110. **Liu-Stratton Y, Roy S, Sen CK.** 2004. DNA microarray technology in nutraceutical and food safety. Toxicology Letters **150:**29-42.

111. **Bodrossy L, Sessitsch A.** 2004. Oligonucleotide microarrays in microbial diagnostics. Current Opinion in Microbiology **7:**245-254.

112. **Zhou J, Thompson DK.** 2002. Challenges in applying microarrays to environmental studies. Current Opinion in Biotechnology **13:**204-207.

113. **Wu LY, Thompson DK, Liu XD, Fields MW, Bagwell CE, Tiedje JM, Zhou JZ.** 2004. Development and evaluation of microarray-based whole-genome hybridization for detection of microorganisms within the context of environmental applications. Environmental Science & Technology **38:**6775-6782.

114. **Bae JW, Rhee SK, Park JR, Chung WH, Nam YD, Lee I, Kim H, Park YH.** 2005. Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. Applied and environmental microbiology **71:**8825-8835.

115. **Treimo J, Vegarud G, Langsrud T, Marki S, Rudi K.** 2006. Total bacterial and species-specific 16S rDNA micro-array quantification of complex samples. Journal of Applied Microbiology **100:**985-998.

116. **Morozova O, Marra MA.** 2008. Applications of next-generation sequencing technologies in functional genomics. Genomics **92:**255-264.

117. **O'Flaherty S, Klaenhammer TR.** 2011. The Impact of Omic Technologies on the Study of Food Microbes. Annual Review of Food Science and Technology, Vol 2 **2:**353-371.

118. **Buermans HPJ, den Dunnen JT.** 2014. Next generation sequencing technology: Advances and applications. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease **1842:**1932-1941.

119. **Mardis ER.** 2008. Next-generation DNA sequencing methods. Annual Review of Genomics and Human Genetics **9:**387-402.

120. **Roh SW, Abell GCJ, Kim KH, Nam YD, Bae JW.** 2010. Comparing microarrays and next-generation sequencing technologies for microbial ecology research. Trends in Biotechnology **28:**291-299.

121. **Stellato G, De Filippis F, La Storia A, Ercolini D.** 2015. Coexistence of Lactic Acid Bacteria and Potential Spoilage Microbiota in a Dairy Processing Environment. Appl Environ Microbiol **81:**7893-7904.

122. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2012. High-throughput sequencing detects subpopulations of bacteria not previously associated with artisanal cheeses. Applied and environmental microbiology doi:10.1128/AEM.00918-12.

123. **Masoud W, Vogensen FK, Lillevang S, Abu Al-Soud W, Sorensen SJ, Jakobsen M.** 2011. The fate of indigenous microbiota, starter cultures,

Escherichia coli, Listeria innocua and Staphylococcus aureus in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. International journal of food microbiology doi:10.1016/j.ijfoodmicro.2011.11.014.

124. **O'Sullivan DJ, Cotter PD, O'Sullivan O, Giblin L, McSweeney PLH, Sheehan JJ.** 2015. Temporal and Spatial Differences in Microbial Composition during the Manufacture of a Continental-Type Cheese. Applied and Environmental Microbiology **81:**2525-2533.

125. **Kim D-S, Choi S-H, Kim D-W, Kim RN, Nam S-H, Kang A, Kim A, Park H-S.** 2011. Genome Sequence of Lactobacillus cypricasei KCTC 13900. Journal of Bacteriology **193:**5053-5054.

126. **Monnet C, Loux V, Bento P, Gibrat J-F, Straub C, Bonnarme P, Landaud S, Irlinger F.** 2012. Genome Sequence of Corynebacterium casei UCMA 3821, Isolated from a Smear-Ripened Cheese. Journal of Bacteriology **194:**738-739.

127. **Papadimitriou K, Ferreira S, Papandreou NC, Mavrogonatou E, Supply P, Pot B, Tsakalidou E.** 2012. Complete Genome Sequence of the Dairy Isolate Streptococcus macedonicus ACA-DC 198. Journal of Bacteriology **194:**1838-1839.

128. **Schroder J, Maus I, Trost E, Tauch A.** 2011. Complete genome sequence of Corynebacterium variabile DSM 44702 isolated from the surface of smear-ripened cheeses and insights into cheese ripening and flavor generation. Bmc Genomics **12**.

129. **Zhang J, Chiodini R, Badr A, Zhang GF.** 2011. The impact of next-generation sequencing on genomics. Journal of Genetics and Genomics **38:**95-109.

130. **Lazarevic V, Whiteson K, Huse S, Hernandez D, Farinelli L, Østerås M, Schrenzel J, François P.** 2009. Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. Journal of Microbiological Methods **79:**266-271.

131. **Wolfe Benjamin E, Button Julie E, Santarelli M, Dutton Rachel J.** 2014. Cheese Rind Communities Provide Tractable Systems for In Situ and In Vitro Studies of Microbial Diversity. Cell **158:**422-433.

132. **Reuter Jason A, Spacek DV, Snyder Michael P.** 2015. High-Throughput Sequencing Technologies. Molecular Cell **58:**586-597.

133. **Di Bella JM, Bao Y, Gloor GB, Burton JP, Reid G.** 2013. High throughput sequencing methods and analysis for microbiome research. Journal of Microbiological Methods **95:**401-414.

134. **Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y.** 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics **13:**341.

135. **Milani C, Hevia A, Foroni E, Duranti S, Turroni F, Lugli GA, Sanchez B, Martín R, Gueimonde M, van Sinderen D, Margolles A, Ventura M.** 2013. Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. PLoS ONE **8:**e68739.

136. **O'Sullivan DJ, Fallico V, O'Sullivan O, McSweeney PL, Sheehan JJ, Cotter PD, Giblin L.** 2015. High-throughput DNA sequencing to survey

bacterial histidine and tyrosine decarboxylases in raw milk cheeses. BMC Microbiol **15:**266.

137. **Torriani S, Gatto V, Sembeni S, Tofalo R, Suzzi G, Belletti N, Gardini F, Bover-Cid S.** 2008. Rapid detection and quantification of tyrosine decarboxylase gene (tdc) and its expression in gram-positive bacteria associated with fermented foods using PCR-based methods. Journal of Food Protection **71:**93-101.

138. **Coton E, Coton M.** 2005. Multiplex PCR for colony direct detection of Gram-positive histamine- and tyramine-producing bacteria. Journal of Microbiological Methods **63:**296-304.

Table 1: Food associated amines and their effects

Amine Structure	Amine	Effect
Monoamines	Tyramine	Hypertensive reactions, migraines, increased blood sugar levels
	Histamine	Respiratory distress, heart palpitations
	Tryptamine	Increased blood pressure
	β-phenylethlyamine	Increased blood pressure & migraines
Diamines	Putrescine	Hypotension, bradycardia, carcinogenic effects, potentiate effects of other amines
	Cadaverine	Hypotension, bradycardia, potentiate effects of other amines
Polyamines	Agmatine, Spermine, Spermidine	Cell growth and differentiation

Table 2: Non-exhaustive list of genotyping methods to study microbiota of cheese and milk

Authors	Method	Substrate	Type of Study: Microorganisms Detected/Genes Targeted
Rossi *et al*, 1999	Conventional Nested	Raw Milk	Propionibacteria (*P. freudenreichii, P. jensenii, P. acidipropionici*)
Herman *et al*, 1997	Conventional PCR	Hard/Semi hard cheeses	*C. tyrobutyricum*
Ladero *et al*, 2008	qPCR	French/Spanish Commercial Cheeses	*hdc* gene
Fernandez *et al*, 2006	qPCR	Milk, Cabrales Cheese	*hdcA G*ene
Ladero *et al*, 2010	qPCR	Raw/Pasteurised Milk	*tdcA* Gene
Lopez-Enriquez et al, 2	qPCR	Innoculated raw and pasteurised milk chee	*fla* gene of *C. tyrobutyricum*
Falentin *et al*, 2010	qPCR and RT-PCR	Emmental Cheese	*P. freudenreichii* and *L. paracasei*
Graber *et al*, 2007	qPCR	Bovine milk cheese	*Staphylococcus aureus*
Hagi *et al*, 2010	qPCR	Raw milk cheese	*Listeria monocytogenes*
Cocolin *et al*, 2004	PCR-DGGE	Grana Padano cheese	*Clostridium* species
Randazzo *et al*, 2002	PCR-DGGE	Ragusano Cheese	*Lactobacillus* species
Randazzo *et al*, 2006	PCR-DGGE	Pecorino Siciliano cheese	Microbial biodiversity studies
Alegria *et al*, 2009	PCR-DGGE	Casín cheese	Lactic Acid Bacteria profiles

Giannino *et al*, 2009	PCR-DGGE	Fontina cheese	Microbial biodiversity studies
Bonetta *et al*, 2008	PCR-DGGE	Robiola di Roccaverno cheese	Microbial biodiversity studies
Florez *et al*, 2006	PCR-DGGE	Cabrales cheese	Microbial diversity and succession
Alegria *et al*, 2012	PCR-DGGE	Oscypek cheese	Microbial biodiversity studies
Barbieri *et al*, 2012	PCR-DGGE	Fossa cheese	NSLAB biodiversity
Ogier *et al*, 2002	PCR-TTGE	Washed curd cheese	Differentiation between dominant microbes
Abriouel *et al*, 2008	PCR-TTGE	Alberquilla	LAB identification
Duthoit *et al*, 2003	SSCP	Salers cheese	Profile community dynamics
Saubusse *et al,* 2007	SSCP	Raw milk cheese	*L. monocytogenes* inhibition
Ercolini *et al*, 2003	FISH	Stilton cheese	Microbe visualisation studies
Bunthof *et al,* 2001	FISH	Bovine milk cheese	LAB viability studies
Rademaker *et al*, 2005	T-RFLP	Tilsit cheese	Microbial dynamics studies
Cardinale *et al*, 2004	RISA	Goats milk	Microbial biodiversity studies
Ercolini *et al,* 2008	D-HPLC	Caciocavallo Silano cheese	Whey culture profiles
Treimo *et al,* 2006	DNA Microarray	Liquid cheese model	Lactococcus and Propionibacteria studies

Quigley *et al,* 2012	Pyrosequencing	Artisanal cheeses	Microbial community analysis
Masoud *et al,* 2011	Pyrosequencing	Danish raw milk and cheese	Microbial dynamics studies
Alegria *et al,* 2012	Pyrosequencing	Oscypek cheese	Microbial biodiversity studies
Wolfe *et al,* 2014	Illumina HiSeq	Cheese rinds	Microbial Diversity and Functionality
O'Sullivan *et al,* 2015	Pyrosequencing	Continental Type Cheese	Microbial dynamics and diversity studies
Stellato et al, 2015	Pyrosequencing	Cheese/Dairy Processing Facilities	Co-Existence of LAB and spoilage bacteria
O'Sullivan *et al,* 2015	Ion PGM	Artisanal Cheeses	Decarboxylase Genes (*hdc* and *tdc)*

Table 3: List of Bases/Read and Yield/Run of the most common NGS platforms

Instrument	**Read Length (bp)**	**Yield (Mb)/Run**
Roche 454 GS Junior	400	50 Mb
Roche 454 FLX Titanium XL+	700	700 Mb
Roche 454 FLX+	650	650 Mb
Illumina MiSeq	2 x 300	15 Gb
Illumina HiSeq 2000/2500	2 x 150	600 Gb
Helicos Heliscope	~ 30	15 Gb
Life Technologies Ion Torrent (318 Chip)	200 - 400	1.5 – 2 Gb
Life Technologies Proton (Ion P1 Chip)	125	8 – 10 Gb
Life Technologies Abi/Solid	75 + 35	300 Gb

Figure 1: Microbe associated cheese quality defects

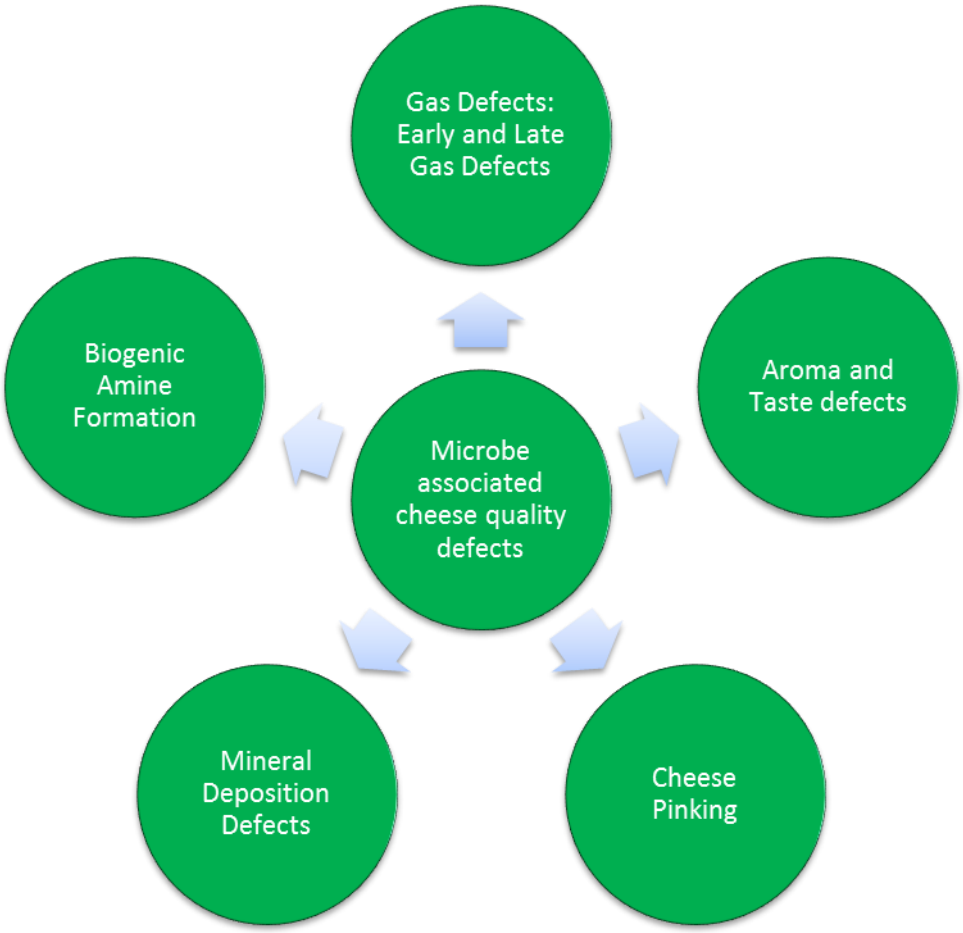
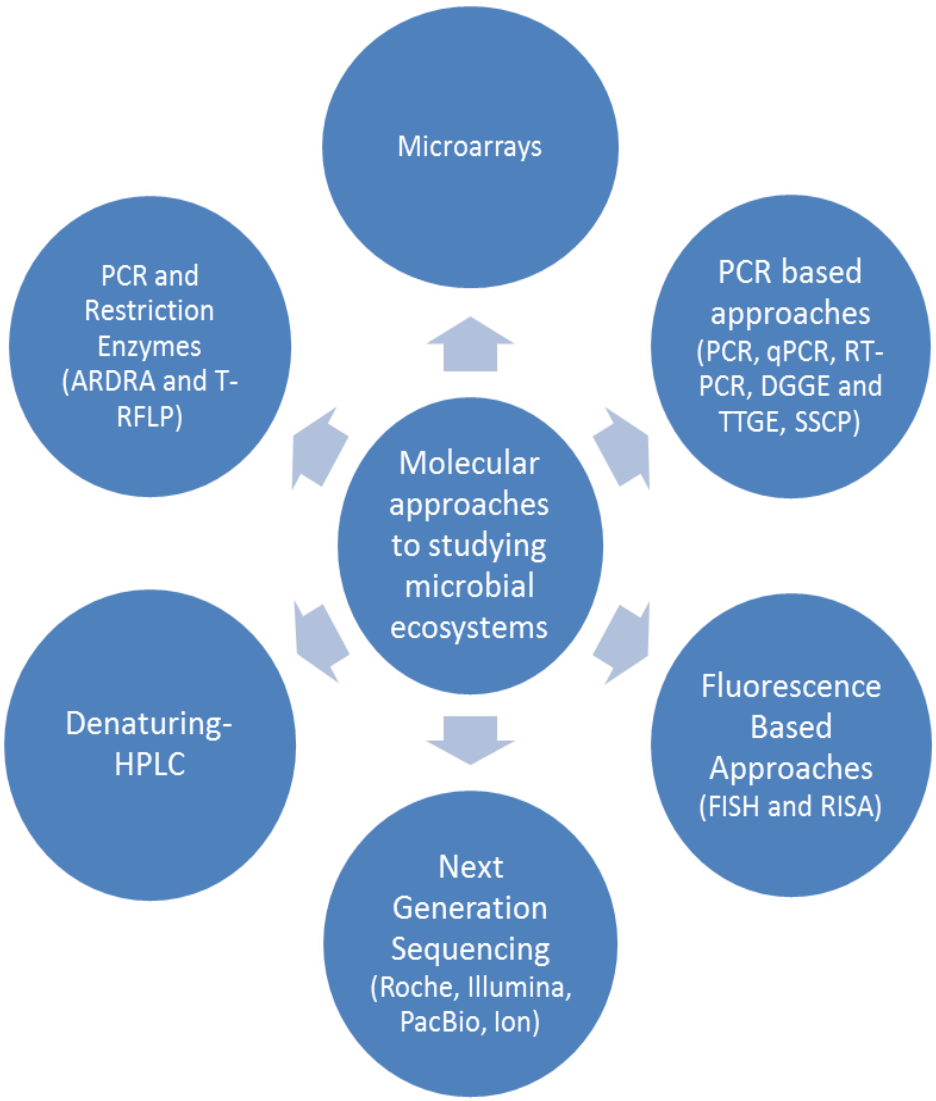


Figure 2: Methods of profiling complex microbial ecosystems



Chapter 2

Temporal and spatial differences in microbial composition during the manufacture of a Continental-type cheese

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2.0. Abstract

We sought to determine if the time, within a production day, that a cheese is manufactured has an influence on the microbial community present within that cheese. To facilitate this, 16S rRNA amplicon sequencing was used to elucidate the microbial community dynamics of brine salted Continental-type cheese in cheeses produced early and late in the production day. Differences in microbial composition of the core and rind of the cheese were also investigated.

Throughout ripening, it was apparent that late production day cheeses had a more diverse microbial population than their early day equivalents. Spatial variation between the cheese core and rind was also noted in that cheese rinds were found to initially have a more diverse microbial population but thereafter the opposite was the case. Interestingly, the genera *Thermus*, *Pseudoalteromonas* and *Bifidobacterium*, not routinely associated with a Continental-type cheese produced from pasteurised milk were detected. The significance, if any, of the presence of these genera will require further attention. Ultimately, the use of high throughput sequencing has facilitated a novel and detailed analysis of the temporal and spatial distribution of microbes in this complex cheese system and established that the period during a production cycle at which a cheese is manufactured can influence its microbial composition.

2.1. Introduction

Commercial cheeses produced with defined starter/adjunct strains often suffer from variations in cheese flavour profile and microbial content (1). This is thought to be primarily due to batch variations in milk quality and storage time as well as manufacturing practices (2) and the adventitious microbial populations present (3, 4). Indeed, in the latter case, aroma and taste defects, along with biogenic amine formation, mineral deposition (calcium lactate) issues and irregular gas formation are common defects associated with a variety of microorganisms (5).

Analysis of the bacterial composition of cheese has traditionally involved the use of culture based techniques which, while effective for quantifying common starter/non-starter bacteria as well as certain spoilage bacteria (*Clostridium*, *Staphylococus*), do not always accurately reflect the total microbiota present (6, 7). PCR based molecular profiling techniques targeting either particular populations or select taxonomic communities are also routinely used and have been extensively reviewed (8-10). PCR based methods cannot, however, provide comprehensive coverage of total microbial populations.

The advent of high throughput next generation sequencing (NGS) has advanced the field of microbial ecology by providing a powerful means of analysing dominant and sub-dominant populations and their dynamics in highly complex ecosystems (2). NGS has been applied extensively to a variety of environments including the sea (11), soil (12) as well as the gut (13). More recently, NGS of bacterial 16S rRNA amplicons has been used to characterise the microbial communities of a variety of fermented foods and beverages (14-20), as well as of raw milk and raw milk cheeses (21-26). Indeed, this approach has led to identification of a number of genera previously not

associated with cheese ecosystems (*Prevotella, Helcococcus*) or with particular cheese types (*Arthrobacter* in goat's milk cheese). Microbial content has also been shown to vary with milk source, processing (raw or pasteurised) and addition of various ingredients (27). Ultimately, NGS platforms offer significantly increased detection sensitivity over more traditional molecular methods with respect to the study of bacterial communities (2, 26, 28, 29). NGS based approaches have also been used to profile communities present in production facilities providing a unique insight into possible microbial reservoirs important for cheese sensory characteristics or for identifying potential biofilm forming genera (2).

Both culture and molecular based approaches have been used to better understand the spatial distribution of microbes in cheese. Microbial composition varies throughout the cheese block due to several factors including salt, moisture, pH and the availability of oxygen (30). The effect of salt is particularly important in brine-salted cheese varieties as salt migrates to the core of the cheese over the ripening process, affecting moisture levels and microbial growth (31). To date the majority of studies examining the spatial distribution of microbial populations in cheese have relied on two methods. One involves non-destructive fluorescent microscopy, based on production of a gel cassette system (32) or via cryosectioning, followed by fluorescence in situ hybridization (FISH) using rRNA targeted probes (33, 34). The second involves destructive sampling of selected regions of cheese followed by an assessment of the microbiota by culture-dependent and/or independent methods (3, 30, 35-37). More recently an NGS approach was used by Wolfe *et al*. to reveal both the microbial composition and functional potential of 137 cheese rind communities. In this case, 16S rDNA and Internal Transcribed Spacer (ITS) amplicon

sequencing allowed for characterisation of microbial communities while 'shotgun' metagenomics permitted an in-depth analysis of pathways involved in flavour formation (38).

In this study, 16S rRNA amplicon sequencing was used to describe, from both a spatial and a temporal perspective, the microbiota present in a brine-salted continental-type cheese produced within a single production day. This study builds on results from a previous study which reported a significant interaction between time of day of manufacture and stage of ripening on mean viable counts of Non Starter Lactic Acid Bacteria (NSLAB) ($p < 0.04$), with cheeses ($n=42$), produced late (in comparison to those produced early or middle in the day of manufacture) having significantly higher mean viable NSLAB counts (39). We assess if production of the cheese early or later during the daily cheese-making cycle impacts on the subsequent development of its bacterial community, investigate how these populations change throughout the ripening process and examine variance in microbial spatial distribution between the cheese core and rind. In each case noteworthy variations in the microbial composition, resulting from differences in production phase, stage of ripening or the part of the cheese being studied, are apparent.

2.2. Materials and Methods

2.2.1. Cheese Production, Sampling and Nucleic Acid Extraction

Four blocks of semi-hard brine salted Continental-type cheese produced from pasteurised milk were sourced, one day post production. The blocks were produced in a single production day, from separate vats and corresponded to early day (morning sampling; [ED], n=2) and late day (afternoon sampling; [LD], n=2) production with 6-8 hours separating ED and LD manufacture. Furthermore, two blocks were received from each respective vat. Cheeses were produced based on a Swiss-type model using the thermophilic starters *Streptococcus thermophilus* and *Lactobacillus helveticus. Propionibacterium freudenreichii* was added as an adjunct. Post production, cheeses were subjected to ripening at 10°C for 10 days prior to hot-room ripening (20°C) from day 10 to day 40. Cheeses were then stored at 6°C for the remainder of ripening.

Each individual block was sampled aseptically, using a cheese trier, at 4 stages; 1 day post production (TP1), 10 days post production (TP2), 40 days post production (TP3) and after maturation at 64 days post production (TP4). Internal (core) and external (rind/1cm segment) regions of the cheese, at each time point, were also sampled. 1g of cheese was homogenised in 9ml of a 2% tri-sodium citrate buffer (VWR, Dublin, Ireland). Enzymatic lysis treatment on homogenised cheese samples was conducted prior to DNA extraction and included treatment with lysozyme (1mg/ml), mutanolysin (50U/ml) and proteinase K (800µg/ml) and incubation for 1 hour at 55°C as per Quigley *et al.* (40). DNA was extracted using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, USA). Grated samples from cheeses were analysed for salt (41), moisture (42) and pH (43) at TP4.

2.2.2. PCR amplification of the microbial 16S rRNA gene

Extracted DNA was amplified using universal primers targeting the V4 region of the bacterial 16S gene (239nt) (4, 44). Primers, predicted to bind to 94.6% of all bacterial 16S genes, consisted of a forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). Primers also included a 19-mer sequence (GCCTGCCAGCCCGCTCAG) at the 5' end to allow emulsion based clonal amplification for the 454-Pyrosequencing system. Identification of individual sequences from the pooled samples was achieved by incorporating molecular identifier tags between the primer sequence and the adaptamer.

PCR reactions were carried out in triplicate and contained 25µl BioMix Red Master Mix (Bioline, London, UK), 1µl of each primer (200 nmol l^{-1}), 5µl of the DNA template (standardised to 100ng DNA/sample) and nuclease free water to a final volume of 50µl. PCR amplification was carried out using a G-Storm Thermal Cycler (Gene Technologies, UK). Amplification consisted of an initial denaturation at 94°C for 10 minutes followed by 40 cycles of; denaturation at 94° for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final elongation step at 72°C for 2 minutes. PCR amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, UK). DNA quantity was assessed using the Quant-It Picogreen dsDNA reagent (Invitrogen, USA) in accordance with manufacturer's guidelines and in conjunction with the NanoDrop

3300 Fluorospectrometer (Thermo-Fisher Scientific, Wilmington, USA). Furthermore, DNA was standardised to equi-molar concentrations prior to library preparation and sequencing.

2.2.3. High-throughput sequencing and bioinformatic analysis

16S rRNA amplicons from the V4 region were sequenced on a Roche 454 FLX platform (Roche Diagnostics Ltd, West Sussex, UK) as previously described (17, 44) and according to protocols. Reads were quality filtered using the RDP sequencing pipeline (45). Reads with low quality scores (below 40), short lengths (less than 150bp), and reads lacking exact matches with respect to primer sequence were discarded. Reads were clustered, aligned and chimeras removed also within QIIME (46). All assigned OTUs were considered. A phylogenetic tree was generated using the FastTree software and subsequently alpha and beta diversities were calculated. Principle coordinate analysis (PCoA), measuring dissimilarities at phylogenetic differences based on weighted/unweighted Unifrac analysis were carried out using the QIIME suite of programs (46). Resultant PCoA plots were visualised with KiNG. Each trimmed FASTA sequence was assessed using the BLAST programme (47) against the SILVA 16S database (version 1.06). The resultant BLAST programme output was parsed using MEGAN (48). Bit scores were used for filtering the results prior to tree construction and summarization (absolute cut-off, BLAST bit score of 86, relative cut-off, 10% of top hit). Reads were deposited in the SRA database under the accession number PRJEB8181.

2.3. Results

2.3.1. α and β diversity of microbial populations in early and late day production cheeses

Blocks of brine salted Continental-type cheese, manufactured early or late during a production cycle, were sampled at various stages throughout the ripening process. Post DNA extraction, amplicons corresponding to the V4 region of the bacterial 16S rRNA gene were generated by PCR. These amplicons were then subjected to NGS, generating 294,853 reads. This corresponded to 87,156 reads for TP1, 97,045 reads for TP2, 62,248 reads from TP3 and 48,404 reads from TP4 (full list of reads/individual sample and associated bar graphs located in Table S1/Figure S2). Species diversity (α-diversity) and richness were calculated for each time point as well as for time of manufacture (early/late day) and the location (core or rind) from which the samples were collected. These are presented in Table 1. Chao1 values, reflective of Operational Taxonomic Unit richness, ranged from 237.8 to 529.38, while the Shannon index, used to measure overall sample diversity, ranged from 2.51 to 3.82. Analysis of this data reveals that α-diversity decreases throughout the ripening process. Cheeses produced early in the production day had a less diverse microbiota than those produced late in the production day. Diversity appeared greatest in the rinds of the samples at TP1 whereas, for all subsequent time points, core populations were more diverse. These observations held true regardless of whether the samples were from ED or LD manufacture. Rarefaction curves, used to determine species richness from sampling, were calculated at 97% similarity. These revealed that bacterial diversity was well represented as samples are nearing parallel with the x-axis (Figure S1).

β diversity, based on the Unweighted UniFrac matrix, and represented in the form of a PCoA plot, was used to determine if samples grouped with respect to ripening point, time of manufacture (early/late) and internal/external regions of the cheese (Figure 1A/B). Notably, samples from the same time point during the cheese ripening process generally grouped together, with data points from TP1/TP2 and TP3/TP4 also forming distinct clusters. In addition, samples clustered according to time of cheese production with those produced early in the production day clustering together and away from a more diffuse cluster of data points corresponding to samples from cheeses manufactured later in the production cycle (Fig. 1A). Core and rind samples also formed distinct clusters. The distinction between the core and rind populations was more apparent in samples manufactured later in the production cycle (Fig. 1B).

2.3.2. Cheese composition

Cheese pH, salt and S/M was determined at TP4 for both ED and LD cheeses. Results were similar with respect to pH (5.39 ED and 5.45 LD), salt (0.59% ED and 0.57% LD) and Salt/Moisture (1.55% ED and 1.51% LD).

2.3.3. High throughput sequencing reveals differences in microbial taxa between cheeses produced early and late in the production day

Phylogenetic assignment of high throughput sequence data revealed the presence of bacteria corresponding to 5 phyla; *Firmicutes, Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus* and *Actinobacteria.* As expected the *Firmicutes* dominated throughout the study representing 93.46 – 99.75% of reads in the ED samples. The percentages of the reads that corresponded with *Firmicutes* were lower in the LD samples and ranged from 72.26 – 85.56%. *Deinococcus-Thermus* was detected in

both the ED and LD samples but at higher percentage populations in LD samples. Less dominant populations, corresponding to *Actinobacteria* and *Proteobacteria*, were also detected. *Proteobacteria* populations were highest at TP1 in both ED and LD samples.

At genus level, a number of differences were noted between cheese produced early and late in the production day (Fig. 2). *Lactobacillus* and *Streptococcus* populations dominated in both ED and LD samples throughout the study. Percentage populations of *Lactobacillus* were similar in both ED and LD samples at TP1 (64.4% ED and 63.5% LD), thereafter it was noticed that populations were consistently higher in the ED samples. Proportions of *Streptococcus* were greater in the ED samples (31.1%) than the LD samples (18.3%), a trend that continued throughout the study. *Thermus* was detected in both ED and LD samples but at consistently greater proportions in the LD samples (0.1% – 5% in ED and 10.9% – 24.4% in LD).

Among the sub-dominant populations, there were a number of other notable observations. At TP1 and 2, *Acinetobacter* and *Pseudomonas* were detected exclusively in the ED samples while *Brevibacterium* and *Corynebacterium* were detected only in the LD samples at TP1. *Clostridium* was identified at TP2 in both ED and LD samples and was consistently detected throughout the remainder of the study. In all instances, *Clostridium* was present at higher proportions in ED samples. *Staphylococcus*, a genus commonly associated with food spoilage, was detected in both ED and LD samples at TP2 only. Of the other sub-dominant populations detected, *Vibrio*, *Lactococcus* and *Psychrobacter* were present in both ED and LD samples recurrently, while *Pseudoalteromonas* was present in ED and LD samples up

until TP4. A full list of both dominant and subdominant genera present is located in Table S2.

2.3.4. Distribution of microbial communities present in the core and rind of a brine salted continental-type cheese

Although the majority of genera detected in this study were localised in both the core and rind of the cheese sampled (Fig. 3 and 4), differences in proportions were noted. This is most obvious when examining populations corresponding to the genus *Lactobacillus* which were consistently higher in the core of the cheeses than in the rind throughout the ripening process. In contrast, *Streptococcus* populations were consistently higher in the respective rinds than in the core. *Thermus* populations were also noticeably higher in the rinds than the core. This difference was particularly apparent in the LD samples (i.e. the samples in which *Thermus* levels were highest). Populations including *Lactococcus*, *Vibrio* and *Psychrobacter* were consistently detected in both the core and the rind throughout the ripening process. Similarly *Pseudomonas* and *Pseudoalteromonas* were identified in the core and rind at initial ripening stages but not at TP4. Of the other subdominant populations, *Clostridium*, present in TP2, 3 and 4, was only detected in the respective cheese cores. Similarly, *Ruminococcaceae Incertae Sedis, Bifidobacterium* and *Arthrobacter* were sporadically detected in core regions only. *Brevibacterium and Corynebacterium,* genera commonly associated with surface ripened cheeses, were located in the rind as were *Staphylococcus* and *Weisella*. A full list of both dominant and subdominant genera present is located in Table S3.

2.5. Discussion

In this study, NGS of 16S rRNA amplicons provided a detailed insight into the microbiota present in a brine salted continental-type cheese produced with thermophilic starter bacteria. As expected, bacterial diversity was found to decrease throughout the ripening process. Interestingly, bacterial diversity in late production day cheeses were determined to be greater than those produced early in the production day. Differences in microbial populations present in the respective cores and rinds were noted while several genera not usually associated with cheese produced from pasteurised milk were also detected.

Microbial diversity (α diversity) was greatest at TP1 (1d post production) in both early and late production day samples. While diversity may seem low in comparison to gut or soil communities (12, 49), it is comparable to that seen in studies of similar cheese types (27). Cheeses that were produced later during the initial manufacturing day ultimately had a more diverse microbial population than their early day equivalents. This trend persisted throughout ripening demonstrating, for the first time, that the time of day at which production occurs impacts on the microbiota present not only in the final product but throughout ripening. Greater diversity in terms of microbial populations present in LD cheeses may be due to accumulating microbial load during the manufacturing process or as a result of longer milk storage times. The significance of this phenomenon with respect to cheese quality will be the focus of further investigations.

Prior studies have described differences in the spatial distribution of microbial communities between the rind and core of several cheeses produced from both raw and pasteurised milk. Variation is likely due to the abiotic characteristics of the

cheese including O_2, pH, salt, a_w, redox potential and temperature fluctuations (30, 50). In this study greater initial diversity in the rind may be due to the high cook temperatures associated with some continental-type cheeses. Dependent on block size, cheese cores may hold higher temperatures longer than the rind, consequently reducing microbial growth. Increased diversity in the rind, at TP1, may also be due to the presence of halophiles (*Vibrio*, *Pseudoalteromonas*) associated with the salting process. Aerobic and aerotolerant microbes, including *Streptococcus, Pseudoalteromonas*, *Psychrobacter*, *Vibrio*, and *Brevibacterium*, were detected more often and at greater percentage populations in the cheese rind than in the core. This is likely due to the oxygen concentration present at/near the surface of the cheese in contrast to the more anaerobic core (35). Prior studies have shown that Gram-positive LAB are more likely to be distributed in the core than the rind of smear ripened and Swiss-type cheeses (Comté, Morbier, Langres) (3). In agreement, we observed consistently higher proportions of *Lactobacillus* in the core than the rind, throughout ripening possibly due to their preference for a micro-anaerobic environment. In contrast, *Streptococcus*, present in both the core and rinds throughout ripening, were found at higher percentages in the rind. In samples from TPs 2 – 4, the cores of both ED and LD cheeses had higher microbial diversity than the rinds. This difference was particularly evident in the late production day samples. Reduced diversity in the rind may be due to several factors including substrate competition, availability of O_2 as well as pH/salt micro-gradients (50). Aerobic staphylococci were also identified in the rinds of both early and late day samples at TP2 in agreement with Maher and Murphy, who described rinds of smear ripened

cheeses as providing conditions that are complimentary for the survival of spoilage microbes (51).

Gram-negative bacteria were detected throughout this study, many of which would not generally be associated with a commercial cheese produced from pasteurised milk. *Thermus* was detected throughout ripening and at higher percentage populations in the late day samples (10.9% at TP1 up to 24.4% at TP4). The presence of *Thermus* was confirmed by subsequent PCR using *Thermus* specific primers (data not shown). This aerobic, marine associated thermophilic and heterotrophic genus was originally isolated from alkaline hot springs in Yellowstone National Park (52, 53). As *Thermus* has previously been identified in two separate hot water systems, it is conceivable that this bacterium was introduced *via* a water source (53, 54). No negative health effects have been reported from with consumption of these cheeses but further studies will be required to assess the effect of *Thermus* on cheese quality. Other Gram-negative genera detected include *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter*, *Vibrio* and *Flavobacterium*. *Vibrio* and *Pseudoalteromonas* are marine-associated, halophilic genera and therefore may have gained access to the cheese *via* the brining process. While it is not yet clear what the significance of the presence of these populations is, particularly at the levels present in the cheese, they may play a role in ripening (38, 50, 55). Psychrotrophic bacteria including *Psychrobacter* and *Pseudomonas* have previously been isolated from a variety of cheeses as well as raw milk and are particularly adapted to low temperature milk storage conditions (50, 56, 57).

Many genera more commonly associated with artisanal and surface ripened cheeses were detected. *Brevibacterium* and *Corynebacterium* were identified immediately

post-production and are associated with flavour and colour development in smear ripened cheese (22, 58-60). *Arthrobacter, Weissella* and *Acinetobacter*, previously isolated from a variety of artisanal cheeses, were also identified, although their impact on cheese quality is unknown (61-68). The significance of the presence of gut associated genera, including *Bifidobacterium* and *Ruminococcaceae Incertae Sedis*, is also unclear.

Clostridium was consistently identified in all time points aside from TP1. The percentages of clostridia present, with respect to early production day samples, increased throughout ripening to 3.1% in TP4 ED cheeses. While the presence of *Clostridium* is a particular issue due its association with late gas production in various cheeses (5), in this instance no defects were noted at the time of sampling. Finally, *Propionibacterium* populations were not detected despite their addition as adjuncts. Further investigation of this revealed that *Propionibacterium* species are one of the very few species that are not successfully amplified by the degenerate primers used in this study.

In conclusion, the use of high throughput amplicon sequencing to profile the microbiota present in a brine-salted, continental-type cheese has revealed distinct differences in bacterial diversity, throughout ripening, between cheeses produced early and late in the production day. As mentioned, the differences between ED and LD cheeses may be due to increased microbial load and/or increased milk storage time between production runs and therefore adapting these practices may allow for a more microbiologically consistent product. Spatial variation due to environmental factors present in the core and rind was also described in this study. Furthermore, the presence of genera that would usually not be traditionally associated with this

cheese type (*Thermus*, *Bifidobacterium*, *Ruminococcaceae Incertae Sedis, Psychrobacter, Pseudoalteromonas*) were described. The significance of the presence of these genera requires further investigation.

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2.7. References:

1. **Rehman S-U, McSweeney PLH, Banks JM, Brechany EY, Muir DD, Fox PF.** 2000. Ripening of Cheddar cheese made from blends of raw and pasteurised milk. International Dairy Journal **10:**33-44.

2. **Bokulich NA, Mills DA.** 2013. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl Environ Microbiol **79:**5214-5223.

3. **Ogier J-C, Lafarge V, Girard V, Rault A, Maladen V, Gruss A, Leveau J-Y, Delacroix-Buchet A.** 2004. Molecular Fingerprinting of Dairy Microbial Ecosystems by Use of Temporal Temperature and Denaturing Gradient Gel Electrophoresis. Applied and Environmental Microbiology **70:**5628-5643.

4. **Quigley L, McCarthy R, O'Sullivan O, Beresford TP, Fitzgerald GF, Ross RP, Stanton C, Cotter PD.** 2013. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. Journal of Dairy Science **96:**4928-4937.

5. **O'Sullivan DJ, Giblin L, McSweeney PL, Sheehan JJ, Cotter PD.** 2013. Nucleic acid-based approaches to investigate microbial-related cheese quality defects. Frontiers in microbiology **4:**1.

6. **Peláez C, Requena T.** 2005. Exploiting the potential of bacteria in the cheese ecosystem. International Dairy Journal **15:**831-844.

7. **Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM.** 2001. Recent advances in cheese microbiology. International Dairy Journal **11:**259-274.

8. **Jany J-L, Barbier G.** 2008. Culture-independent methods for identifying microbial communities in cheese. Food Microbiology **25:**839-848.

9. **Cocolin L, Alessandria V, Dolci P, Gorra R, Rantsiou K.** 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. International Journal of Food Microbiology **167:**29-43.

10. **Bokulich NA, Mills DA.** 2012. Next-generation approaches to the microbial ecology of food fermentations. BMB reports **45:**377-389.

11. **Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ.** 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences **103:**12115-12120.

12. **Nacke H, Thurmer A, Wollherr A, Will C, Hodac L, Herold N, Schoning I, Schrumpf M, Daniel R.** 2011. Pyrosequencing-based assessment of bacterial community structure along different management types in German forest and grassland soils. PLoS One **6:**e17000.

13. **Claesson MJ, O'Sullivan O, Wang Q, Nikkilä J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW.** 2009. Comparative Analysis of Pyrosequencing and a Phylogenetic Microarray for Exploring Microbial Community Structures in the Human Distal Intestine. PLoS One **4:**e6669.

14. **Humblot C, Guyot J-P.** 2009. Pyrosequencing of Tagged 16S rRNA Gene Amplicons for Rapid Deciphering of the Microbiomes of Fermented Foods Such as Pearl Millet Slurries. Applied and Environmental Microbiology **75:**4354-4361.

15. **Nam Y-D, Yi S-H, Lim S-I.** 2012. Bacterial diversity of cheonggukjang, a traditional Korean fermented food, analyzed by barcoded pyrosequencing. Food Control **28:**135-142.

16. **Połka J, Rebecchi A, Pisacane V, Morelli L, Puglisi E.** 2015. Bacterial diversity in typical Italian salami at different ripening stages as revealed by high-throughput sequencing of 16S rRNA amplicons. Food Microbiology **46:**342-356.

17. **Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD.** 2013. Sequence-based analysis of the microbial composition of water kefir from multiple sources. FEMS Microbiology Letters **348:**79-85.

18. **Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD.** 2014. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. Food Microbiology **38:**171-178.

19. **Jung JY, Lee SH, Kim JM, Park MS, Bae J-W, Hahn Y, Madsen EL, Jeon CO.** 2011. Metagenomic Analysis of Kimchi, a Traditional Korean Fermented Food. Applied and Environmental Microbiology **77:**2264-2274.

20. **Roh SW, Kim K-H, Nam Y-D, Chang H-W, Park E-J, Bae J-W.** 2009. Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. ISME J **4:**1-16.

21. **Masoud W, Vogensen FK, Lillevang S, Abu Al-Soud W, Sørensen SJ, Jakobsen M.** 2012. The fate of indigenous microbiota, starter cultures, Escherichia coli, Listeria innocua and Staphylococcus aureus in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. International Journal of Food Microbiology **153:**192-202.

22. **Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2013. The complex microbiota of raw milk. FEMS Microbiology Reviews **37:**664-698.

23. **Aldrete-Tapia A, Escobar-Ramírez MC, Tamplin ML, Hernández-Iturriaga M.** 2014. High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese. Food Microbiology **44:**136-141.

24. **Delgado S, Rachid CTCC, Fernández E, Rychlik T, Alegría Á, Peixoto RS, Mayo B.** 2013. Diversity of thermophilic bacteria in raw, pasteurized and selectively-cultured milk, as assessed by culturing, PCR-DGGE and pyrosequencing. Food Microbiology **36:**103-111.

25. **Masoud W, Takamiya M, Vogensen FK, Lillevang S, Al-Soud WA, Sørensen SJ, Jakobsen M.** 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. International Dairy Journal **21:**142-148.

26. **Alegría Á, Szczesny P, Mayo B, Bardowski J, Kowalczyk M.** 2012. Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. Applied and Environmental Microbiology **78:**1890-1898.

27. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2012. High-Throughput Sequencing for Detection of Subpopulations of Bacteria Not Previously Associated with Artisanal Cheeses. Applied and Environmental Microbiology **78:**5717-5723.

28. **Ercolini D.** 2013. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology. Applied and Environmental Microbiology **79:**3148-3155.

29. **Fuka MM, Wallisch S, Engel M, Welzl G, Havranek J, Schloter M.** 2013. Dynamics of Bacterial Communities during the Ripening Process of Different Croatian Cheese Types Derived from Raw Ewe's Milk Cheeses. PLoS One **8:**e80734.

30. **Sheehan A, O'Cuinn G, FitzGerald RJ, Wilkinson MG.** 2009. Distribution of microbial flora, intracellular enzymes and compositional indices throughout a 12kg Cheddar cheese block during ripening. International Dairy Journal **19:**321-329.

31. **Guinee TP.** 2004. Salting and the role of salt in cheese. International Journal of Dairy Technology **57:**99-109.

32. **Malakar PK, Brocklehurst TF, Mackie AR, Wilson PDG, Zwietering MH, van't Riet K.** 2000. Microgradients in bacterial colonies: use of fluorescence ratio imaging, a non-invasive technique. International Journal of Food Microbiology **56:**71-80.

33. **Ercolini D, Hill PJ, Dodd CER.** 2003. Development of a fluorescence in situ hybridization method for cheese using a 16S rRNA probe. Journal of Microbiological Methods **52:**267-271.

34. **Fleurot I, Aigle M, Fleurot R, Darrigo C, Hennekinne J-A, Gruss A, Borezée-Durant E, Delacroix-Buchet A.** 2014. Following Pathogen Development and Gene Expression in a Food Ecosystem: the Case of a Staphylococcus aureus Isolate in Cheese. Applied and Environmental Microbiology **80:**5106-5115.

35. **Monfredini L, Settanni L, Poznanski E, Cavazza A, Franciosi E.** 2012. The spatial distribution of bacteria in Grana-cheese during ripening. Systematic and Applied Microbiology **35:**54-63.

36. **Fitzsimons NA, Cogan TM, Condon S, Beresford T.** 2001. Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. Journal of Applied Microbiology **90:**600-608.

37. **Gobbetti M, Burzigotti R, Smacchi E, Corsetti A, De Angelis M.** 1997. Microbiology and biochemistry of gorgonzola cheese during ripening. International Dairy Journal **7:**519-529.

38. **Wolfe Benjamin E, Button Julie E, Santarelli M, Dutton Rachel J.** 2014. Cheese Rind Communities Provide Tractable Systems for In Situ and In Vitro Studies of Microbial Diversity. Cell **158:**422-433.

39. **Daly DFM.** 2014. Studies on factors relating to the development of defects in commercial cheese. PhD. University College Cork.

40. **Quigley L, O'Sullivan O, Beresford TP, Paul Ross R, Fitzgerald GF, Cotter PD.** 2012. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. Journal of Applied Microbiology **113:**96-105.

41. **IDF.** 1988. Cheese and processed cheese: Determination of chloride content (potentiometric titration method). International Standards 4a Brussels, Belgium: International Dairy Federation.

42. **IDF.** 1982. Determination of total solids content (cheese and processed cheese). International Standards 4a Brussels, Belgium: International Dairy Federation.

43. **Standards IB.** 1976. British Standard Methods for Chemical Analysis of Cheese: Determination of pH value. London: British Standards Institute.

44. **Quigley L, O'Sullivan O, Beresford T, Ross R, Fitzgerald G, Fitzgerald G, Cotter P, Cotter P.** 2012. High-throughput sequencing for detection of

subpopulations of bacteria not previously associated with artisanal cheeses. Appl Environ Microbiol **78:**5717 - 5723.

45. **Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM.** 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res **37:**D141-145.

46. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods **7:**335-336.

47. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-410.

48. **Huson DH, Auch AF, Qi J, Schuster SC.** 2007. MEGAN analysis of metagenomic data. Genome Res **17:**377-386.

49. **Stearns JC, Lynch MDJ, Senadheera DB, Tenenbaum HC, Goldberg MB, Cvitkovitch DG, Croitoru K, Moreno-Hagelsieb G, Neufeld JD.** 2011. Bacterial biogeography of the human digestive tract. Sci. Rep. **1**.

50. **Montel M-C, Buchin S, Mallet A, Delbes-Paus C, Vuitton DA, Desmasures N, Berthier F.** 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. International Journal of Food Microbiology **177:**136-154.

51. **Maher MM, Murphy PM.** 2000. Microbiological Changes during Ripening in Two Irish Smear-Ripened, Farmhouse Cheeses Produced from Raw Milk. Irish Journal of Agricultural and Food Research **39:**107-121.

52. **Spanevello MD, Patel BKC.** 2004. The phylogenetic diversity of Thermus and Meiothermus from microbial mats of an Australian subsurface aquifer runoff channel. FEMS Microbiology Ecology **50:**63-73.

53. **Pask-Hughes R, Williams Rad.** 1975. Extremely Thermophilic Gram-negative Bacteria from Hot Tap Water. Journal of General Microbiology **88:**321-328.

54. **Bagh LK, Albrechtsen H-J, Arvin E, Ovesen K.** 2004. Distribution of bacteria in a domestic hot water system in a Danish apartment building. Water Research **38:**225-235.

55. **Bleicher A, Neuhaus K, Scherer S.** 2010. Vibrio casei sp. nov., isolated from the surfaces of two French red smear soft cheeses. International Journal of Systematic and Evolutionary Microbiology **60:**1745-1749.

56. **Delcenserie V, Taminiau B, Delhalle L, Nezer C, Doyen P, Crevecoeur S, Roussey D, Korsak N, Daube G.** 2014. Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. Journal of Dairy Science **97:**6046-6056.

57. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2011. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. International Journal of Food Microbiology **150:**81-94.

58. **Brennan NM, Brown R, Goodfellow M, Ward AC, Beresford TP, Simpson PJ, Fox PF, Cogan TM.** 2001. Corynebacterium mooreparkense sp. nov. and

Corynebacterium casei sp. nov., isolated from the surface of a smear-ripened cheese. International Journal of Systematic and Evolutionary Microbiology **51:**843-852.

59. **Brennan NM, Ward AC, Beresford TP, Fox PF, Goodfellow M, Cogan TM.** 2002. Biodiversity of the Bacterial Flora on the Surface of a Smear Cheese. Applied and Environmental Microbiology **68:**820-830.

60. **Mounier J, Rea MC, O'Connor PM, Fitzgerald GF, Cogan TM.** 2007. Growth Characteristics of Brevibacterium, Corynebacterium, Microbacterium, and Staphylococcus spp. Isolated from Surface-Ripened Cheese. Applied and Environmental Microbiology **73:**7732-7739.

61. **Addis E, Fleet GH, Cox JM, Kolak D, Leung T.** 2001. The growth, properties and interactions of yeasts and bacteria associated with the maturation of Camembert and blue-veined cheeses. International Journal of Food Microbiology **69:**25-36.

62. **Fuka MM, Engel M, Skelin A, Redžepović S, Schloter M.** 2010. Bacterial communities associated with the production of artisanal Istrian cheese. International Journal of Food Microbiology **142:**19-24.

63. **Irlinger F, Bimet F, Delettre J, Lefèvre M, Grimont PAD.** 2005. Arthrobacter bergerei sp. nov. and Arthrobacter arilaitensis sp. nov., novel coryneform species isolated from the surfaces of cheeses. International Journal of Systematic and Evolutionary Microbiology **55:**457-462.

64. **Mounier J, Gelsomino R, Goerges S, Vancanneyt M, Vandemeulebroecke K, Hoste B, Scherer S, Swings J, Fitzgerald GF, Cogan TM.** 2005. Surface Microflora of Four Smear-Ripened Cheeses. Applied and Environmental Microbiology **71:**6489-6500.

65. **Feurer C, Vallaeys T, Corrieu G, Irlinger F.** 2004. Does Smearing Inoculum Reflect the Bacterial Composition of the Smear at the End of the Ripening of a French Soft, Red-Smear Cheese? Journal of Dairy Science **87:**3189-3197.

66. **Williams AG, Banks JM.** 1997. Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from cheddar cheese manufactured in the United Kingdom. International Dairy Journal **7:**763-774.

67. **Porcellato D, Østlie HM, Brede ME, Martinovic A, Skeie SB.** 2013. Dynamics of starter, adjunct non-starter lactic acid bacteria and propionic acid bacteria in low-fat and full-fat Dutch-type cheese. International Dairy Journal **33:**104-111.

68. **Litopoulou-Tzanetaki E, Tzanetakis N.** 2011. Microbiological characteristics of Greek traditional cheeses. Small Ruminant Research **101:**17-32.

Table 1: Alpha diversity of continental-type cheeses segregated according to time of production day (Early day [ED] and Late day [LD]) and spatial distribution (Core/Rind)

Production Day	Chao1	Simpson	Shannon Index	Phylogenetic Diversity	Observed OTUs
Early Day Production					
TP1 ED	401.77	0.69	2.88	13.25	222.50
TP2 ED	328.80	0.65	2.62	11.13	198.25
TP3 ED	345.91	0.73	3.17	12.19	210.00
TP4 ED	304.11	0.66	2.63	9.72	165.25
Late Day Production					
TP1 LD	523.31	0.80	3.56	16.15	310.25
TP2 LD	478.63	0.75	3.29	14.58	292.75
TP3 LD	397.96	0.82	3.60	12.69	236.75
TP4 LD	357.94	0.78	3.34	12.46	215.33
Core and Rind					
Early Day Production					
TP1 Core	372.24	0.67	2.80	12.18	194.00
TP2 Core	294.59	0.62	2.51	11.37	182.00
TP3 Core	417.14	0.72	3.16	13.00	238.50
TP4 Core	370.37	0.61	2.56	11.65	183.50
TP1 Rind	431.30	0.70	2.96	14.32	251.00
TP2 Rind	363.00	0.67	2.72	10.89	214.50
TP3 Rind	274.69	0.75	3.18	11.37	181.50
TP4 Rind	237.84	0.71	2.70	7.78	147.00
Late Day Production					
TP1 Core	517.23	0.80	3.52	14.80	290.00
TP2 Core	471.02	0.75	3.32	14.86	295.00
TP3 Core	412.17	0.83	3.60	12.71	244.50
TP4 Core	405.02	0.83	3.82	15.40	241.00
TP1 Rind	529.38	0.79	3.60	17.51	330.50
TP2 Rind	486.25	0.76	3.26	14.29	290.50
TP3 Rind	383.75	0.81	3.60	12.67	229.00
TP4 Rind	334.40	0.76	3.10	10.99	202.50

Figure 1: Principal Coordinate analysis of the β diversity (unweighted Unifrac) of cheese samples. (A) Co-ordinates reflect early and late day samples and are colour coded to reflect the ripening phase of the cheese (B) The same data is depicted but in this instance core and rind samples are distinguished.

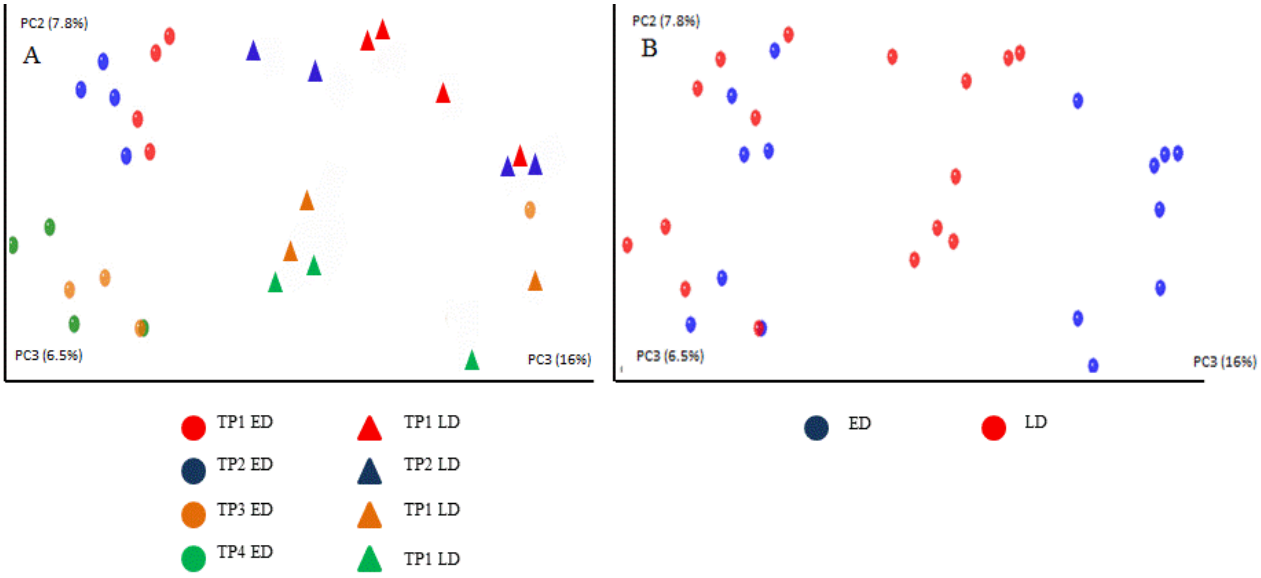


Figure 2: Relative abundance of bacteria at genus level for a Continental-type cheese produced early and late (ED and LD) in the production day. Results depicted are mean values of reads generated from individual core/rind samples from each respective cheese block and were standardised to equi-molar concentrations prior to library preparation.

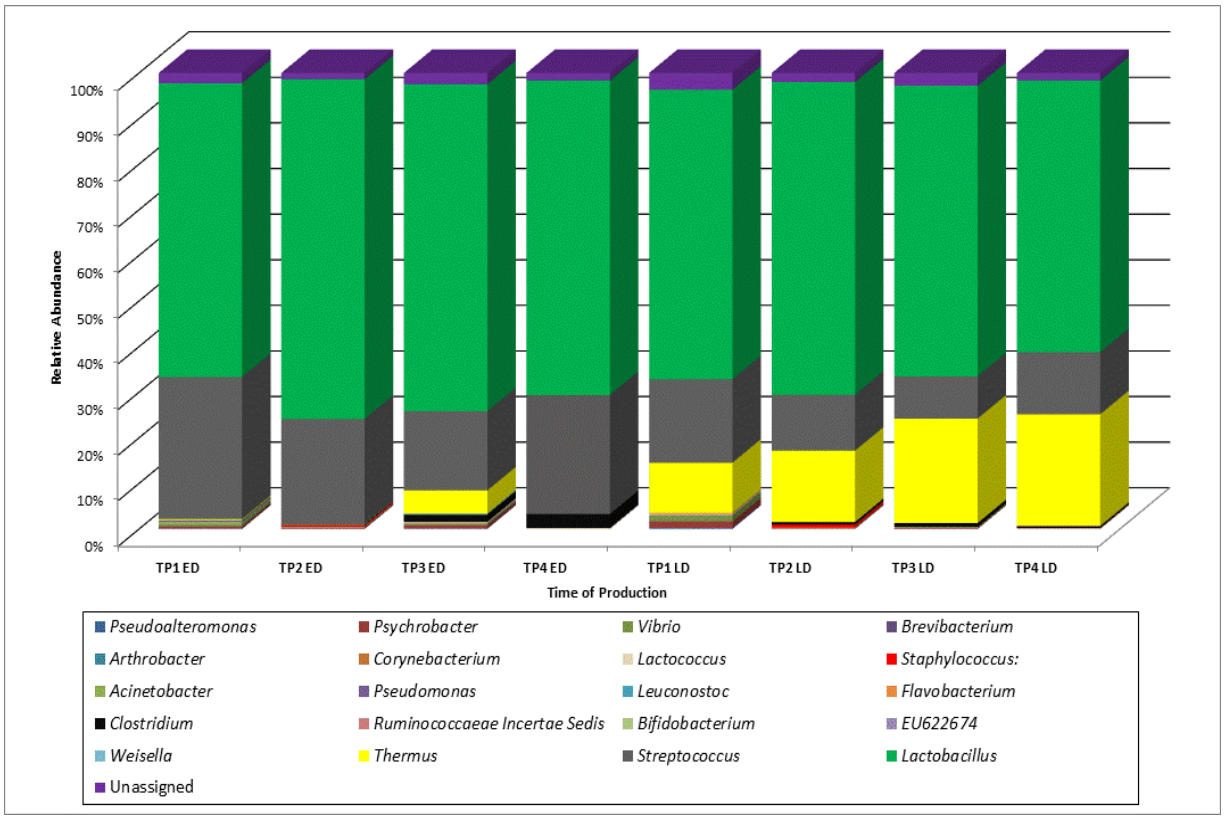


Figure 3: Relative abundance of bacteria at genus level for each TP according to sample location (Core/Rind). Data presented are mean values of respective reads from individual cheese samples and were standardised to equi-molar concentrations prior to library preparation.

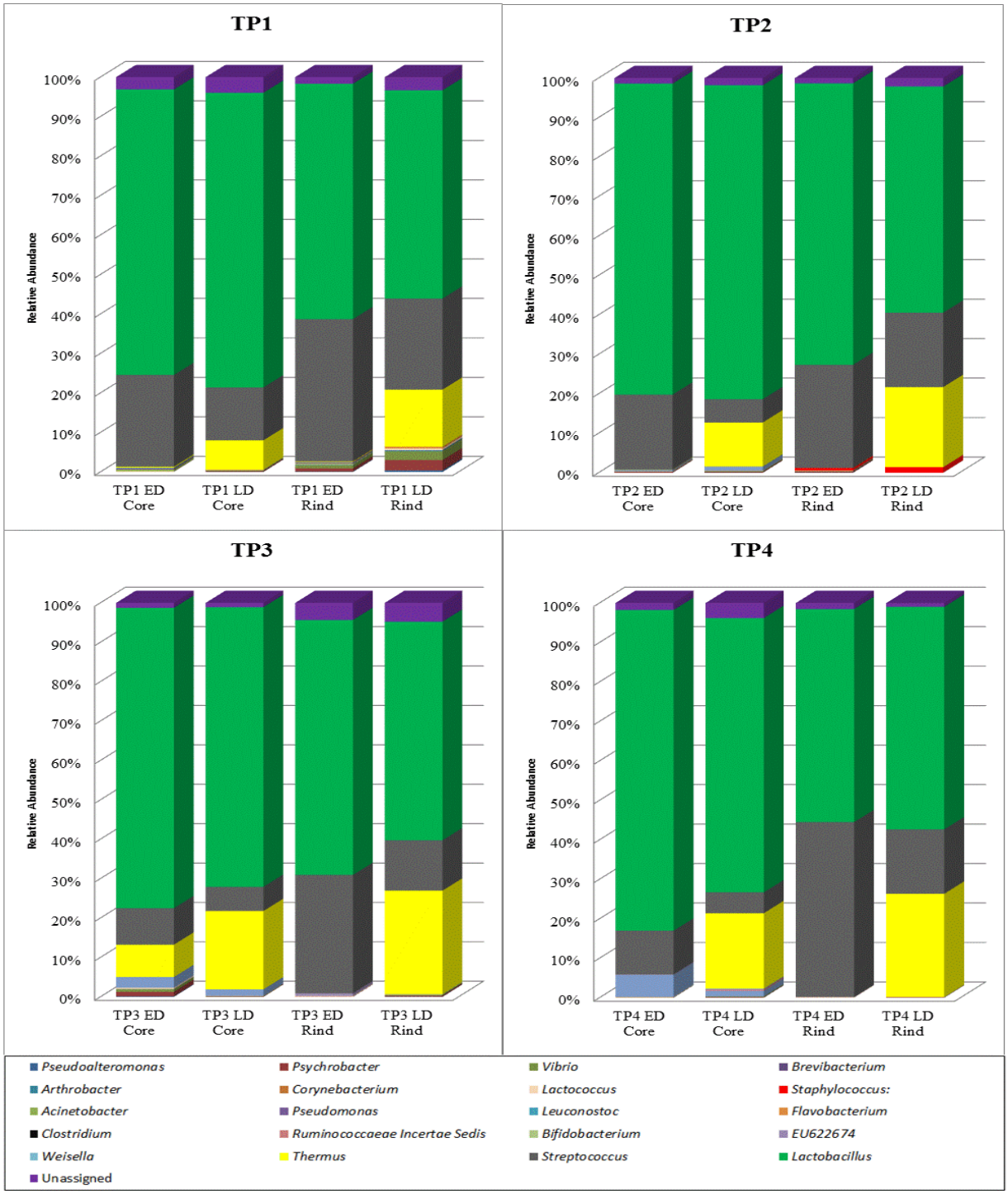
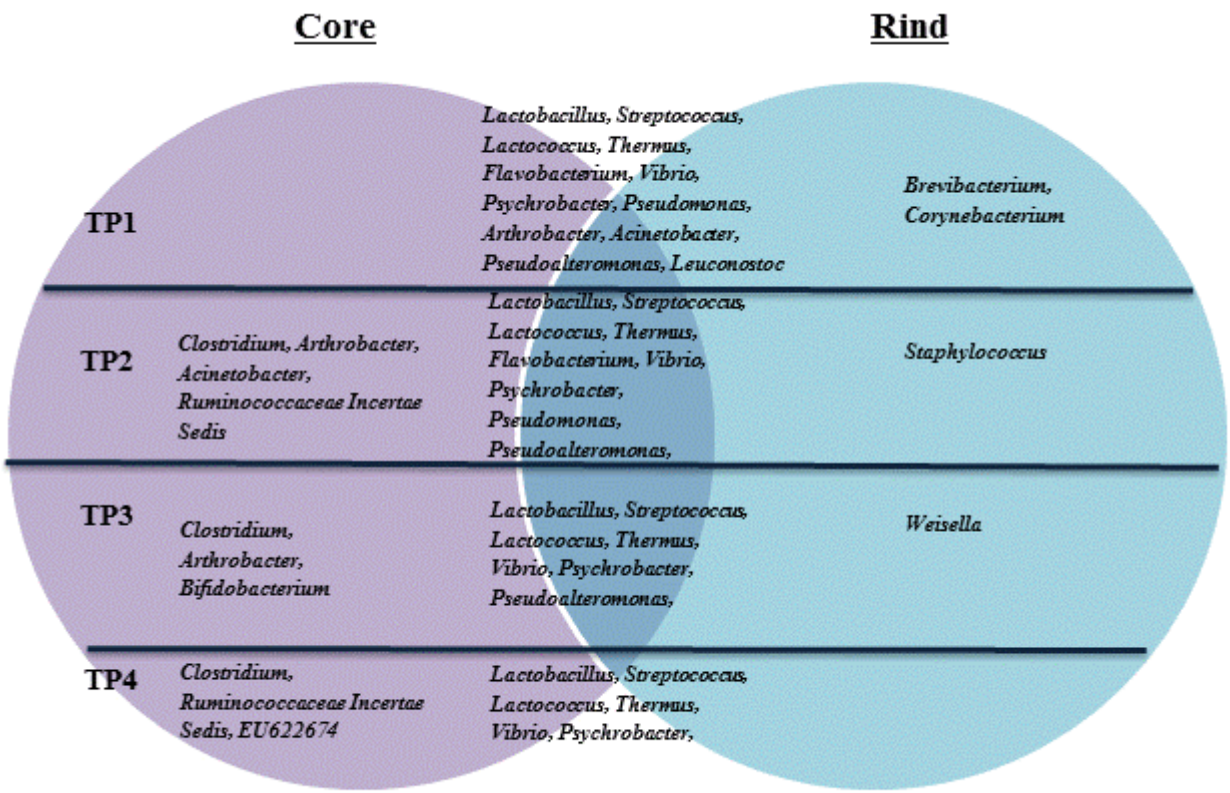


Figure 4: Venn diagram depicting spatial differences in microbial composition at each time point. Genera located in the intersecting region were detected in both the core and the rind while those located on the periphery were detected exclusively in the core/rind.



2.8. Appendices

Supplementary Tables:

Table S1: Summary of reads generated for each individual sample, at Phylum, Family and Genus level, post quality filtering

	TP1, LD, B1, Rind	TP1, LD, B2, Rind	TP1, LD, B1, Core	TP1, LD, B2, Core	TP1, ED, B1, Rind	TP1, ED, B2, Rind	TP1, ED, B1, Core	TP1, ED, B2, Core	Total
Phylum	13736	9403	6724	7422	14682	10614	9204	15371	87156
Family	13649	9327	6669	7352	14618	10562	9174	15322	86673
Genus	13548	9202	6575	7127	14297	10157	8713	14884	84503
	TP2, LD, B1, Rind	**TP2, LD, B2, Rind**	**TP2, LD, B1, Core**	**TP2, LD, B2, Core**	**TP2, ED, B1, Rind**	**TP2, ED, B2, Rind**	**TP2, ED, B1, Core**	**TP2, ED, B2, Core**	
Phylum	16797	11854	11097	18525	16528	5690	7248	9306	97045
Family	16746	11794	11080	18484	16485	5663	7231	9247	96730
Genus	16521	11518	10912	18168	16374	5549	7162	9161	95365
	TP3, LD, B1, Rind	**TP3, LD, B2, Rind**	**TP3, LD, B1, Core**	**TP3, LD, B2, Core**	**TP3, ED, B1, Rind**	**TP3, ED, B2, Rind**	**TP3, ED, B1, Core**	**TP3, ED, B2, Core**	
Phylum	8694	6553	11510	5735	5604	6015	9528	8609	62248
Family	8672	6547	11500	5727	5579	5988	9508	8554	62075
Genus	8192	6327	11403	5655	5435	5679	9465	8442	60598
	TP4, LD, B1, Rind	**TP4, LD, B2, Rind**	**TP4, LD, B1, Core**	**TP4, LD, B2, Core**	**TP4, ED, B1, Rind**	**TP4, ED, B2, Rind**	**TP4, ED, B1, Core**	**TP1, ED, B2, Core**	
Phylum	6885	7484	4651	Sequencing Failed	5295	7942	6671	9476	48404
Family	6869	7469	4627		5279	7907	6638	9450	48239
Genus	6817	7419	4474		5205	7830	6515	9346	47606

Table S2: Summary of read percentages and relative abundances, at Phylum, Family and Genus level, for early and late day production continental-type cheese (Early Day [ED], Late Day [LD]). Results depicted are mean values of reads generated from individual core/rind samples from each respective cheese block and were standardised to equi-molar concentrations prior to library preparation.

Phylum (%)	**TP1 ED**	**TP1 LD**	**TP2 ED**	**TP2 LD**	**TP3 ED**	**TP3 LD**	**TP4 ED**	**TP4 LD**
Proteobacteria	2.14	3.02	0.61	0.33	1.50	0.46	0.25	0.28
Bacteroidetes	0.08	0.25	0	0.06	0	0	0	0.04
Actinobacteria	0.07	0.17	0.02	0.01	0.04	0.03	0	0
Firmicutes	97.53	85.56	99.33	83.96	93.46	76.62	99.75	75.26
Deinococcus-Thermus	0.18	10.99	0.04	15.64	5.01	22.89	0	24.42
Relative Abundance (%)	100	100	100	100	100	100	100	100
Family (%)	**TP1 ED**	**TP1 LD**	**TP2 ED**	**TP2 LD**	**TP3 ED**	**TP3 LD**	**TP4 ED**	**TP4 LD**
Pseudoalteromonadaceae:	0.07	0.22	0.03	0.01	0.09	0.02	0	0
Moraxellaceae:	0.80	1.45	0.26	0.11	0.73	0.21	0.05	0.10
Vibrionaceae:	0.53	1.25	0.11	0.10	0.41	0.14	0.03	0.09
Brevibacteriaceae:	0	0.01	0	0	0	0	0	0
Micrococcaceae:	0.07	0.13	0.02	0.01	0.03	0	0	0
Corynebacteriaceae:	0	0.01	0	0	0	0	0	0

Streptococcaceae:	32.59	18.57	24.03	12.35	18.50	9.34	27.06	13.70
Lactobacillaceae:	64.42	63.51	74.61	68.76	72.62	65.44	69.10	59.77
Staphylococcaceae:	0	0	0.35	0.66	0	0	0	0
Lachnospiraceae:	0.06	0.01	0.01	0	0.14	0.15	0.14	0.62
Ruminococcaceae:	0.03	0	0.01	0	0.04	0.07	0.05	0.19
Thermaceae	0.18	10.99	0.04	15.64	5.01	22.89	0	24.42
Pseudomonadaceae	0.26		0.03	0	0	0	0	0
Leuconostocaceae	0.08	0.01	0	0	0.26	0.02	0	0
Enterobacteriaceae	0.06		0	0	0	0.03	0	0
Flavobacteriaceae	0.08	0.25	0	0.05	0	0	0	0
Bacillaceae	0	3.20	0	1.49	0.11	0.65	0	0.42
Clostridiaceae	0	0	0.12	0.53	1.64	0.87	3.20	0.38
Bifidobacteriaceae	0	0	0	0	0	0.03	0	0
Acetobacteraceae	0	0	0	0	0	0	0	0.03
Unassigned	0.77	0.39	0.38	1.06	0.43	0.14	0.37	0.29
Relative Abundance (%)	99.23	99.23	99.62	99.71	99.57	99.86	99.63	99.71
Genus (%)	**TP1 ED**	**TP1 LD**	**TP2 ED**	**TP2 LD**	**TP3 ED**	**TP3 LD**	**TP4 ED**	**TP4 LD**
Pseudoalteromonas:	0.0697	0.2166	0.0335	0.0137	0.0907	0.02	0	0
Psychrobacter:	0.4881	1.4457	0.2295	0.1047	0.7091	0.21	0.0374	0.0894
Vibrio:	0.5257	1.2352	0.1057	0.0995	0.4134	0.14	0.0272	0.0894

Brevibacterium:	0	0.0120	0	0	0	0	0	0
Arthrobacter:	0.0697	0.1283	0.0232	0.0103	0.0302	0	0	0
Corynebacterium:	0	0.0120	0	0	0	0	0	0
Streptococcus:	31.1788	18.3072	23.0914	12.2715	17.3746	9.26	26.0686	13.6225
Lactococcus:	0.1180	0.2346	0.0800	0.0618	0.2924	0.04	0.1225	0.0421
Lactobacillus:	64.4227	63.5119	74.5100	68.5789	71.7267	63.71	68.9729	59.5899
Staphylococcus:	0	0	0.3508	0.6590	0	0	0	0
Thermus:	0.1797	10.9883	0.0413	15.6436	5.0074	22.89	0	24.4217
Acinetobacter	0.3031	0	0.0206	0	0	0	0	0
Pseudomonas	0.2575	0	0.0310	0	0	0	0	0
Leuconostoc	0.0751	0.0100	0	0	0	0	0	0
Flavobacterium	0.0778	0.2486	0	0.0480	0	0	0	0
Clostridium	0	0	0.1135	0.5285	1.6333	0.87	3.0901	0.3733
Ruminococcaeae Incertae Sedis	0	0	0.0129	0	0	0	0.0204	0.1104
Bifidobacterium	0	0	0	0	0	0.03	0	0
Weisella	0	0	0	0	0.2521	0.02	0	0
EU622674	0	0	0	0	0	0	0	0.0315
Unassigned	2.2341	3.6494	1.3566	1.9803	2.4701	2.82	1.6608	1.6299
Relative Abundance (%)	97.7659	96.3506	98.6434	98.0197	97.5299	97.18	98.3392	98.3701

Table S3: Summary of read percentages and relative abundances, at genus level, for core and rind samples of continental-type cheese (Early Day [ED], Late Day [LD]). Results depicted are mean values of reads generated from individual core/rind samples from each respective cheese block and were standardised to equi-molar concentrations prior to library preparation.

Genus	TP1 ED Core	TP1 ED Rind	TP1 LD Core	TP1 LD Rind	TP2 ED Core	TP2 ED Rind	TP2 LD Core	TP2 LD Rind	TP3 ED Core	TP3 ED Rind	TP3 LD Core	TP3 LD Rind	TP4 ED Core	TP4 ED Rind	TP4 LD Core	TP4 LD Rind
Pseudoalteromonas	0	0.112	0.028	0.399	0	0.059	0.027	0	0.149	0	0	0.033	0	0	0	0
Psychrobacter	0	0.787	0.256	2.601	0.205	0.248	0.206	0	1.130	0.052	0.128	0.302	0.068	0	0.129	0.077
Vibrio	0	0.847	0.187	2.253	0.060	0.140	0.196	0	0.678	0	0.081	0.203	0.050	0	0.194	0.056
Brevibacterium:	0	0	0	0.024	0	0	0	0	0	0	0	0	0	0	0	0
Arthrobacter	0	0.112	0.020	0.233	0	0.041	0.020	0	0.050	0	0	0	0	0	0	0
Corynebacterium	0	0	0	0.024	0	0	0	0	0	0	0	0	0	0	0	0
Streptococcus	23.215	36.047	13.400	23.075	19.011	26.132	5.898	18.861	9.268	30.028	6.153	12.783	11.086	44.345	5.289	16.320
Lactococcus	0.163	0.091	0.057	0.407	0.060	0.095	0.064	0.059	0.342	0.215	0.052	0.033	0.111	0.136	0	0.056
Lactobacillus	72.303	59.605	74.604	52.736	78.791	71.321	79.515	57.272	76.220	64.713	70.977	55.486	81.253	53.993	69.490	56.385
Staphylococcus	0	0	0	0	0	0.612	0	1.340	0	0	0	0	0	0	0	0
Thermus	0.276	0.121	7.438	14.437	0.060	0.027	11.164	20.275	8.215	0	19.884	26.281	0	0	19.093	26.147
Acinetobacter	0.467	0.203	0	0	0.048	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas	0.339	0.207	0	0	0.072	0	0	0	0	0	0	0	0	0	0	0
Leuconostoc	0.099	0.061	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Flavobacterium	0.000	0.125	0.028	0.463	0	0	0.041	0.056	0	0	0	0	0	0	0	0
Clostridium	0	0	0	0	0.266	0	1.040	0	2.680	0	1.641	0	5.623	0	1.419	0

Ruminococcaeae																
Incertae Sedis	0	0	0	0	0.030	0	0	0	0	0	0	0	0.037	0	0.452	0
Bifidobacterium	0	0	0	0	0	0	0	0	0	0	0	0.066	0	0	0	0
Weisella	0	0	0	0	0	0	0	0	0	0.645	0	0.039	0	0	0	0
EU622674	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.129	0
Unassigned	3.139	1.681	3.980	3.329	1.395	1.328	1.830	2.136	1.268	4.346	1.084	4.775	1.771	1.526	3.806	0.926
Relative																
Abundance (%)	96.861	98.319	96.020	96.671	98.605	98.672	98.170	97.864	98.732	95.654	98.916	95.225	98.229	98.474	96.194	99.074

Supplementary Figures:

Figure S1: Rarefaction curves of microbial populations using the Shannon, Simpson and Chao1 indices

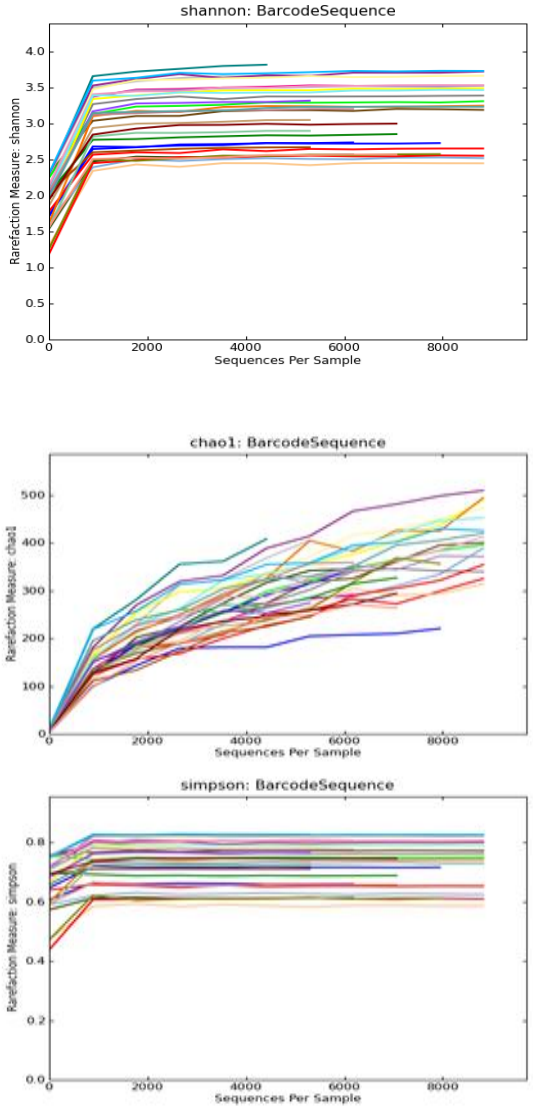
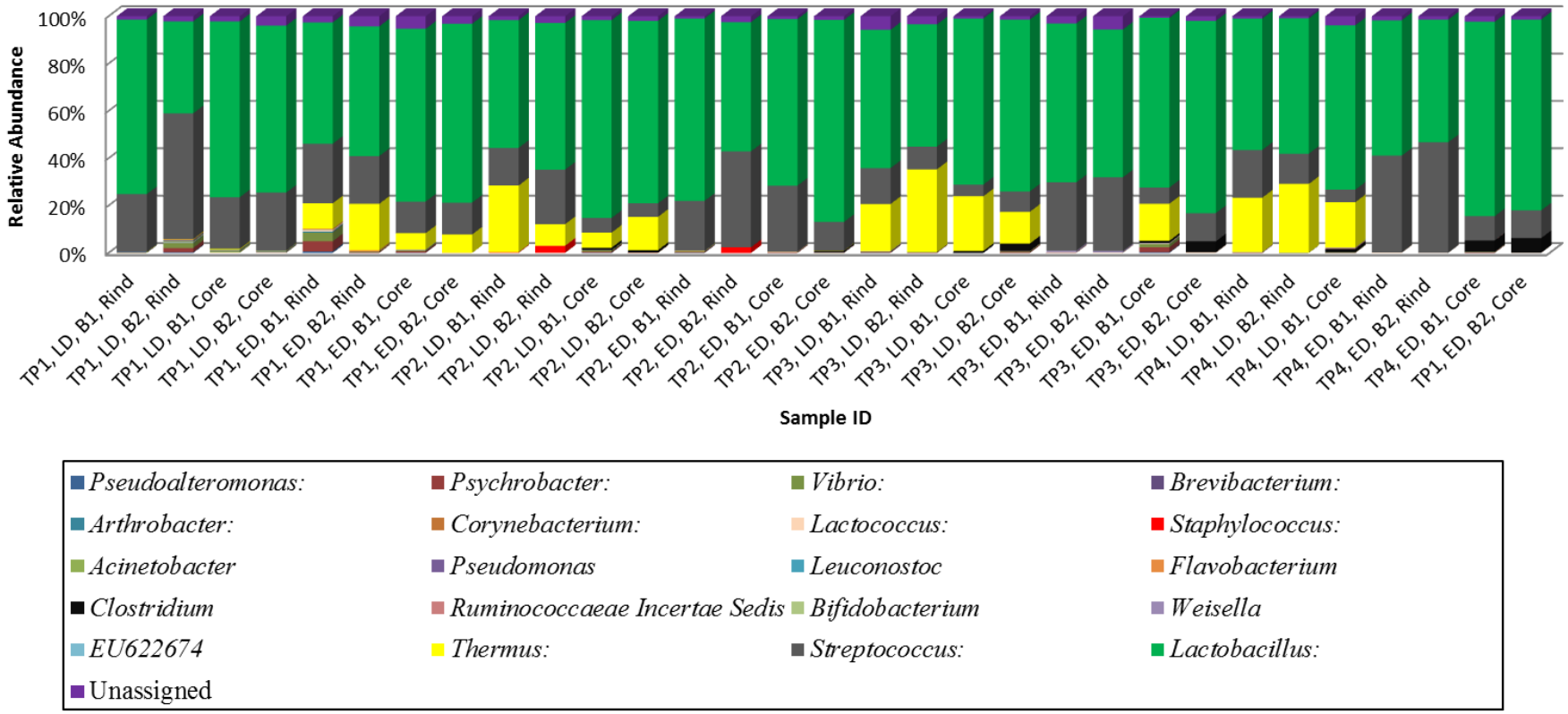


Figure S2: Relative abundance of individual samples at genus level for a Continental-type cheese produced early and late (ED and LD) in the production day



Chapter 3

High-throughput DNA sequencing to survey bacterial histidine and tyrosine decarboxylases in raw milk cheeses

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3.0. Abstract

The aim of this study was to employ high-throughput DNA sequencing to assess the incidence of bacteria with biogenic amine (BA; histamine and tyramine) producing potential from among 10 different cheeses varieties. To facilitate this, a diagnostic approach using degenerate PCR primer pairs that were previously designed to amplify segments of the histidine (*hdc*) and tyrosine (*tdc*) decarboxylase gene clusters were employed. In contrast to previous studies in which the decarboxylase genes of specific isolates were studied, in this instance amplifications were performed using total metagenomic DNA extracts. Amplicons were initially cloned to facilitate Sanger sequencing of individual gene fragments to ensure that a variety of *hdc* and *tdc* genes were present. Once this was established, high throughput DNA sequencing of these amplicons was performed to provide a more in-depth analysis of the histamine- and tyramine-producing bacteria present in the cheeses. High-throughput sequencing resulted in generation of a total of 1,563,764 sequencing reads and revealed that *Lactobacillus curvatus*, *Enterococcus faecium* and *E. faecalis* were the dominant species with tyramine producing potential, while *Lb. buchneri* was found to be the dominant species harbouring histaminogenic potential. Commonly used cheese starter bacteria, including *Streptococcus thermophilus* and *Lb. delbreueckii,* were also identified as having biogenic amine producing potential in the cheese studied. Molecular analysis of bacterial communities was then further complemented with HPLC quantification of histamine and tyramine in the sampled cheeses. In this study, high-throughput DNA sequencing successfully identified populations capable of amine production in a variety of cheeses. This approach also gave an insight into the broader *hdc* and *tdc*

complement within the various cheeses. This approach can be used to detect amine producing communities not only in food matrices but also in the production environment itself.

3.1. Introduction

High-throughput sequencing (HTS) has significantly enhanced our ability to profile complex microbial ecosystems such as those in the sea (1), soil (2), gut (3) and various foods including cheese (4-7). While most of these studies rely on amplifying regions of the bacterial 16S rRNA or fungal ITS genes to study the microbial composition of these communities, it is also possible to use HTS to sequence select non-16S based genes (8). With reference to this, HTS-based methods are currently being explored to improve food safety by targeting specific undesirable populations/genes (9, 10), and the potential exists to target genes involved in biogenic amine (BA) formation. BAs are low molecular weight organic bases with biological activity produced, primarily, by decarboxylation of precursor amino acids. BAs are classified according to their chemical structures and can be aromatic (tyramine), heterocyclic (histamine and tryptamine) or aliphatic (putrescine and cadaverine) (11-14). In eukaryotes BAs are generally associated with a variety of biological processes including blood pressure regulation, neurotransmission, cellular growth and allergic responses. In prokaryotes, however, BA formation is generally linked with cell survival, particularly in low pH conditions where it serves as a stress response mechanism. Up-regulation of decarboxylase gene expression has previously been shown to occur in the presence of the precursor amino acid and in low pH environments, such as those encountered in fermented foods. The amino acid/amine transporter system also acts to generate energy in the form of proton motive force, thus providing a further competitive advantage under such stress conditions (15, 16). Microbial BA formation is encountered in a variety of fermented foods and beverages including cheese, fish, beer, wine, meat products

and fermented vegetables (17). The most commonly occurring BAs detected in foods include histamine, tyramine, putrescine and cadaverine (18). The accumulation of histamine and/or tyramine at high levels may produce toxicological effects including hypertension, headaches, palpitations and vomiting in certain individuals, particularly those with reduced mono/di-amine oxidase activity, due to either genetic or pharmacological reasons. The European Food Safety Authority regard histamine and tyramine as the most important BAs from a toxicological viewpoint (19). Additionally, the presence of di-amines, such as putrescine and cadaverine, can further promote toxicological effects as they act as potentiators of histamine and tyramine toxicity by competing for detoxifying enzymes (20-24). As the detrimental effects associated with consumption of BAs varies depending on the amine in question and the susceptibility of the individual, it is particularly difficult to set defined limits for BAs in food products (25). Consequently, regulatory limits describing BA concentrations have yet to be established for the cheese industry. Notably, ripened cheeses are second only to fish as the most commonly implicated source of dietary BAs (19, 26, 27), which has led to the coining of the term the “cheese reaction” (28).

BAs can be formed by a variety of cheese associated lactic acid bacteria (LAB) including *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* (15, 17, 18, 23). Several factors are associated with the accumulation of BAs in cheese including low pH, milk processing parameters (raw/pasteurised), the presence of amine forming species (starter or non-starter/contaminating bacteria), availability of precursor amino acids, ripening temperature/time and salt content, among other factors (29). While the majority of cheese is produced from

pasteurised milk, raw milk cheeses are also popular due to their unique flavour characterisitics (27). High levels of secondary proteolysis as a result of starter and non-starter bacterial action, together with higher microbial load and, in many cases, long ripening times make raw milk cheeses particularly susceptible to BA formation (13, 14, 27, 28, 30, 31). The presence of BAs can also be used as an indicator of overall product hygiene in the form of biogenic amine indices (19).

Methods employed to detect BAs in dairy products have been extensively reviewed (15, 20, 29, 32, 33). Essentially, detection is either direct, i.e., detection of the respective amines or indirect, i.e., based on identifying amine forming bacteria. Amine detection methods rely primarily on chromatographic techniques such as thin layer and high performance liquid chromatography (HPLC) (29). While initial approaches for identifying responsible bacteria were based on differential chromogenic agars and enzymatic methods, more recently, molecular based methods such as DNA hybridisation, polymerase chain reaction (PCR) and quantitative (q)PCR have been used (20, 32, 34). PCR based approaches are of particular use for establishing the aminogenic potential of various isolates from food products. In this instance, strains associated with raw materials, production equipment and, in the case of cheese, starter bacteria can be pre-emptively screened for decarboxylase biomarkers leading to a potential reduction of amines in the final product. A review published by Landete et al (20) describes several sets of PCR primers for detecting producers of the major food-associated amines (20).

In this study a range of raw milk, speciality cheeses were screened for the presence of histidine decarboxylase (*hdc*) and tyrosine decarboxylase (*tdc*) genes associated with the production of histamine and tyramine, respectively. Previously optimised

PCR primer pairs amplifying regions of the Gram-positive *hdc* and *tdc* gene clusters were employed and the resultant amplicons were cloned and subjected to Sanger sequencing in order to establish that that there was sufficient heterogeneity among the decarboxylases present to merit a more detailed HTS analysis. The Ion PGM platform was selected for HTS analysis as its rapid run time and varied chips sizes (314, 316 and 318) allow for flexibility with respect to cost, bp yield and read length, therefore making it potentially relevant for the dairy industry. HTS revealed the dominant and sub-dominant species with tyramine and histamine producing potential, in these raw milk cheeses. More importantly, the value of employing HTS to survey decarboxylase genes within a microbial population is established.

3.2. Materials and Methods

3.2.1. Sample collection

Ten speciality cheeses were purchased from a local market. Raw milk cheeses with long ripening times (3 – 24 months) were selected and divided into 2 groups (hard and semi-hard). Cheeses originated from several European countries including two Irish artisanal cheeses (A and B), Reblochon, Manchego, Morbier, Tête de Moine, Pecorino Sardo, Ossau-Iraty, Comté and Gorgonzola. Cheeses were vacuum packed and stored at 4°C for 3 days prior to DNA extraction. Table 1 provides a description of the cheeses selected for this study. These particular cheeses were selected due to their potential to accumulate BAs and are not reflective of all cheese within the respective categories.

3.2.2. Determination of BA content of cheese

BAs were acid extracted, derivatised and quantified, in duplicate, using the method described by Özoğul (35) with modifications for a cheese matrix. Five grams of cheese was weighed into a sterile bag containing 20 ml 0.013N H_2SO_4. The suspension was homogenised in a stomacher (Iul Instruments, Barcelona, Spain) for 10 min. The liquid phase was transferred to a sterile 50 ml tube while the remaining cheese homogenate was subjected to a second acid extraction with 20 ml 0.013 N H_2SO_4. The liquid phases were pooled and centrifuged at 5,000 g, 4°C for 15 min. After centrifugation, the solution was brought to a final volume of 50 ml with 0.013 N H_2SO_4. A 10 ml aliquot was filtered using 0.2 µm cellulose acetate filters (Chromacol, Welwyn Garden, Herts, UK).

Extracted BAs were then derivatised by mixing 1 ml of each respective extract with 1 ml 2 N NaOH and 1 ml 2% benzoyl chloride (Sigma-Aldrich, Wicklow, Ireland) in

glass test tubes. The mixture was vortexed and allowed to stand for 15 min prior to the addition of 2 ml saturated NaCl. Two ml of diethyl ether was then added. A plastic pipette was used to transfer the top layer of the extract to a second glass test tube with a further 2 ml diethyl ether added and the resultant top layers pooled. Diethyl ether was evaporated off using a stream of nitrogen at 45°C for 20 min. The BA residue was dissolved by adding 1 ml acetonitrile.

BAs were separated using a Luna C18 RF 5 µm, 100 Å column 250 x 4.6mm (Phenomenex Queens Avenue, Macclesfield, UK) and were eluted at an initial flow rate of 1.6 ml/min for 30 min with Acetonitrile (A) and H_2O (B), using the following gradients:

0-1 min	1.6 ml/min	40% A + 60% B
1-10 min	1.8 ml/min	50% A + 50% B
10-20 min	2.0 ml/min	60% A + 40% B
20-25 min	2.0 ml/min	70% A + 30% B
25-26 min	1.6 ml/min	40% A + 60% B
26-30 min	1.6 ml/min	40% A + 60% B

BAs were quantified using 5 data points on calibration curves against standard solutions of histamine (100-2000 µg/ml), tyramine (5-100 µg/ml), putrescine and cadaverine (Sigma-Aldrich, Dublin, Ireland) (Table S1). Data was presented as mg of individual BA per kg of cheese.

3.2.3. Determination of cheese pH, salt and moisture contents

Grated samples of each cheese were analysed for salt content (36), moisture (37) and pH (38) using previously described methods.

3.2.4. DNA extraction from selected cheeses

Five grams of each cheese was homogenised in 45 ml of a 2% tri-sodium citrate buffer (VWR, Dublin, Ireland). Cheese homogenate was then subjected to enzymatic lysis using lysozyme (1 mg/ml), mutanolysin (50 U/ml) (Sigma Aldrich, Dublin, Ireland) and proteinase k (800 µg/ml) and incubated at 55°C for 30 min as per Quigley et al (39). DNA was extracted using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, CA USA). After extraction, DNA was concentrated via ethanol precipitation. DNA was re-suspended in 20 µl TE buffer (Sigma-Aldrich, Dublin, Ireland). Quality and purity of extracted DNA was assessed using the NanoDrop 1000 Spectrophotometer (Thermo-Fisher Scientific, Wilmington, VA, USA), as per manufacturers guidelines.

3.2.5. PCR detection of *hdc* and *tdc* gene fragments using selected primer sets

PCR based detection of decarboxylase genes was achieved using primers specific for regions of the Gram-positive and Gram-negative *hdc* operon, respectively, as well as for the *tdc* operon. Primers for the *hdc* operon of Gram-positive bacteria comprised of a forward (HDC3 5'- GATGGTATTGTTTCKTATGA-3') and a reverse primer (HDC4 5' CAAACACCAGCATCTTC-3') targeting a 435 bp fragment of the *hdcA* gene (18). Primers targeting the Gram-negative *hdc* operon comprised of a forward (HIS2-F 5'-AAYTSNTTYGAYTTYGARAARGARGT-3') and a reverse primer (HIS2-R 5'-TANGGNSANCCDATCATYTTRTGNCC-3'), and generated a 531 bp product (40). The *tdc* primers, comprised of a forward (TD5 '5- CAAATGGAAGAAGAAGTAGG-3') and a reverse primer (TD2 '5- ACATAGTCAACCATRTTGAA-3'), amplified an 1100 bp fragment of the *tdc* gene as described by Coton et al (24). PCR reactions were carried out in triplicate and contained 25 µl BioMix Red Master Mix (Bioline, London, UK), 1 µl of each primer (200 nmol l^{-1}), 5 µl DNA template (standardised to

100 ng DNA/reaction) and nuclease free water to a final volume of 50 µl. PCR amplification was carried out using a G-Storm Thermal Cycler (Gene Technologies, Oxfordshire, UK). Amplification consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of; denaturation at 95°C for 45 s, annealing at 48°C for 1 min and extension at 72°C for 90 s. This was followed by a final elongation step at 72°C for 7 min. PCR amplicons were pooled and cleaned using the AMPure XP magnetic bead-based purification system (Beckman Coulter, Takeley, UK).

3.2.6. Cloning of PCR amplicons

Cleaned PCR amplicons were subjected to TOPO cloning reactions using the TOPO TA cloning kit (Invitrogen, CA, USA). TOP10 *E. coli* (Invitrogen) were transformed with the resultant plasmids and plated on LB agar (Merck) containing 50 µg/ml kanamycin (Sigma Aldrich, Dublin, Ireland). Transformants were selected from each cloning reaction and cultured overnight in LB broth and 50 µg/ml kanamycin. Plasmids were then extracted from overnight cultures using the QIAprep Spin Mini Prep kit (Qiagen, Crawley, Sussex, UK) according to the manufacturer's guidelines. Extracted plasmids were quantified and assessed for quality using the NanoDrop 1000 Spectrophotometer (Thermo-Fisher Scientific, Wilmington, VA, USA) prior to Sanger sequencing (Source BioSciences, Dublin, Ireland). The *hdc* amplicons were sequenced using the M13 forward primer while *tdc* amplicons were sequenced using both the M13 forward and reverse primers supplied with the TOPO TA cloning kit.

3.2.7. High Throughput Sequencing

Prior to HTS, *tdc* amplicon libraries were prepared using the Ion Xpress Plus Fragment Library Kit (Life Technologies, Dublin, Ireland). The *hdc* libraries, for which

fragmentation was not required, were prepared using the Ion Plus Fragment Library Kit (Life Technologies, Dublin, Ireland). Libraries were then barcoded, prior to sequencing, using the Ion Xpress Barcode Adaptors (Life Technologies, Dublin, Ireland). Amplicons libraries were assessed for size distribution and concentration using a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA USA). Following library quantification and equimolar pooling, the Ion OneTouch 2 system was used to prepare template positive Ion Sphere Particles (ISP) containing the clonally amplified DNA libraries using the Ion PGM Template OT2 400 kit which allows for < 400 bp reads. Enrichment of the template positive ISP's was performed using the Ion OneTouch ES. An enrichment percentage of 18% was obtained. Sequencing was performed on the Ion Torrent PGM (Life Technologies, Dublin, Ireland) using an Ion 318v2 chip and the Ion PGM Sequencing 400 kit (Life Technologies, Dublin, Ireland) at the Teagasc Next Generation Sequencing suite as per the manufacturer's guidelines.

3.2.8. Bioinformatic Analysis

Following Sanger sequencing, *hdc* reads were analysed using the NCBI nucleotide database (BlastN; http://blast.ncbi.nlm.nih.gov/). Sanger sequencing of the *tdc* amplicons did not provide forward and reverse reads of the complete 1100 bp, therefore, only the overlap (approximately 800 bp), aligned using the MegAlign programme was analysed using the BlastN database.

Raw Ion PGM reads were quality filtered with the fastq_filter script in USEARCH. For both *tdc* and *hdc* amplicons, a length cut-off of 170 bp was used. Reads were then clustered into operational taxonomical units (OTUs) at 97 % identity and chimeras removed with the 64-bit version of USEARCH (41). Subsequently OTUs

were aligned with MUSCLE (42) and a phylogenetic tree generated with the FASTREE package within Qiime (43). Alpha diversity metrics (Shannon, Simpson, CHAO1, Phylogenetic diversity and Observed species) was also calculated within Qiime. For taxonomic assignment OTUs were blasted against the NCBI-NR database and parsed through MEGAN (44).

3.3. Results

This study used previously published PCR primers, designed based on alignments of conserved regions of decarboxylase gene clusters from known BA producing isolates (20). In order to be sure that the variety of decarboxylase genes within the selected cheeses was sufficiently heterogeneous to merit culture-independent HTS analysis, an initial Sanger sequencing-based investigation of cloned PCR amplicons was undertaken. This was then followed by HTS to profile the dominant and subdominant histamine and tyramine producing populations present in the respective cheeses.

3.3.1. Sanger sequencing reveals the identity of bacteria with histaminogenic potential

The selected *hdc* primers targeted a 435 bp fragment of the Gram-positive *hdcA* gene. Six of the 10 cheeses sampled generated PCR amplicons corresponding to the *hdc* operon (Reblochon, Irish artisanal cheese B, Morbier, Tête de Moine, Pecorino Sardo, Ossau-Iraty). No amplicons were generated, across all cheese varieties, when using the selected Gram-negative *hdc* primers (20). The Gram-positive *hdc* amplicons were cloned via the TOPO TA cloning method and a subset of 46 clones were subjected to Sanger sequencing. Table 2 contains a summary of BLAST output for each cheese sample while table S2 contains a complete BLAST analysis of each respective cheese including scores generated, query cover and accession numbers. BLAST output indicated that 35 of the 46 clones sequenced (76.1%) contained a *hdc* fragment corresponding to the *Lactobacillus buchneri hdc* operon. Other *hdc* sequences identified corresponded to the *hdc* operon that is conserved across *Lactobacillus sakei/Tetragenococcus halophilus/T. muriaticus/Oenococcus*

oeni/Lactobacillus hilgardii hdc operon (hereafter referred to as the *Lb. sakei* group of *hdc* operon; 23.4%). In the Reblochon and Tête de Moine cheeses, all of the sequenced *hdc* clones (8 and 8, respectively) corresponded to the *Lb. buchneri hdc* operon. In the Ossau-Iraty cheese all of the *hdc* positive clones were identified as corresponding to the *hdc* operon of the *Lb. sakei* group. The *hdc* genes from *Lb. buchneri* and the *Lb. sakei* group were identified from among the Irish artisanal cheese B, Morbier and Pecorino Sardo cheeses while clones corresponding to the *Lb. sakei* group *hdc* operon were identified from among the Ossau-Iraty cheese.

3.3.2. Sanger sequencing reveals the identity of bacteria with tyraminogenic potential

PCR amplification, using primers designed based on alignments of tyrosine decarboxylases from known producers (20), detected the presence of an 1100 bp fragment of the *tdc* gene in 6 of the 10 cheeses tested (Irish artisanal cheese A, Reblochon, Irish artisanal cheese B, Tête de Moine, Pecorino Sardo, Ossau-Iraty). Table 3 depicts a summary of the BLAST output for each positive cheese samples while Table S3 contains a complete BLAST analysis of samples including top hits, scores generated, query cover and accession numbers. Resultant amplicons were cloned and subjected to Sanger sequencing. In this instance, a subset of 44 clones was sequenced across the six positive cheese types. BLAST analysis revealed the presence of *tdc* fragments corresponding to several species, including *Enterococcus faecalis* which accounted for 19 of the 44 clones sequenced (43.1%). The *tdc* fragments from *Lactobacillus curvatus/Streptococcus thermophilus* (which share high identity with one another; 36%), *E. faecium* (18%) and *Lactobacillus plantarum/brevis* (which, again, are not easily distinguished; 2.3%) were also

identified across the 6 cheese types. With respect to the Pecorino Sardo cheese, all clones contained *tdc* genes corresponding to that of and *E. faecium*. In contrast, *tdc* genes corresponding to those of enterococci, streptococci and lactobacilli were detected across all other cheese varieties.

3.3.3. α-diversity of artisanal cheese microbiota with BA-producing potential as revealed by next generation DNA sequencing

Sanger sequencing established that several cheese samples contained multiple microbial sources of decarboxylase genes. As a result it was apparent that the use of a culture-independent HTS-based approach to provide an in-depth insight into the diversity of the populations present was justified. The previously generated PCR amplicons were used for HTS sequencing (n=6 for gram-positive *hdc* primers and n=6 for *tdc* primers). Amplicons were subjected to HTS using the Ion PGM platform, generating 938,971 *hdc* reads and 624,793 *tdc* reads, after quality filtering (refer to Table S4 for the complete list of assigned reads/cheese). Mean read length across both *tdc* and *hdc* samples was 245 bp. Operational Taxonomic Unit (OTU) diversity (α-diversity) was calculated for both *hdc* and *tdc* samples and is displayed in Table 4a/b. For *hdc* α-diversity, Chao1 values, indicative of taxonomic richness, ranged from 41.75 – 90 while the Shannon index, used to measure the overall sample diversity of Gram-positive bacteria with histamine-producing potential, ranged from 2.57 – 3.23. Irish artisanal cheese B displayed the greatest sample diversity while Tête de Moine exhibited the lowest diversity. The *hdc* α-diversity was observed to be lower than that of the *tdc* samples. For *tdc* samples, Chao1 values ranged from 224.25 – 279.62 while the Shannon index ranged from 5.48 – 6.4. Ossau-Iraty displayed the greatest sample diversity while Irish artisanal cheese B

displayed the lowest sample diversity. The phylogenetic diversity value and number of observed OTU (97% similarity) matrices also indicated that α-diversity was considerably greater in *tdc* samples than *hdc* samples.

3.3.4. High-throughput Ion PGM sequencing reveals the presence of amine forming communities in different cheese varieties

Phylogenetic assignment of high-throughput sequence data revealed *tdc* sequences corresponding to representatives of both the Firmicutes (99.84 – 100% of all *tdc* sequences) and Actinobacteria (0.16% of *tdc* sequences) phyla. All the *hdc* sequences belonged to the Firmicutes phylum (Table S5a/b). The small proportion of *tdc* reads assigned to the phylum Actinobacteria corresponded to the cheese Ossau-Iraty. While reads were successfully allocated at phylum level, there was an expected, progressive reduction in the numbers of assigned reads at order, genus and species levels respectively. Reads successfully allocated, at phylum, order, genus and species levels, are displayed in Figures 1 and 2. At the order level, *Lactobacillales* accounted for 33.14 – 95.11% of reads assigned in the *tdc* samples. The Actinobacteria-assigned *tdc* reads in Ossau-Iraty corresponded to *Actinomycetales* at the order level and to *Micrococcinaeae* at family level but could not be assigned at the genus level. With respect to the *hdc* samples, *Lactobacillales* accounted for 13.7 – 42.3% of the reads assigned at the order level.

At the genus and species levels, the numbers of reads that could be unambiguously assigned was low in all cases (depicted in Table S4) and this was particularly evident when analysing the *hdc* samples. With respect to *hdc* samples, *Lactobacillus* accounted for 62.5% to 100% of all reads assigned at the genus level. Populations corresponding to *Staphylococcus* (37.5% of reads assigned at genus level) were

present in Irish artisanal cheese B, while *Streptococcus* (6.93% of reads assigned at genus level) was identified in the Pecorino Sardo cheese. At the species level, a small cohort of the *Staphylococcus* population was identified as *S. saprophyticus* (5.97% of reads successfully assigned at species level) while *Streptococcus* populations were successfully classified as *S. thermophilus* (6.94% of reads successfully assigned at species level). *Lb. buchneri* accounted for the majority of reads assigned (93.06 – 100%) at species level and was detected across all cheeses except for Ossau-Iraty (Figure 1). With respect to the Ossau-Iraty cheese, no genus or species level assignment was possible.

For the *tdc* samples, reads were assigned primarily to the genus *Enterococcus* and ranged from 7.67 – 99.65% of reads assigned at genus level. *Lactobacillus* populations were also present and accounted for 0.35 – 92.33% of reads assigned at genus level. At the species level, *E. faecalis* accounted for the majority (2.29 - 100%) of reads successfully assigned at species level. Other subdominant populations identified included *E. faecium, Lb. curvatus, Lb. brevis* and *Lb. delbrueckii* (Figure 2). Percentage populations of reads assigned exclusively at genus and species levels are shown in Table S6.

3.3.5. Cheese characterisation

BAs were detected, at various concentrations, in all cheeses sampled and were found to range from 13.8 – 736.5 mg/kg (Table 5). The average histamine content of the positive samples was 34.48 mg/kg while the average tyramine concentration was 108.69 mg/kg. In all cases more than one BA was present in the cheeses sampled. Although not as toxicologically important as histamine and tyramine, putrescine and cadaverine levels were also measured to give a total BA

concentration in each cheese. As expected, tyramine, generally regarded as the most common BA present in cheese (16, 19), was present in 9 cheese samples at concentrations ranging from 4.5 to 323.4 mg/kg. Histamine was present in 8 cheeses (8.4 – 85.1 mg/kg). Cadaverine was detected in all cheese samples at concentrations ranging from 1.2 – 267.4 mg/kg, while putrescine was detected in 7 cheeses (3.9 – 212.7 mg/kg). The presence or concentration of BAs in the respective cheeses did not appear to be influenced by milk type, source or age. The Morbier cheese contained the highest concentration of total BAs (736.5 mg/kg) while the Comté cheese contained only 13.8 mg/kg total BAs. Histamine was not detected by HPLC in the Manchego and Comté cheeses. Similarly, tyramine was not detected in the Gorgonzola cheese by HPLC.

Compositional analyses of the cheeses are presented in Table 6. Salt concentrations ranged from 0.65 – 1.99%, while cheese pH values extended from 5.3 to 7.1. Cheese salt in moisture levels ranged from 2.1 to 6.48.

3.4. Discussion

In this study, a novel, targeted sequencing-based approach was used to screen a range of different cheese varieties for the presence of microbial populations capable of producing the major toxic BAs histamine and tyramine. Initially, Sanger sequencing identified common BA producers (*Lb. buchneri*, *E. faecium* and *E. faecalis*) (23, 45) but more importantly provided proof of heterogeneity, justifying the use of NGS. The longer read lengths associated with the Sanger approach (up to approximately 800bp in the case of the *tdc* amplicon) also allowed, in certain instances, successful identification at genus and species levels. However, the highly conserved nature of decarboxylase genes often reduced the capacity for

distinguishing between certain species. This was particularly evident with respect the *Lb. sakei*/*T. halophilus*/*T. muriaticus*/*O.oeni/Lb. hilgardii hdc* operons and the *Lb. curvatus/S. thermophilus* and *Lb. plantarum/Lb. brevis tdc* operons identified. In the aforementioned cases, when conducting a BLAST analysis, the query cover and % identity are identical while the maximum scores differ slightly. This is as a result of single nucleotide changes in the analysed sequences (described in tables S2 and S3). In the case of the *Lb. curvatus/S. thermophilus tdc* operons identified, it likely that both of these cheese associated species are present within the samples tested. With respect to the difficulty differentiating *Lb. sakei/T. halophilus/T. muriaticus/O. oeni/Lb. hilgardii hdc* operons, it is difficult to predict the exact species present.

A further 1,563,764 sequence reads were generated by high-throughput DNA sequencing of amplicons (post quality filtering). HTS allowed for greater population coverage but, in many cases, the short read length led to reduced resolution Decarboxylases from common BA producers such as *E. faecalis, Lb. buchneri*, *Lb. brevis*, and *Lb. curvatus* were again identified. Subdominant populations, for example *Staphylococcus saprophyticus* and *Lactobacillus delbrueckii*, which were not observed *via* Sanger sequencing, were also present at less than 1% of total reads. The shorter read lengths (mean read length of 245bp) associated with using high-throughput sequencing, meant that, in some cases, the assignment of reads at genus and species levels was challenging (Fig. 1 and 2). This is particularly relevant with respect to the highly conserved *hdc* operon. The absence of decarboxylase gene specific databases, as compared to the well annotated 16S rRNA databases, also affected the identification by BLAST analysis. Thus the combination of reduced read length and the lack of specific databases reduced the identification capacity of

the HTS-based approach. This issue is particularly noticeable when analysing the microbial composition of the raw sheep milk cheese Ossau-Iraty. With reference to Ossau-Iraty, Sanger sequencing allowed for successful identification of genes assigned to *E. faecalis, Lb. curvatus/S. thermophilus* (both *tdc*), and *Lb. sakei/T. halophilus/T. muriaticus/O. oeni/Lb. hilgardii* (*hdc*), however the high-throughput approach did not permit assignment of the *hdc* samples at the genus or species level. In the case of *tdc* analysis, the identification of *E. faecalis*-associated *tdc* was possible. Furthermore, while deep sequencing allowed the identification of *tdc* genes corresponding to *Actinomycetales* (0.16%) (Figure 2), which were assigned to the *Micrococcinaeae*, the shorter read length prevented assignment of these decarboxylases at genus or species levels. In order to overcome the issues of reduced read length, HTS platforms such as the Roche Pyrosequencer and Illumina MiSeq could be employed as they allow for increased read length and consequently greater resolution. With particular respect to the Illumina MiSeq platform, paired end reads (i.e.2 x 300bp) and longer read lengths allow for greater accuracy and more specific taxonomic assignments, particularly with MEGAN software (46).

HPLC results established the presence of various BAs across all cheeses sampled. However, the presence of histamine and/or tyramine did not always correlate with the presence of the corresponding decarboxylase gene fragment. This was most evident in the case of the Morbier cheese, which exhibited the highest total BA concentration in this study. Despite a tyramine concentration of 171.3 mg/kg, no *tdc* amplicons were generated by PCR. This discrepancy may be attributable to the fact that the primers selected for this study were designed to target Gram-positive LAB and were based on alignments with common (type-strains) species including

Lb. sakei, *Lb. buchneri*, *Lactobacillus* 30a, *O. oeni*, *C. perfringens* and *T. muraticus* (*hdc*) and *Lb. brevis*, *C. divergens*, *C. piscicola*, *E. faecalis* and *E. faecium* (*tdc*) (18, 24). Therefore, the primers may not bind to all histamine and tyramine decarboxylase determinants present within the cheeses. With respect to this, primers designed to include a wider taxonomic grouping (i.e. not only LAB) may have allowed for identification of more genera. Additionally, certain yeast species including strains of *Y. lipolytica* (*tdc*), *D. hansenii* and *G. candidum* (*hdc*) are recognised BA producers associated with artisanal cheeses, and may have contributed to the amine content, but would not be detected using the primers employed (13).

In this study, the identification of decarboxylase genes, using HTS, from bacteria commonly used as cheese starter cultures, including *Lb. delbrueckii* and *S. thermophilus* was of particular interest (47). In agreement with previous reports (23, 48), *S. thermophilus* was identified as having histidine decarboxylation capacity in the Pecorino Sardo cheese. The origin of these bacteria, i.e., whether they were added as cheese starters or gained access to the cheese *via* raw milk or during processing or ripening is not known. This highlights the importance of screening starter and adjunct bacteria for aminogenic potential, using molecular methods that can rapidly detect the presence of decarboxylase genes. *S. saprophyticus,* not commonly associated with BA formation in cheese, was identified in this study and has previously been associated with BA formation in fermented meat products (49, 50).

Of the cheeses selected for this study, both Pecorino-Sardo and Manchego have a well-established association with BAs. In particular, Pecorino Sardo, identified in

this study as containing several *hdc* and *tdc* positive bacteria (*Lb. buchneri, E. faecium, E. faecalis*), has previously been shown to contain conditions (microbiota, ripening time, physio-chemical factors) complementary to BA production (30, 51). Manchego has also previously been shown to contain tyrosine decarboxylating microorganisms; however, in this study the Manchego cheese sampled had a low level of total BA concentrations (21.9 mg/kg) and no *tdc* or *hdc* positive amplicons were generated (52). Comté and Gorgonzola have also previously been shown to contain various BAs (53) but in our study BA levels were low and no *hdc* or *tdc* amplicons were generated. Interestingly, blue cheeses such as Gorgonzola are often regarded as having optimal conditions for BA production, due to milk processing and proteolytic activity (presence of molds), for BA formation, however, in this study the Gorgonzola sample exhibited among the lowest total BA concentrations (33, 54).

Ultimately, this study shows for the first time, that sequencing based technologies (Ion PGM platform) have the potential to profile the diversity of histaminogenic and tyraminogenic bacteria present in ripening cheese. A similar approach could also be applied to reduce risk factors associated with BA accumulation. This can be achieved by screening starter cultures, milk and manufacturing/storage facilities with a view to reducing/controlling not only populations associated with BA formation, but potential sources of these populations (13, 55-57). In this way, a pre-emptive approach using existing (refrigeration, preservatives, additives) and/or emerging (microbial modelling, high hydrostatic pressure, irradiation) control measures can be implemented (55, 58-61). This method cannot determine the activity of the respective genes and an RNA based approach would be required to

determine transcriptional potential. In addition, while NGS reads indicate, proportionally, the levels of bacterial populations within the cheese matrix, it does not accurately quantify the numbers of bacteria present. While further optimisation is required, sequencing based approaches have the potential to eventually replace labour intensive culture-based methods which often require primary culturing followed by molecular methods to identify responsible genera.

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3.6. References

1. **Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ.** 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences **103:**12115-12120.

2. **Nacke H, Thurmer A, Wollherr A, Will C, Hodac L, Herold N, Schoning I, Schrumpf M, Daniel R.** 2011. Pyrosequencing-based assessment of bacterial community structure along different management types in German forest and grassland soils. PloS one **6:**e17000.

3. **Claesson MJ, O'Sullivan O, Wang Q, Nikkilä J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW.** 2009. Comparative Analysis of Pyrosequencing and a Phylogenetic Microarray for Exploring Microbial Community Structures in the Human Distal Intestine. PloS one **4:**e6669.

4. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2012. High-Throughput Sequencing for Detection of Subpopulations of Bacteria Not Previously Associated with Artisanal Cheeses. Applied and Environmental Microbiology **78:**5717-5723.

5. **Alegría Á, Szczesny P, Mayo B, Bardowski J, Kowalczyk M.** 2012. Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. Applied and Environmental Microbiology **78:**1890-1898.

6. **Wolfe Benjamin E, Button Julie E, Santarelli M, Dutton Rachel J.** Cheese Rind Communities Provide Tractable Systems for In Situ and In Vitro Studies of Microbial Diversity. Cell **158:**422-433.

7. **O'Sullivan DJ, Cotter PD, Sullivan OO, Giblin L, McSweeney PLH, Sheehan JJ.** 2015. Temporal and spatial differences in microbial composition during the manufacture of a Continental-type cheese. Applied and Environmental Microbiology.

8. **Chang F, Li MM.** 2013. Clinical application of amplicon-based next-generation sequencing in cancer. Cancer Genetics **206:**413-419.

9. **Diaz-Sanchez S, Hanning I, Pendleton S, D'Souza D.** 2013. Next-generation sequencing: The future of molecular genetics in poultry production and food safety. Poultry Science **92:**562-572.

10. **Mayo B, T. C. C. Rachid C, Alegria A, M. O. Leite A, S. Peixoto R, Delgado S.** 2014. Impact of Next Generation Sequencing Techniques in Food Microbiology. Current Genomics **15:**293-309.

11. **Silla Santos MH.** 1996. Biogenic amines: their importance in foods. International journal of food microbiology **29:**213-231.

12. **Shalaby AR.** 1996. Significance of biogenic amines to food safety and human health. Food Research International **29:**675-690.

13. **Linares DM, Martin MC, Ladero V, Alvarez MA, Fernandez M.** 2011. Biogenic amines in dairy products. Critical reviews in food science and nutrition **51:**691-703.

14. **Novella-Rodríguez S, Veciana-Nogués MT, Izquierdo-Pulido M, Vidal-Carou MC.** 2003. Distribution of Biogenic Amines and Polyamines in Cheese. Journal of Food Science **68:**750-756.

15. **Spano G, Russo P, Lonvaud-Funel A, Lucas P, Alexandre H, Grandvalet C, Coton E, Coton M, Barnavon L, Bach B, Rattray F, Bunte A, Magni C, Ladero V, Alvarez M, Fernandez M, Lopez P, de Palencia PF, Corbi A, Trip H,**

Lolkema JS. 2010. Biogenic amines in fermented foods. European journal of clinical nutrition **64 Suppl 3:**S95-100.

16. **Linares DM, Fernández M, Martín MC, Álvarez MA.** 2009. Tyramine biosynthesis in Enterococcus durans is transcriptionally regulated by the extracellular pH and tyrosine concentration. Microbial Biotechnology **2:**625-633.

17. **Burdychova R, Komprda T.** 2007. Biogenic amine-forming microbial communities in cheese. FEMS Microbiology Letters **276:**149-155.

18. **Coton E, Coton M.** 2005. Multiplex PCR for colony direct detection of Gram-positive histamine- and tyramine-producing bacteria. Journal of Microbiological Methods **63:**296-304.

19. 2011. EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. Available online: www.efsa.europa.eu/efsajournal EFSA Journal 2011;9(10):2393 [93 pp.]. doi:10.2903/j.efsa.2011.2393;.

20. **Landete JM, de las Rivas B, Marcobal A, Muñoz R.** 2007. Molecular methods for the detection of biogenic amine-producing bacteria on foods. International journal of food microbiology **117:**258-269.

21. **Bunkova L, Bunka F, Mantlova G, Cablova A, Sedlacek I, Svec P, Pachlova V, Kracmar S.** 2010. The effect of ripening and storage conditions on the distribution of tyramine, putrescine and cadaverine in Edam-cheese. Food Microbiol **27:**880-888.

22. **Ladero V, Fernández M, Alvarez MA.** 2009. Effect of post-ripening processing on the histamine and histamine-producing bacteria contents of different cheeses. International Dairy Journal **19:**759-762.

23. **Linares DM, del Río B, Ladero V, Martínez N, Fernández M, Martín MC, Álvarez MA.** 2012. Factors influencing biogenic amines accumulation in dairy products. Frontiers in microbiology **3**.

24. **Coton M, Coton E, Lucas P, Lonvaud A.** 2004. Identification of the gene encoding a putative tyrosine decarboxylase of Carnobacterium divergens 508. Development of molecular tools for the detection of tyramine-producing bacteria. Food Microbiology **21:**125-130.

25. **Ladero V, Calles M, Fernández M, Alvarez MA.** Toxicological Effects of Dietary Biogenic Amines.

26. **Valsamaki K, Michaelidou A, Polychroniadou A.** 2000. Biogenic amine production in Feta cheese. Food Chemistry **71:**259-266.

27. **Guarcello R, Diviccaro A, Barbera M, Giancippoli E, Settanni L, Minervini F, Moschetti G, Gobbetti M.** 2015. A survey of the main technology, biochemical and microbiological features influencing the concentration of biogenic amines of twenty Apulian and Sicilian (Southern Italy) cheeses. International Dairy Journal **43:**61-69.

28. **Fernandez M, Linares DM, Del Rio B, Ladero V, Alvarez MA.** 2007. HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. The Journal of dairy research **74:**276-282.

29. **Loizzo MR, Menichini F, Picci N, Puoci F, Spizzirri UG, Restuccia D.** 2013. Technological aspects and analytical determination of biogenic amines in cheese. Trends in Food Science & Technology **30:**38-55.

30. **Schirone M, Tofalo R, Fasoli G, Perpetuini G, Corsetti A, Manetta AC, Ciarrocchi A, Suzzi G.** 2013. High content of biogenic amines in Pecorino cheeses. Food Microbiology **34:**137-144.

31. **Montel M-C, Buchin S, Mallet A, Delbes-Paus C, Vuitton DA, Desmasures N, Berthier F.** 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. International journal of food microbiology **177:**136-154.

32. **Marcobal A, de las Rivas B, Muñoz R.** 2006. Methods for the Detection of Bacteria Producing Biogenic Amines on Foods: A Survey. J. Verbr. Lebensm. **1:**187-196.

33. **O Sullivan D, Cotter P, Giblin L, Sheehan J, McSweeney P.** 2013. Nucleic acid-based approaches to investigate microbial-related cheese quality defects. Frontiers in Microbiology **4**.

34. **Ladero V, Linares DM, Fernández M, Alvarez MA.** 2008. Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: Relation with histamine content. Food Research International **41:**1015-1019.

35. **Özoğul F.** 2004. Production of biogenic amines by Morganella morganii, Klebsiella pneumoniae and Hafnia alvei using a rapid HPLC method. Eur Food Res Technol **219:**465-469.

36. **IDF.** 1988. Cheese and processed cheese: Determination of chloride content (potentiometric titration method). International Standards 4a Brussels, Belgium: International Dairy Federation.

37. **IDF.** 1982. Determination of total solids content (cheese and processed cheese). International Standards 4a Brussels, Belgium: International Dairy Federation.

38. **Standards IB.** 1976. British Standard Methods for Chemical Analysis of Cheese: Determination of pH value. London: British Standards Institute.

39. **Quigley L, O'Sullivan O, Beresford TP, Paul Ross R, Fitzgerald GF, Cotter PD.** 2012. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. Journal of Applied Microbiology **113:**96-105.

40. **de las Rivas B, Marcobal A, Carrascosa AV, Munoz R.** 2006. PCR detection of foodborne bacteria producing the biogenic amines histamine, tyramine, putrescine, and cadaverine. Journal of food protection **69:**2509-2514.

41. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26:**2460-2461.

42. **Edgar R.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.

43. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nature methods **7:**335-336.

44. **Huson DH, Auch AF, Qi J, Schuster SC.** 2007. MEGAN analysis of metagenomic data. Genome Research **17:**000.

45. **Stratton JE, Hutkins RW, Taylor SL.** 1991. Biogenic Amines in Cheese and other Fermented Foods: A Review. Journal of food protection **54:**460-470.

46. **Mitra S, Schubach M, Huson DH.** 2010. Short clones or long clones? A simulation study on the use of paired reads in metagenomics. BMC Bioinformatics **11 Suppl 1:**S12.

47. **Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM.** 2001. Recent advances in cheese microbiology. International Dairy Journal **11:**259-274.

48. **Rossi F, Gardini F, Rizzotti L, La Gioia F, Tabanelli G, Torriani S.** 2011. Quantitative Analysis of Histidine Decarboxylase Gene (hdcA) Transcription and Histamine Production by Streptococcus thermophilus PRI60 under Conditions Relevant to Cheese Making. Applied and Environmental Microbiology **77:**2817-2822.

49. **Bover-Cid S, Hugas M, Izquierdo-Pulido M, Vidal-Carou MC.** 2001. Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. International journal of food microbiology **66:**185-189.

50. **Santos MH.** 1998. Amino acid decarboxylase capability of microorganisms isolated in Spanish fermented meat products. International journal of food microbiology **39:**227-230.

51. **Martuscelli M, Gardini F, Torriani S, Mastrocola D, Serio A, Chaves-López C, Schirone M, Suzzi G.** 2005. Production of biogenic amines during the ripening of Pecorino Abruzzese cheese. International Dairy Journal **15:**571-578.

52. **Fernández-García E, Tomillo J, Núñez M.** 1999. Effect of added proteinases and level of starter culture on the formation of biogenic amines in raw milk Manchego cheese. International journal of food microbiology **52:**189-196.

53. **Mayer HK, Fiechter G, Fischer E.** 2010. A new ultra-pressure liquid chromatography method for the determination of biogenic amines in cheese. Journal of Chromatography A **1217:**3251-3257.

54. **Herrero-Fresno A, Martinez N, Sanchez-Llana E, Diaz M, Fernandez M, Martin MC, Ladero V, Alvarez MA.** 2012. Lactobacillus casei strains isolated from cheese reduce biogenic amine accumulation in an experimental model. International journal of food microbiology **157:**297-304.

55. **Naila A, Flint S, Fletcher G, Bremer P, Meerdink G.** 2010. Control of biogenic amines in food--existing and emerging approaches. J Food Sci **75:**R139-150.

56. **Komprda T, Smělá D, Novická K, Kalhotka L, Šustová K, Pechová P.** 2007. Content and distribution of biogenic amines in Dutch-type hard cheese. Food Chemistry **102:**129-137.

57. **Bokulich NA, Mills DA.** 2013. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl Environ Microbiol **79:**5214-5223.

58. **Leuschner RG, Heidel M, Hammes WP.** 1998. Histamine and tyramine degradation by food fermenting microorganisms. International journal of food microbiology **39:**1-10.

59. **Leuschner RG, Hammes WP.** 1998. Degradation of histamine and tyramine by Brevibacterium linens during surface ripening of Munster cheese. Journal of food protection **61:**874-878.

60. **García-Ruiz A, González-Rompinelli EM, Bartolomé B, Moreno-Arribas MV.** 2011. Potential of wine-associated lactic acid bacteria to degrade biogenic amines. International journal of food microbiology **148:**115-120.

61. **Fadda S, Vignolo G, Oliver G.** 2001. Tyramine degradation and tyramine/histamine production by lactic acid bacteria and Kocuria strains. Biotechnology Letters **23:**2015-2019.

Table 1: Description of cheeses used in this study (age, origin and rind type). HPLC results as well as presence of the respective decarboxylases detected by PCR are also included.

Cheese	**Milk Type and Source**	**Age**	**Type**	**Region**	**Rind**	**Total BA by HPLC (mg/kg)**	***Hdc* gene presence by PCR**	***Tdc* gene presence by PCR**
Irish Artisanal Cheese A	Raw, Cow	12 – 18 months	Hard	Ireland	Waxed	290.3	N	Y
Reblochon	Raw, Cow	4 - 12 weeks	Semi-hard	France	Washed, smear ripened	104.1	Y	Y
Irish Artisanal Cheese B	Raw, Cow	12 - 18 months	Hard	Ireland	Cloth bound natural	456.6	Y	Y
Manchego	Raw, Sheep	6 -12 months	Semi-hard	Spain	Waxed	21.9	N	N
Morbier	Raw, Cow	2 – 3 months	Semi-hard	France	Natural	736.5	Y	N
Tête de Moine	Raw, Cow	3 – 6 months	Hard	Switzerland	Washed	131.9	Y	Y
Pecorino Sardo	Raw, Sheep	6 – 10 months	Hard	Italy	Natural	134.2	Y	Y
Ossau-Iraty	Raw, Sheep	3 – 6 months	Semi-hard	France	Natural	393.8	Y	Y
Comté	Raw, Cow	6 – 12 months	Hard	France	Natural	13.8	N	N
Gorgonzola	Raw, Cow	3 – 4 months	Semi-hard	Italy	Natural	34.2	N	N

Table 2: Summary of homologues of histidine decarboxylase (*hdc*) gene fragments detected in *hdc* positive cheeses using Sanger sequencing of cloned amplicons

Cheese	# of Clones	BLAST Output	E-Value	% Identity
Reblochon	8	*Lb. buchneri* histidine decarboxylase operon (*hdcA* gene, *hdcB* gene, *hdcC* gene and *hisS* gene)	0	99%
Irish Artisanal Cheese B	5	*Lb. buchneri* histidine decarboxylase operon (*hdcA* gene, *hdcB* gene, *hdcC* gene and *hisS* gene)	0	99%
	1	*Lb. sakei hdc* gene, partial cds/*T. halophilus hdc* operon/*T. muriaticus hdc/O. oeni hdc* operon*/Lb. hilgardii hdc* operon	0	99%
Morbier	7	*Lb. buchneri* histidine decarboxylase operon (hdcA gene, hdcB gene, *hdcC* gene and hisS gene)	0	99%
	1	*Lb.sakei* hdc gene, partial cds/*T. halophilus hdc* operon/*T. muriaticus* p*hdc/O. oeni hdc* operon*/Lb. hilgardii hdc* operon	0	96%
Tête De Moine	8	*Lb. buchneri* histidine decarboxylase operon (*hdcA* gene, *hdcB* gene, *hdcC* gene and *hisS* gene)	0	99%
Pecorino Sardo	7	*Lb. buchneri* histidine decarboxylase operon (*hdcA* gene, *hdcB* gene, *hdcC* gene and *hisS* gene)	0	99%
	1	*Lb.sakei* hdc gene, partial cds/*T. halophilus hdc* operon/*T. muriaticus* p*hdc/O. oeni hdc* operon*/Lb. hilgardii hdc* operon	0	99%
Ossau-Iraty	8	*Lb.sakei* hdc gene, partial cds/*T. halophilus hdc* operon/*T. muriaticus* p*hdc/O. oeni hdc* operon*/Lb. hilgardii hdc* operon	0	99%

Table 3: Summary of homologues of tyrosine decarboxylase (*tdc*) gene fragments detected in *tdc* positive cheeses using Sanger sequencing of cloned amplicons

Cheese	# of Clones	BLAST output	E-Value	% Identity
Irish Artisanal Cheese A	5	*E. faecalis tdc* operon complete cds	0	99%
	1	*E. faecalis tdc* operon complete cds	1.0E-141	99%
	2	*Lb. curvatus tdc* complete cds/*S. thermophilus tdcA* gene	0	99%
Reblochon	5	*Lb. curvatus tdc* /*S. thermophilus tdcA* gene complete cds	0	99%
	1	*E. faecalis tdc* gene, complete cds	0	100%
Irish Artisanal Cheese B	8	*Lb. curvatus tdc*, complete cds/*S. thermophilus tdcA* gene	0	99%
Tête de Moine	7	*E. faecalis tdc* gene, complete cds	0	98%
	1	*Lb. plantarum/Lb. brevis tdc* gene cds	0	99%
Pecorino Sardo	6	*E. faecium tyrS gene, tyrdc* gene complete cds	0	99%
	1	*E. faecium tyrS gene, tyrdc* gene complete cds	2E-70	79%
	1	*E. faecium tyrS gene, tyrdc* gene complete cds	0	89%
Ossau-Iraty	2	*E. faecalis*, *tdc* gene complete cds	0	98%
	2	*E. faecalis* complete genome	0	97%
	1	*E. faecalis* complete genome	0	99%
	1	*Lb. curvatus tdc* gene complete cds/*S. thermophilus tdcA*	0	99%

gene

Table 4a/b: α-diversity of artisanal cheeses post Ion PGM sequencing. Table 4a details diversity of *hdc* positive samples while Table 4b presents *tdc* positive sample diversity.

Table 4a

***hdc* α-diversity**					
Cheese	**Chao1 value**	**Simpson value**	**Shannon Index value**	**Phylogenetic Diversity value**	**No. of observed OTU's (97% Similarity)**
Reblochon	55	0.80	2.85	21.96	52
Irish Artisanal Cheese B	90	0.82	3.23	27.71	75
Morbier	57.5	0.76	2.73	20.38	57
Tête de Moine	41.75	0.67	2.39	18.25	38
Pecorino Sardo	69.5	0.75	2.73	21.66	67
Ossau-Iraty	52	0.78	2.57	23.48	50

Table 4b

***tdc* α-diversity**					
Cheese	**Chao1 value**	**Simpson value**	**Shannon Index value**	**Phylogenetic Diversity value**	**No. of observed OTU's (97% Similarity)**
Irish Artisanal Cheese A	249.06	0.98	6.40	145.48	246
Reblochon	247.96	0.97	5.48	143.93	225
Irish Artisanal Cheese B	224.25	0.97	5.51	126.47	188
Tête de Moine	273.50	0.97	5.78	171.53	270
Pecorino Sardo	270.18	0.97	5.81	152.71	259
Ossau-Iraty	279.62	0.98	5.96	150.62	256

Table 5: Average concentrations of biogenic amines (mg/kg of cheese) detected as determined by HPLC

Cheese	Histamine (mg/kg)	Tyramine (mg/kg)	Putrescine (mg/kg)	Cadaverine (mg/kg)	Total BA (mg/kg)
Irish Artisanal Cheese A	22.9	140.4	122.0	5.0	290.3
Reblochon	8.4	45.1	28.2	22.3	104.1
Irish Artisanal Cheese B	34.4	190.6	157.2	74.4	456.6
Manchego	n.d.	17.9	n.d.	4.0	21.9
Morbier	85.1	171.3	212.7	267.4	736.5
Tête de Moine	51.6	44.6	n.d.	35.7	131.9
Pecorino Sardo	23.4	40.4	66.9	3.5	134.2
Ossau-Iraty	20.8	323.4	40.1	9.4	393.8
Comté	n.d.	4.5	n.d.	9.3	13.8
Gorgonzola	29.2	n.d.	3.9	1.2	34.2

Table 6: Compositional analysis of cheeses (Salt %, pH and Salt in Moisture)

Cheese	Salt (%)	pH	Salt in Moisture levels
Irish Artisanal Cheese A	1.59	5.3	6.26
Reblochon	1.08	6.4	2.10
Irish Artisanal Cheese B	1.99	5.4	6.48
Manchego	1.44	5.7	5.24
Morbier	1.36	6.9	4.32
Tête de Moine	1.49	7.1	4.46
Pecorino Sardo	1.72	5.6	6.44
Ossau-Iraty	1.42	6.4	4.73
Comté	0.65	6.1	2.34
Gorgonzola	1.96	7.1	4.32

Figure 1: Phylogenetic assignement, using MEGAN, of *hdc* reads across cheeses at Phylum, Order, Genus and Species level. Note that no genus or species level assignement was possible for the Ossau-Iraty cheese.

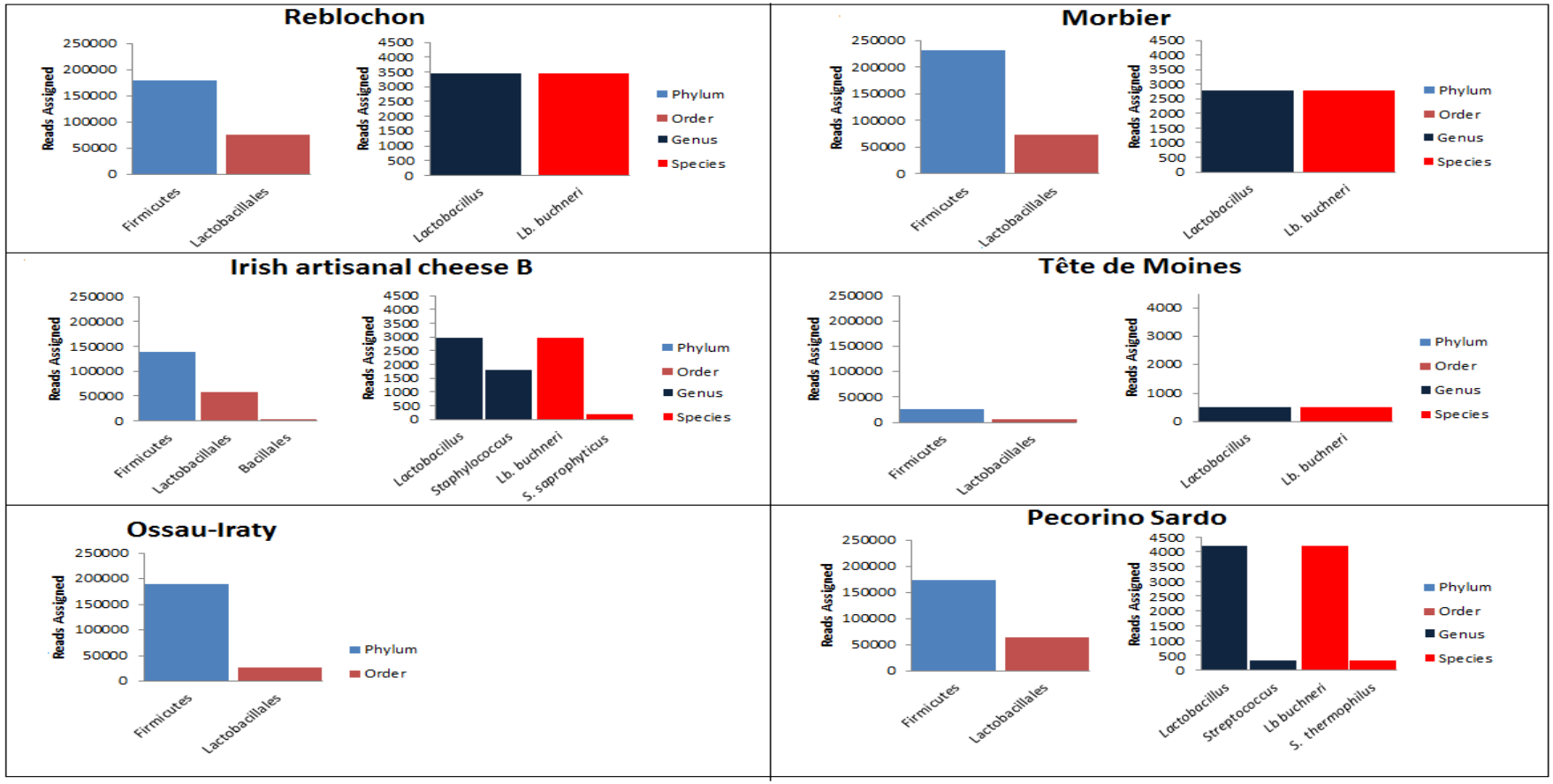
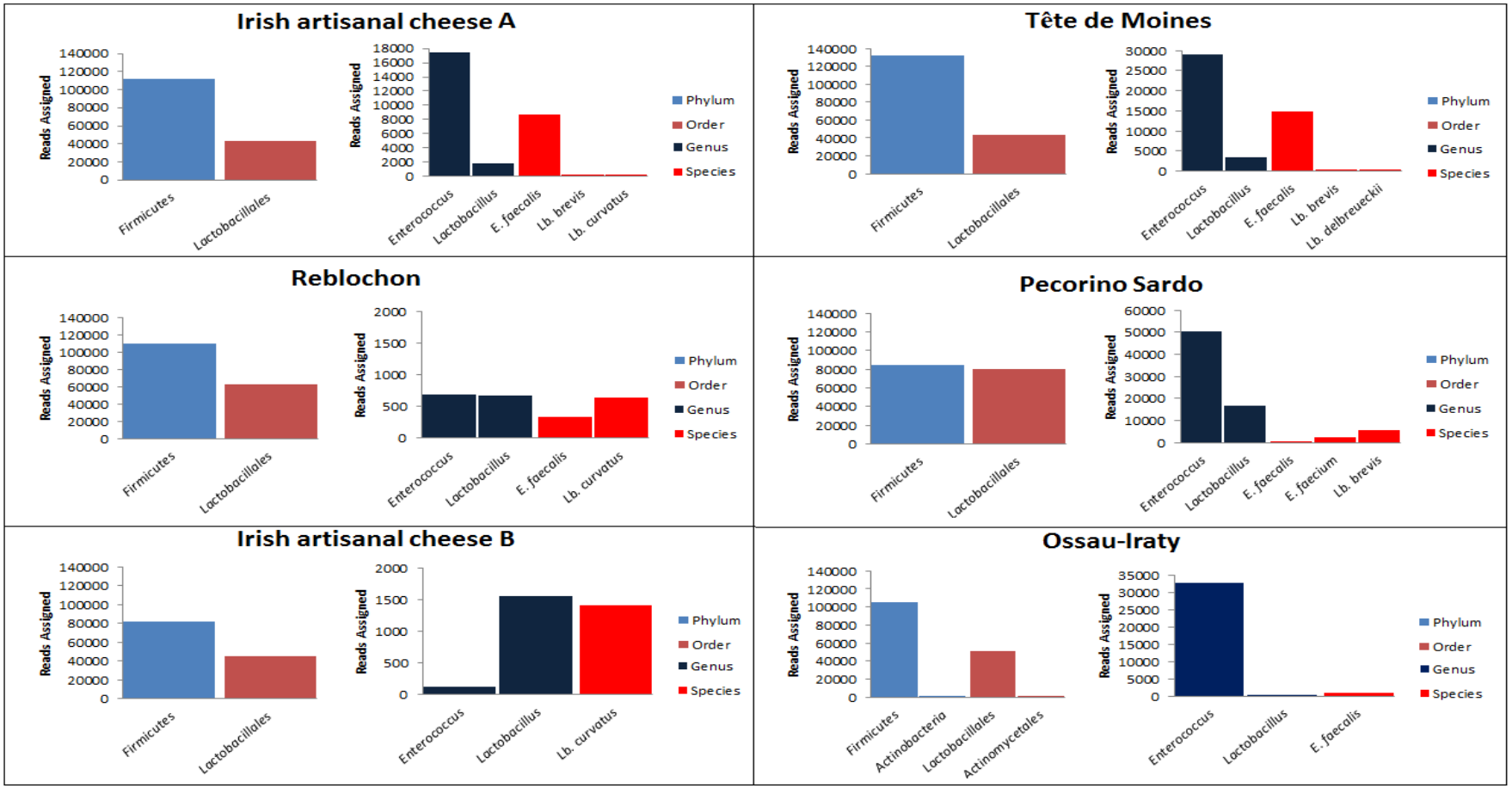


Figure 2: Phylogenetic assignement, using MEGAN, of *tdc* reads across cheeses at Phylum, Order, Genus and Species level



3.6. Appendices

Supplementary Tables

Table S1: Composition of reference standard mixes used for HPLC quantification of biogenic amines in cheese

Mix No.	Histamine (µg/ml)	Putrescine (µg/ml)	Cadaverine (µg/ml)	Tyramine (µg/ml)
1	100	5	10	50
2	200	10	20	100
3	500	25	50	250
4	1000	50	100	500
5	2000	100	200	1000

Table S2: Description of the BLAST analysis conducted on *hdc* clones subjected to Sanger sequencing. Max score, query cover, % identity and the relevant accession numbers are included.

Cheese	Clone	Top BLAST Hits	Max score	Query cover	E value	Identity (%)	Accession
Reblochon	1	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Reblochon	2	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Reblochon	3	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Reblochon	4	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Reblochon	5	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Reblochon	6	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Reblochon	7	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Reblochon	8	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Irish Artisanal Cheese B	1	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	AJ749838.1
Irish Artisanal Cheese B	2	*Lb. buchneri hdc* gene, partial cds	791	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	791	100%	0	99%	AJ749838.1
Irish Artisanal Cheese B	3	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1

Irish Artisanal Cheese B	4	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	AJ749838.1
Irish Artisanal Cheese B	5	*Lb. sakei hdc operon*	782	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	782	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	776	100%	0	99%	AB670117.1
		O. oeni Hdc operon	776	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	776	100%	0	99%	NG_036021.1
Irish Artisanal Cheese B	6	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Morbier	1	*Lb. buchneri hdc* gene, partial cds	713	100%	0	100%	DQ132890.1
		Lb. buchneri hdc operon	713	100%	0	100%	AJ749838.1
Morbier	2	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1
Morbier	3	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1
Morbier	4	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Morbier	5	*Lb. buchneri hdc* gene, partial cds	782	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	782	100%	0	99%	AJ749838.1
Morbier	6	*Lb. sakei hdc operon*	699	100%	0	96%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	699	100%	0	96%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	693	100%	0	95%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	693	100%	0	95%	AB710473.1
		O. oeni Hdc operon	693	100%	0	95%	DQ132887.1
		Lb. hilgardii hdc operon	693	100%	0	95%	NG_036021.1
Morbier	7	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1
Morbier	8	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1

Tête De Moine	1	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Tête De Moine	2	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Tête De Moine	3	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Tête De Moine	4	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1
Tête De Moine	5	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1
Tête De Moine	6	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Tête De Moine	7	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Tête De Moine	8	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Pecorino Sardo	1	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Pecorino Sardo	2	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Pecorino Sardo	3	*Lb. buchneri hdc* gene, partial cds	793	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	793	100%	0	99%	DQ132890.1
Pecorino Sardo	4	*Lb. buchneri hdc* gene, partial cds	787	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	787	100%	0	99%	AJ749838.1
Pecorino Sardo	5	*Lb. sakei hdc* operon	787	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	787	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	782	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	782	100%	0	99%	AB710473.1
		O. oeni Hdc operon	782	100%	0	99%	DQ132887.1

		Lb. hilgardii hdc operon	782	100%	0	99%	NG_036021.1
Pecorino Sardo	6	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Pecorino Sardo	7	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Pecorino Sardo	8	*Lb. buchneri hdc* gene, partial cds	798	100%	0	99%	DQ132890.1
		Lb. buchneri hdc operon	798	100%	0	99%	AJ749838.1
Ossau-Iraty	1	*Lb. sakei hdc* operon	798	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	798	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	793	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	793	100%	0	99%	AB710473.1
		O. oeni Hdc operon	793	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	793	100%	0	99%	NG_036021.1
Ossau-Iraty	2	*Lb. sakei hdc* operon	798	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	798	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	793	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	793	100%	0	99%	AB710473.1
		O. oeni Hdc operon	793	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	793	100%	0	99%	NG_036021.1
Ossau-Iraty	3	*Lb. sakei hdc* operon	793	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	793	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	787	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	787	100%	0	99%	AB710473.1
		O. oeni Hdc operon	787	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	787	100%	0	99%	NG_036021.1
Ossau-Iraty	4	*Lb. sakei hdc* operon	798	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	798	100%	0	99%	AY800122.1

		T. halophilus hdc gene *c*omplete and partial cds	793	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	793	100%	0	99%	AB710473.1
		O. oeni Hdc operon	793	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	793	100%	0	99%	NG_036021.1
Ossau-Iraty	5	*Lb. sakei hdc* operon	795	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	795	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	789	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	789	100%	0	99%	AB710473.1
		O. oeni Hdc operon	789	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	789	100%	0	99%	NG_036021.1
Ossau-Iraty	6	*Lb. sakei hdc* operon	798	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	798	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	793	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	793	100%	0	99%	AB710473.1
		O. oeni Hdc operon	793	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	793	100%	0	99%	NG_036021.1
Ossau-Iraty	7	*Lb. sakei hdc* operon	793	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	793	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	787	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	787	100%	0	99%	AB710473.1
		O. oeni Hdc operon	787	100%	0	99%	DQ132887.1
		Lb. hilgardii hdc operon	787	100%	0	99%	NG_036021.1
Ossau-Iraty	8	*Lb. sakei hdc* operon	793	100%	0	99%	DQ132888.1
		Lb. sakei strain *hdc* gene, partial cds	793	100%	0	99%	AY800122.1
		T. halophilus hdc gene *c*omplete and partial cds	787	100%	0	99%	AB670117.1
		T. muriaticus plasmid pHDC-I-1 DNA, complete sequence	787	100%	0	99%	AB710473.1

	O. oeni Hdc operon	787	100%	0	99%	DQ132887.1
	Lb. hilgardii hdc operon	787	100%	0	99%	NG_036021.1

Table S3: Description of the BLAST analysis conducted on *tdc* clones subjected to Sanger sequencing. Max score, query cover, % identity and the relevant accession numbers are included.

Cheese	Clone	Top BLAST Hits	Max score	Query cover	E value	Identity (%)	Accession
Irish Artisanal Cheese A	1	*E. faecalis* complete genome	1356	100%	0	99%	CP008816.1
		E. faecalis tdc operon, complete sequence; and putative amino acid transporter gene, complete cds	1356	100%	0	99%	AF354231.1
		E faecalis tdc complete cds	1345	100%	0	99%	KF195933.1
Irish Artisanal Cheese A	2	*E. faecalis*, complete genome	512	99%	1.00E-141	99%	CP008816.1
		E. faecalis tdc operon, complete sequence; and putative amino acid transporter gene, complete cds	512	99%	1.00E-141	99%	AF354231.1
Irish Artisanal Cheese A	3	*Lb. curvatus tdc* gene, complete cds	1286	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1280	100%	0	99%	FR682467.1
Irish Artisanal Cheese A	4	*E. faecalis,* complete genome	1400	100%	0	99%	AE016830.1
		E. faecalis , complete genome	1395	100%	0	99%	CP008816.1
		E. faecalis strain *tdc* gene, complete cds	1395	100%	0	99%	KF195933.1
Irish Artisanal Cheese A	5	*E. faecalis* ATCC 29212, complete genome	1306	100%	0	99%	CP008816.1
		E. faecalis, complete genome	1306	100%	0	99%	CP002621.1
		E. faecalis tdc operon, complete sequence; and putative amino acid transporter gene, complete cds	1306	100%	0	99%	AF354231.1
Irish Artisanal Cheese A	6	*Lb. curvatus tdc* gene, complete cds	1467	100%	0	100%	AB086652.1
		S. thermophilus tdcA gene	1461	100%	0	99%	FR682467.1

Irish Artisanal Cheese A	7	*E. faecalis* complete genome	1168	100%	0	99%	CP008816.1
		E. faecalis tdc operon, complete sequence; and putative amino acid transporter gene, complete cds	1168	100%	0	99%	AF354231.1
		E faecalis tdc complete cds	1157	100%	0	99%	KF195933.1
Irish Artisanal Cheese A	8	*E. faecalis* complete genome	1411	100%	0	99%	CP008816.1
		E. faecalis tdc operon, complete sequence; and putative amino acid transporter gene, complete cds	1411	100%	0	99%	AF354231.1
		E faecalis tdc complete cds	1400	100%	0	99%	KF195933.1
Reblochon	1	*Lb. curvatus tdc* gene, complete cds	1471	100%	0	100%	AB086652.1
		S. thermophilus tdcA gene	1465	100%	0	99%	FR682467.1
Reblochon	2	*E. faecalis tdc* gene, complete cds	1519	100%	0	100%	KF195933.1
		E. faecalis, complete genome	1519	100%	0	100%	HF558530.1
		E. faecalis, complete genome	1513	100%	0	99%	AE016830.1
Reblochon	3	*Lb. curvatus tdc* gene, complete cds	1330	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1325	100%	0	99%	FR682467.1
Reblochon	4	*S. thermophilus tdcA* gene	1772	99%	0	99%	FR682467.1
		Lb. curvatus tdc gene, complete cds,	1772	99%	0	99%	AB086652.1
Reblochon	5	*Lb. curvatus tdc* gene, complete cds	1528	99%	0	100%	AB086652.1
		S. thermophilus tdcA gene	1522	99%	0	99%	FR682467.1
Reblochon	6	*Lb. curvatus tdc* gene, complete cds	1585	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1580	100%	0	99%	FR682467.1
Irish Artisanal Cheese B	1	*Lb. curvatus tdc* gene, complete cds	1495	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1489	100%	0	99%	FR682467.1
Irish Artisanal Cheese B	2	*Lb. curvatus tdc* gene, complete cds	1351	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1345	100%	0	99%	FR682467.1
Irish Artisanal Cheese B	3	*S. thermophilus tdcA* gene	1402	100%	0	99%	FR682467.1

		Lb. curvatus tdc gene, complete cds,	1402	100%	0	99%	AB086652.1
Irish Artisanal Cheese B	4	*Lb. curvatus tdc* gene, complete cds	1600	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1594	100%	0	99%	FR682467.1
		Lb. curvatus partial *tdc* gene	972	65%	0	97%	FN392115.1
Irish Artisanal Cheese B	5	*Lb. curvatus tdc* gene, complete cds	1493	100%	0	100%	AB086652.1
		S. thermophilus tdcA gene	1487	100%	0	99%	FR682467.1
Irish Artisanal Cheese B	6	*Lb. curvatus tdc* gene, complete cds	1546	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1541	100%	0	99%	FR682467.1
Irish Artisanal Cheese B	7	*Lb. curvatus tdc* gene, complete cds	1472	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1467	100%	0	99%	FR682467.1
Irish Artisanal Cheese B	8	*Lb. curvatus tdc* gene, complete cds	1469	100%	0	100%	AB086652.1
		S. thermophilus tdcA gene	1463	100%	0	99%	FR682467.1
Tête de Moine	1	*E. faecalis tdc* gene, complete cds	1476	100%	0	100%	KF195933.1
		E. faecalis, complete genome	1476	100%	0	100%	HF558530.1
		E. faecalis, complete genome	1471	100%	0	99%	AE016830.1
Tête de Moine	2	*Lb. brevis* genome	1373	100%	0	99%	CP005977.1
		Lb. brevis, complete genome	1373	100%	0	99%	AP012167.1
		*Lb.plantarum tyr*DC and *tyr*P genes, complete cds	1373	100%	0	99%	JQ040309.1
Tête de Moine	3	*E. faecalis tdc* gene, complete cds	1567	100%	0	100%	KF195933.1
		E. faecalis, complete genome	1567	100%	0	100%	HF558530.1
		E. faecalis, complete genome	1561	100%	0	99%	AE016830.1
Tête de Moine	4	*E. faecalis tdc* gene, complete cds	970	100%	0	99%	KF195933.1
		E. faecalis, complete genome	970	100%	0	99%	HF558530.1
		E. faecalis, complete genome	965	100%	0	99%	AE016830.1
Tête de Moine	5	*E. faecalis tdc* gene, complete cds	1587	100%	0	99%	KF195933.1
		E. faecalis, complete genome	1587	100%	0	99%	HF558530.1

		E. faecalis, complete genome	1581	100%	0	99%	AE016830.1
Tête de Moine	6	*E. faecalis tdc* gene, complete cds	1417	100%	0	99%	KF195933.1
		E. faecalis, complete genome	1417	100%	0	99%	HF558530.1
		E. faecalis, complete genome	1411	100%	0	99%	AE016830.1
Tête de Moine	7	*E. faecalis tdc* gene, complete cds	1448	100%	0	99%	KF195933.1
		E. faecalis, complete genome	1448	100%	0	99%	HF558530.1
		E. faecalis, complete genome	1443	100%	0	99%	AE016830.1
Tête de Moine	8	*E. faecalis tdc* gene, complete cds	1421	100%	0	99%	KF195933.1
		E. faecalis, complete genome	1421	100%	0	99%	HF558530.1
		E. faecalis, complete genome	1415	100%	0	99%	AE016830.1
Pecorino Sardo	1	*E. faecium*, complete genome	1275	100%	0	99%	CP006620.1
		E. faecium, complete genome	1275	100%	0	99%	CP004063.1
		E. faecium tyrS gene, partial cds; *tyrDC* genes, complete cds, *tyrP* gene, partial cds	1269	100%	0	99%	HM921050.1
Pecorino Sardo	2	*E. faecium*, complete genome	1544	100%	0	99%	CP006620.1
		E. faecium, complete genome	1544	100%	0	99%	CP006620.2
		E. faecium tyrS gene, partial cds; *tyrDC* genes, complete cds, *tyrP* gene, partial cds	1533	100%	0	99%	HM921050.1
Pecorino Sardo	3	*E. faecium tyrS* gene, partial cds, *tyrDC* genes, complete cds, *tyrP* gene, partial cds	276	85%	2.00E-70	79%	HM921050.1
		E. faecium, complete genome	270	85%	1.00E-68	79%	CP006620.1
		E. faecium, complete genome	270	85%	1.00E-68	79%	CP004063.1
Pecorino Sardo	4	*E. faecium tyrS* gene, partial cds, *tyrDC* genes, complete cds, *tyrP* gene, partial cds	859	97%	0	89%	HM921050.1
		E. faecium, complete genome	843	97%	0	89%	CP006620.1
		E. faecium, complete genome	843	97%	0	89%	CP004063.1
Pecorino Sardo	5	E. faecium, complete genome	1419	100%	0	99%	CP006620.1
		E. faecium, complete genome	1419	100%	0	99%	CP004063.1
		E. faecium tyrS gene, partial cds; *tyrDC* genes, complete cds,	1408	100%	0	99%	HM921050.1

		tyrP gene, partial cds					
Pecorino Sardo	6	*E. faecium tyrS* gene, partial cds, *tyrDC* genes, complete cds, *tyrP* gene, partial cds	1142	100%	0	99%	HM921050.1
		E. faecium, complete genome	1131	100%	0	99%	CP006620.1
		E. faecium, complete genome	1131	100%	0	99%	CP004063.1
Pecorino Sardo	7	E. faecium, complete genome	1613	100%	0	99%	CP006620.1
		E. faecium, complete genome	1613	100%	0	99%	CP004063.1
		E. faecium tyrS gene, partial cds; *tyrDC* genes, complete cds, *tyrP* gene, partial cds	1607	100%	0	99%	HM921050.1
Ossau-Iraty	1	*E. faecalis*, complete genome	1467	100%	0	99%	CP008816.1
		E. faecalis, complete genome	1467	100%	0	99%	CP004081.1
		E faecalis tdc, complete sequence; and putative amino acid transporter gene, complete cds	1467	100%	0	99%	AF354231.1
Ossau-Iraty	2	*E. faecalis*, complete genome	1823	99%	0	99%	CP003726.1
		E. faecalis, complete genome	1823	99%	0	99%	CP002621.1
		E faecalis tdc, complete sequence; and putative amino acid transporter gene, complete cds	1807	99%	0	99%	AF354231.1
Ossau-Iraty	3	*E. faecalis*, complete genome	1201	100%	0	100%	AE016830.1
		E. faecalis, complete genome	1195	100%	0	99%	CP008816.1
		E faecalis tdc, complete sequence; and putative amino acid transporter gene, complete cds	1195	100%	0	99%	KF195933.1
Ossau-Iraty	4	*E. faecalis*, complete genome	1596	100%	0	99%	CP003726.1
		E. faecalis, complete genome	1596	100%	0	99%	CP002621.1
		E faecalis tdc, complete sequence; and putative amino acid transporter gene, complete cds	1585	100%	0	99%	AF354231.1
Ossau-Iraty	5	*E. faecalis tdc* gene, complete cds	1557	100%	0	99%	KF195933.1
		E. faecalis, complete genome	1557	100%	0	99%	HF558530.1
		E. faecalis, complete genome	1552	100%	0	99%	AE016830.1
Ossau-Iraty	6	*Lb. curvatus tdc* gene, complete cds	1227	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1221	100%	0	99%	FR682467.1
		Lb. curvatus partial *tdc* gene	715	61%	0	98%	FN392115.1

Ossau-Iraty	7	*Lb. curvatus tdc* gene, complete cds	1448	100%	0	99%	AB086652.1
		S. thermophilus tdcA gene	1443	100%	0	99%	FR682467.1

Table S4: List of reads assigned at Phylum, Order, Genus and Species level for individual cheeses, post quality filtering

***hdc* Reads Assigned**	**Reblochon**	**Irish Artisanal Cheese B**	**Morbier**	**Tete de Moine**	**Pecorino Sardo**	**Ossau-Iraty**	**Total Reads**
Phylum	179002	139353	231711	26719	173218	188968	**938971**
Order	75790	59451	73165	5371	64645	25895	**304317**
Genus	3445	4764	2770	527	4500	0	**16006**
Species	3444	3147	2770	522	4496	0	**14379**

***tdc* Reads Assigned**	**Irish Artisanal Cheese A**	**Reblochon**	**Irish artisanal cheese B**	**Tete de Moine**	**Pecorino Sardo**	**Ossau-Iraty**	**Total Reads**
Phylum	112469	109410	81689	131959	83961	105478	**624966**
Order	42581	62869	44828	43726	79858	50993	**324855**
Genus	19286	1355	1682	32550	67084	32662	**154619**
Species	9224	972	1403	15495	8297	890	**36281**

Table S5a/b: Microbial composition of bacteria at phylum, order, genus and species levels.

Table 5a reflects *hdc* samples while table 5b depicts *tdc* samples.

Table 5a

***hdc* Microbial Composition**	**Reblochon**	**Irish artisanal cheese B**	**Morbier**	**Tete de Moine**	**Pecorino Sardo**	**Ossau-Iraty**
Phylum						
Firmicutes	100%	100%	100%	100%	100%	100%
Order						
Lactobacillales	42.34%	41.38%	31.58%	20.10%	37.32%	13.70%
Bacillales	0%	1.28%	0%	0%	0%	0%
Unassigned	57.66%	57.34%	68.42%	79.90%	62.68%	86.30%
Sum	100%	100%	100%	100%	100%	100%

Table 5b

***tdc* Microbial Composition**	**Irish artisanal cheese A**	**Reblochon**	**Irish artisanal cheese B**	**Tete de Moine**	**Pecorino Sardo**	**Ossau-Iraty**
Phylum						
Firmicutes	100%	100%	100%	100%	100%	99.84%
Actinobacteria	0%	0%	0%	0%	0%	0.16%
Sum	100%	100%	100%	100%	100%	100%
Order						
Lactobacillales	37.86%	57.46%	54.88%	33.14%	95.11%	48.1%
Actinomycetales	0%	0%	0%	0%	0%	0.16%
Unassigned	62.14%	42.54%	45.12%	66.86%	4.89%	51.74%
Sum	100%	100%	100%	100%	100%	100%

Table S6: Relative abundance of bacteria at Genus and Species levels for individual cheeses.

Relative abundance is expressed as a function of total reads assigned at the genus level.

***hdc* samples**	**Reblochon**	**Irish artisanal cheese B**	**Morbier**	**Tete de Moine**	**Pecorino Sardo**	**Ossau-Iraty**
Genus						
Lactobacillus	100%	62.55%	100%	100%	93.07%	0%
Streptococcus	0%	0%	0%	0%	6.93%	0%
Staphylococcus	0%	37.45%	0%	0%	0%	0%
Species						
Lactobacillus buchneri	100%	94.03%	100%	100%	93.06%	0%
Staphylococcus saprophyticus	0%	5.97%	0%	0%	0%	0%
Streptococcus thermophilus	0%	0%	0%	0%	6.94%	0%

***tdc* samples**	**Irish artisanal cheese A**	**Reblochon**	**Irish artisanal cheese B**	**Tete de Moine**	**Pecorino Sardo**	**Ossau-Iraty**
Genus						
Enterococcus	90.80%	50.63%	7.67%	89.31%	75.32%	99.65%
Lactobacillus	9.20%	49.37%	92.33%	10.69%	24.68%	0.35%
Species						
Enterococcus faecalis	94.53%	34.57%	0%	95.28%	2.29%	100%
Enterococcus faecium	0%	0%	0%	0%	29.37%	0%
Lactobacillus brevis	2.12%	0%	0%	1.94%	68.34%	0%
Lactobacillus curvatus	0.30%	65.43%	100%	0%	0%	0%
Lactobacillus delbrueckii	0%	0%	0%	2.79%	0%	0%

Chapter 4

Thermus and the pink discolouration defect in cheese

Author Contributions

Daniel O'Sullivan Chapter Contributions:

Experimental:

Responsible for whole genome shotgun sequencing, MEGAN and KEGG analysis

Manuscript Preparation:

Generated all graphical representations of whole genome data and KEGG analysis.

Contributor to manuscript preparation

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4.0. Abstract

A DNA sequencing-based strategy was applied to study the microbiology of continental-type cheeses with a pink discolouration defect. The basis for this phenomenon has remained elusive, despite decades of research. The bacterial composition of cheese containing the defect was compared to control cheese using 16S rDNA and shotgun metagenomic sequencing as well as qPCR. Throughout, it was apparent that *Thermus,* a carotenoid-producing genus was present at higher levels in defect, relative to control, cheeses. Prompted by this finding and data confirming the pink discoloration to be associated with the presence of a carotenoid, a culture-based approach was employed and *Thermus thermophilus* was successfully cultured from defect cheeses. The link between *Thermus* and the pinking phenomenon was then established through the cheese defect equivalent of Koch's postulates when the defect was re-created by the reintroduction of a *T. thermophilus* isolate to a test cheese during the manufacturing process.

4.1. Introduction

Pink discolouration defect is a problem that affects the cheese industry worldwide (1). Despite first being noted in the scientific literature in 1933 (2), and the subject of extensive research, the basis for this phenomenon has remained elusive . It particularly impacts a range of ripened cheeses, including Swiss, Cheddar and Italian-type cheese (3-8), resulting in the downgrading or rejection of cheese and a consequential economic loss (1). The defect can manifest in a number of ways depending on the cheese type: at the surface of the cheese block (in patches or over the entire surface), as a uniform pink border occurring below the external surfaces of the cheese block conferring a pinked ring appearance or sporadically distributed within the cheese block (1). Pink discoloration affects both cheeses with and without additional colorants. In cheeses with colorants such as annatto, pink discoloration is thought to be as a results of factors (oxidation, precipitation, temperature and photo-oxidation) affecting the constituents of the colorant itself (1, 5). Contrastingly, in cheeses without colorants, the cause of this defect is unknown. There have been suggestions that it is due to physicochemical factors (Maillard browning) (5, 9-11), while others have proposed a microbial basis (8, 12). In the latter case, it has been claimed that cheeses containing specific starter cultures, and thermophilic strains of lactobacilli and propionic acid bacteria (PAB) in particular, are more likely to have a pink discolouration (6, 8, 13), but this has been the subject of much debate and no clear consensus has been achieved.

High-throughput DNA sequencing technologies have provided a detailed insight into the microbial composition of a wide variety of different ecosystems (14), as well as a selection of food-associated niches (15) including, more recently, dairy-

based foods (16-19), revealing novel, albeit in many cases descriptive, findings. Here we employ a combination of 16S rDNA and shotgun metagenomic sequencing, qPCR, culture based microbiology and cheese manufacture to identify the microbial component responsible for the pink discolouration phenomenon.

4.2. Materials and Methods

4.2.1. DNA extraction from cheeses

Cheese samples (n=18), with (defect cheese n=9) or without (control cheese n=9) pinking discolouration were sourced. For nucleic acid extraction, 1 g of cheese from the defect or control cheese was combined with 9 ml 2% tri-sodium citrate and homogenised before DNA was extracted using the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc., CA, USA) (20) as described previously (20). Additional steps were added to the standard manufacturer's instructions. These included treatment of the homogenate with 50 µg ml^{-1} lysozyme (Sigma-Aldrich Ltd., Arklow, Co. Wicklow, Ireland) and 100 U mutanolysin (Sigma Ltd.) at 37°C for 1 hour followed with protein digestion by adding 250 µg ml^{-1} proteinase K (Sigma Ltd.) and incubating at 55°C for 1 hour.

4.2.2. Generation of 16S rDNA amplicons for high throughput sequencing

DNA extracts were used as a template for PCR amplification of 16S rDNA tags (V4 region; 408 nt long) using universal 16S primers predicted to bind to 94.6% of all 16S genes i.e. the forward primer F1, 5'-AYTGGGYDTAAAGNG, (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) and reverse primer V5, 5-CCGTCAATTYYTTTRAGTTT-3' (21). The primers incorporated the proprietary 19-mer sequences at the 5' end to allow emulsion-based clonal amplification for the 454-pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. The PCR reaction contained 25 µl BioMix Red™ (Bioline Reagents Ltd., London, UK), 1 µl of each primer (10 pmol), 5 µl DNA template and nuclease free

H_2O to give a final reaction volume of 50 µl. PCR amplification was performed using a G-Storm thermal cycler (Somerset Biotechnology Centre, Somerset, UK). The amplification programme consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles; denaturation at 94°C for 1 min, annealing at 52°C for 1min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also included. Amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, UK). The quantity of DNA was assessed using the Quant-It™ Picogreen® dsDNA reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and a Nanodrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). The ND3300 excites in the presence of dsDNA bound with Picogreen® at 470 nm and monitors emission at 525 nm.

4.2.3. 16S rDNA sequencing and bioinformatic analysis

The 16S rDNA V4-V5 amplicons were sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) according to 454 protocols. Read processing was performed using techniques implemented in the RDP pyrosequencing pipeline (22). Sequences not passing the FLX quality controls were discarded, the 454 specific portion of the primer were trimmed, the raw sequences were sorted according to tag sequences and reads with low quality scores (quality scores below 40) and short length (less than 150 bp for the 16S rDNA V4 region) were removed as well as reads that did not have exact matches with the primer sequence. The QIIME suite of programs was used to align, chimera check, cluster and, measure microbial α-diversities and to plot rarefaction curves to determine if sequencing was carried out to sufficient depth (23). Taxonomy was

assigned to trimmed fasta sequences using BLAST (24) against the SILVA version 100 database (25). The resulting BLAST output was parsed using MEGAN version 6.3.0 (26). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results (BLAST bit-score 86,) (27)

The statistical significance of differences in proportions of microbial taxa was determined by the non-parametric Kruskal-Wallis test (28) using the Minitab® statistical package, the level of significance was determined at $P < 0.05$. Sequence data has been deposited to European Nucleotide Archive (ENA) accession number PRSEB6952.

4.2.4. Shotgun metagenomics sequencing and gene function analysis

A selection of defect and control cheeses were shotgun sequenced for metagenomic analysis. This work was carried out by GATC (GATC Biotech, Constance, Germany) including DNA extraction from cheese samples and DNA library preparations followed by sequencing on an Illumina HiSeq 2000 platform (GATC Biotech). Resultant reads were processed using Picard/SAM Tools and assembled using Velvet. Genes were then predicted using MetaGeneMark and annotated using the BLAST programme against the N_R database. Finally sequences were parsed using MEGAN version 5.7.1 (26) and gene function assessed using KEGG (29). Sequence data has been deposited to ENA accession number PRSEB6952.

4.2.5. Raman analysis

Raman spectra were acquired with RISE (Raman Integrated Scanning Electron) microscope integrating TESCAN dual-beam (FIB-SEM) GAIA system with WITec Confocal Raman microscope. The 532 nm green laser was used for spectral acquisition. Integration time per pixel was 0.5 s. Area of interest was imaged with 3 steps per 1 µm (stepsize 1/3 µm). Spectra were processed by ProjectPlus software (WiTec). First the PCA (principle component analysis) procedure was run to find the number of components and then NMF (Non-negative Matrix Factorization) was applied to distinguish spectra of the components.

4.2.6. Culturing of *Thermus*

4.2.6.1. Culture-based Method

Castenholz TYE (Tryptone Yeast Extract) medium was chosen to selectively support the growth of strains from the genus *Thermus* (30). Castenholz TYE medium was prepared by mixing 5 parts 2X Castenholz salts with one part 1% TYE and 4 parts distilled water. An enrichment step, whereby cheese was homogenised in Castenholz medium and incubated at 70°C for 3 days, was employed to encourage the growth of *Thermus*, which are characterised by their highly thermophilic nature, and to prevent the growth of more moderately thermophilic cultures such as those within the starter culture population. A 3% agar was employed to allow incubation at high temperature (55°C) without rapid dehydration of the media. Castenholz Salts, 2X contained 0.2 g nitrilotriacetic acid, 0.12g $CaSO_4.2H_20$, 0.2g $MgSO_4.H_2O$, 0.016g NaCl, 0.21g KNO_3, 1.4g $NaNO_3$, 0.22g Na_2HPO_4, 2.0ml $FeCl_3$ solution (0.03%) and 2.0ml Nitsch's Trace elements {0.5ml H_2SO_4, 2.2g $MnSO_4$, 0.5g $ZnSO_4.7H_2O$, 0.5g H_3BO_3, 0.016g $CuSO_4.5H_2O$, 0.025g $Na_2MoO_4.2H_2O$, 0.046g $CoCl_2.6H_2O$ distilled water 1L}, adjusted to a final volume of 1 L and final pH of 8.2.

1% TYE solution consisted of 10.0 g tryptone, 10.0 g yeast extract dissolved in 1 L distilled water. The final pH of Castenholz TYE medium was 7.6. For preparation of the corresponding agar, 3% (w/v) bacteriological agar was added to the final solution.

4.2.6.2. PCR and qPCR-based detection of Thermus

A set of primers (TpolFor; 5'-AGCCTCCTCCACGAGTTC-3' and TpolRev; 5'-GTAGGCGAGGAGCATGGGGT-3') targeting a region specifically conserved within the polymerase I gene of *Thermus* were designed to facilitate PCR and qPCR-based detection of the genus. The theoretical specificity of these primers was tested using the oligo probe search tools in the BLAST classifier database (Altschul et al., 1990). The PCR reaction contained 25 µl BioMix Red™ (Bioline Reagents Ltd., London, United Kingdom), 1 µl of each primer (10 pmol), 5 µl DNA template and nuclease free H_2O to give a final reaction volume of 50 µl. PCR amplification of the polymerase I gene using these primers was carried out under the following parameters: 95°C for 2 min initial denaturation, followed by 40 cycles of 94°C x 30 s, 63°C x 30 s, 72°C x 45 s, and a final elongation of 72°C for 2 min . The resultant products were visualised by agar gel electrophoresis. Amplicons generated were cleaned using the Roche High Pure PCR clean-up kit and sequenced (Source Bioscience, Dublin, Ireland). The specificity of the primer pair was tested using DNA from a selection of cheese-associated Gram-positive and Gram-negative cultures, i.e., *Streptococcus thermophilus, Lactobacillus helveticus* DPC6865*, Propionibacterium freudenreichii* DPC6451 and *Lactococcus lactis* HP as well as *Escherchia coli* DPC6009*, Listeria monocytogenes* EGDe*, Salmonella typhimurium*

LT2 and *Bifidobacterium longum* DPC5697 (all strains were obtained from the Moorepark Culture Collection, Fermoy, Cork, Ireland).

To facilitate the quantification of *Thermus* by molecular means, a qPCR protocol was designed. Genomic DNA was extracted from *Thermus thermophilus* HB27 (DSMZ Culture Collection, Germany) using the PowerFood Microbial DNA extraction kit (MoBio Laboratories Inc.). A PCR product from within the polymerase I gene was generated using the genus-specific primers, as described above.

Purified amplicons were cloned into the pCR® 2.1-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad, California) in accordance with manufacturer's instructions. Following cloning, the complete construct was transformed into chemically competent TOP-10 *E. coli* cells (Invitrogen) and harvested on LB media containing 100 µg ml^{-1} ampicillin. The accuracy of the cloned amplicon was confirmed by restriction analysis and DNA sequencing. Quantative PCR standards were prepared following the linearization of plasmid DNA with *Pst*I restriction enzyme and quantification with the Nanodrop ND-1000 (Thermo Fisher Scientific Inc). A standard curve was then generated via a series of dilutions from 10^2 to 10^8 copies μl^{-1} DNA. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics Ltd.) was used for quantification according to the manufacturer's instructions. Each PCR reaction contained 5 µl Sybr green master mix (Roche Diagnostics Ltd.), 1 µl of both forward and reverse primer (7.5 pmol), 2 µl of DNA and was made up to a final volume of 10 µl with nuclease free sdH_2O. The PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 61°C for 15 s and elongation 72°C for 20 s. Assays were performed in triplicate. To facilitate quantification by

qPCR, we applied the formula of Quigley et al (31) to convert from copies μl^{-1} to cfu g^{-1} of cheese.

4.2.7. Cheese spiking studies

4.2.7.1. Cheese manufacture and analysis

Cheese manufacture incorporated three replicate trials consisting of four treatments (control and three tests), each of which required 454 kg of milk (i.e. a combined total of 5448 kg of milk). Three 10 kg rounds of cheese were produced per treatment. The scale and conditions used in this study were reflective of those used during commercial cheese manufacture. Starter cultures *S. thermophilus* (Defined Starter Mix, Laboratories Standa, Caen, France) and *Lb. helveticus* DPC6865 (Moorepark Culture Collection), were each grown overnight at 37°C in reconstituted low heat-skim milk powder, which had first been heat-treated at 90°C for 30 min. *Propionibacterium freudenreichii* DPC6451 (Moorepark Culture Collection) was grown for 3 days at 30°C in sodium lactate broth. *T. thermophilus* DPC6866 (Moorepark Culture Collection), obtained from a cheese with a pink defect, was grown in Castenholz broth at 60°C with shaking for 36 hours. Cells were collected by centrifugation at 14,000 *g* for 20 min, washed once to remove trace media and resuspended in sterile water. Raw milk was obtained from Teagasc, Moorepark dairy herd, standardised, pasteurised at 72°C for 15 s and pumped at 32°C into four individual cylindrical stainless steel vats with automated variable speed cutters and stirrers. This milk was employed to manufacture a continental-type cheese at pilot-scale level in Moorepark Technology Ltd (Fermoy, Cork, Ireland). To enumerate specific bacterial components, cheese samples were aseptically removed, placed in a stomacher bag, diluted 1:10 with sterile tri-sodium

citrate (2% w/v, Sigma Ltd., Arklow, Co. Wicklow, Ireland) and homogenised in a Seward Stomacher® 400 Lab System (Seward Ltd., West Sussex, United Kingdom) for 2 min. Further dilutions were prepared as required. Viable *S. thermophilus* were enumerated on M17 agar (Oxoid Ltd., Hampshire, United Kingdom) with 0.5% lactose (Oxoid Ltd.) at 42°C for 3 days. *Lb. helveticus* were enumerated on MRS agar (Oxoid Ltd.) adjusted to pH 5.4 at 37°C for 3 days under anaerobic conditions. PAB levels were enumerated on sodium lactate agar containing 40 μg ml^{-1} kanamycin (Sigma Ltd.) at 30°C for 7 days under anaerobic conditions. Non-starter lactic acid bacteria (NSLAB) were enumerated on *Lactobacillus* Selective Agar (LBS; Difco) at 30°C for 5 days aerobically. Details with respect to the manufacture of control and test cheeses can be found in Table 1. Enumeration of microbiological content, composition of cheeses and proteolysis were measured at various stages of ripening (Table S2). *T. thermophilus* was monitored using qPCR methods. To facilitate this, DNA was extracted from milk, whey or 10 ml cheese homogenate using the PowerFood DNA isolation kit as described above. Grated samples from cheeses were analysed for salt (32), moisture (33) and protein (34) after 11 days of manufacture, pH (35) was measured throughout ripening. The levels of nitrogen soluble at pH 4.6 (pH 4.6SN) were measured as described by Sheehan et al. (36). Free amino acid analysis was carried out on pH 4.6SN extract as described by Fenelon et al. (37).

4.2.7.2. Visual detection of pinking

Cheese wheels were examined visually throughout ripening for the formation of pink discolouration defect. Pink colour formation was quantified using a colorimeter (CR-400 Chroma Meter, Konica Minolta, Osakam, Japan) using Hunter,

L, a, b colour scale. The colour was measured using fresh sliced exposed cheese surface. The colorimeter was standardised using the white Konica Minolta Calibration Plate for the following colour space parameters Y, y, and x, as defined by the International Commission on Illumination. Hunter ***a*** (redness) values were recorded.

4.2.8. Statistical Analysis

A randomised complete block design that incorporated the four treatments and 3 blocks (replicate trials) was used for the analysis of response variables relating to the composition of cheeses, moisture, salt and protein, as well as starter bacteria, PAB, NSLAB, *T. thermophilus*, pH, pH4.6SN, Free Amino Acids (FAA) and apparent colour differences. Analysis of variance was carried out on data using the general linear model procedure of SAS (SAS Institute, Cary, NC, USA). The Tukey honestly significant difference test was used to determine the significance of difference between the means. The level of significance was determined at $p < 0.05$.

4.3. Results

4.3.1. Compositional sequencing reveals higher proportions of the genus *Thermus* in cheeses with a pink defect

Compositional (16S rDNA) sequencing was performed on DNA extracted from control (n=9) and pink defect (n=9) samples of a commercially produced continental-type cheese. Sequencing coverage was satisfactory for all samples (SI Appendix, Figure S1). Phylogenetic analysis established that the sequence reads corresponded to five different bacterial phyla (Figure 1a), i.e. Firmicutes, Proteobacteria, Bacteroides, Actinobacteria and Deinococcus-Thermus. Firmicutes and Deinococcus-Thermus dominated with less than 1% of assigned reads corresponding to other phyla. The proportions of Firmicutes present did not differ between control and defect samples. Reads corresponding to the phylum Deinococcus-Thermus were detected in defect-associated samples only (6%). When reads were assigned at the family level, eleven families were identified (Figure 1b). All reads from the phylum Deinococcus-Thermus were assigned to the family Thermaceae and, again, this was the only taxon for which significant differences were observed, i.e. 6% and 0% in defect and control, respectively. When these reads were assigned at genus level, 10 genera were identified (Figure 1c/SI Appendix, Table S1). Reads corresponding to Deinococcus-Thermus and *Thermaceae* were assigned to the genus *Thermus* and, again, this was the only taxonomic group for which there were significant differences ($P = 0.002$).

4.3.2. Shotgun metagenomic sequencing provides further insight into the *Thermus* population, and associated pathways, that are enriched in pink defect cheeses

A further 10 samples of continental-type cheese, i.e., 2 control cheeses and 8 pink defect cheeses, were selected for shotgun metagenomic sequencing. A total of 231,401,379 reads post quality filtering were obtained. Phylogenetic analysis revealed the presence of bacteria corresponding to three phyla, Firmicutes, Actinobacteria and Deinococcus-Thermus (SI Appendix, Figure S2). Firmicutes were again a dominant component across all samples but, in contrast to the previous compositional data, *Actinobacteria* were also present in high proportions across many samples (reflecting a deficiency in the binding of the 16S rDNA primers used for compositional sequencing to *Propionibacterium*), (SI Appendix, Table S2). *Deinococcus-Thermus* populations were again present in defect samples only (24 – 28% of assigned reads). These corresponded primarily to *Thermus* at the genus level, though sub-dominant populations corresponding to *Meiothermus* and *Deinococcus* were also detected (Figure 2a/SI Table S3). Shotgun analysis also allowed assignment at the species level, which revealed consistently high levels of *Lactobacillus helveticus*, *Streptococcus thermophilus* and, in many cases, *Propionibacterium freudenreichii* (Figure 2b). All three are starters used in the manufacture of this continental-type cheese. Several members of the *Thermus* genus were present, including *T. thermophilus*, *T. aquaticus, T. scotoductus, T. oshimai, T. sp RL* and *T. sp WG*. Of these, *T. thermophilus* dominated, corresponding to 5.9-7.03% of assigned reads (Figure 2b/SI Appendix, Table S4).

Functional analysis of this sequence data was performed with 95,827 genes being assigned across all samples (overview of KEGG pathways present in Figure S3). Unsurprisingly, given the presence of reads corresponding to *Thermus* in the defect samples exclusively, it was noted that genes responsible for the production of

carotenoids were identified in defect samples only (Figure 3). Notably, Raman spectra of samples from regions of pink discoloration within defect cheeses (Figure S4) revealed a peak at 1456 cm^{-1}, characteristic for lycopane (perhydro-transformed carotenoid from lycopene) (38) and is absent from non-pink regions from the same cheese. The pink layer also shows very strong peaks at 877 cm^{-1} and 990 cm^{-1} that are consistent with v1(PO_4^{3-}) of a phosphate salt. The localised distribution of prominent Raman peaks 990 cm^{-1} and 1456 cm^{-1} (carotenoid/phopshate salt; corresponding to red), 1441 cm^{-1} and 2840-2945 cm^{-1} (proteins; corresponding to blue) and 3060 cm^{-1} (lipids; corresponding to green) is shown in Figure 4.

4.3.3. Culture-independent confirmation of the presence of *Thermus* in Cheese

As a consequence of the association between *Thermus* and samples of cheeses containing the pink discolouration defect, attempts were made to isolate this bacterium, which is not regarded as being a typical cheese-associated genus, from the defect cheeses. Castenholz medium was employed as it has previously been shown to support the growth of strains of *Thermus* (39) but, due to its minimal nutrient content, was unlikely to support the growth of other genera associated with cheese. Use of this approach resulted in the successful isolation of a single *Thermus thermophilus* culture from a defect cheese only, however obtaining reliable and consistent counts of this culture, from defective cheeses, was problematic. To address this, a culture-independent quantitative PCR (qPCR)-based method was also developed to detect *Thermus*. A primer pair was designed with a view to selectively amplify the polymerase I gene of *Thermus,* assays with a broad variety of controls established the primers to be specific and confirmed the

absence of residual *Thermus* DNA from *Taq* preparations, thereby removing a potential confounding factor. Quantitative PCR analysis (of the cheeses used for 16S rDNA analysis) confirmed that *Thermus* was absent from the control cheeses and that defect cheeses contained on average 1.77 x 10^3 cfu g^{-1}. Sequencing of PCR amplicons from defect cheeses and from *Thermus* strains isolated from these cheeses revealed that the species in question was *T. thermophilus*. A representative defect cheese isolate, *T. thermophilus* DPC6866, was employed in subsequent studies.

4.3.4. Addition of *T. thermophilus* DPC6866 recreates the pink discolouration defect in cheeses

To establish definitively that *T. thermophilus* is responsible for the formation of pink defects in cheese, we produced cheese, at pilot scale level, following the production protocol typical of this continental cheese-type, to which *T. thermophilus* DPC6866 was added and compared the development of a pink discolouration relative to that of a control cheese. In each instance four cheeses were produced i.e. a control (C) cheese, which did not contain *T. thermophilus*, and three experimental (Exp) cheeses, all of which contained *T. thermophilus* at 10^6 cfu ml^{-1} but which contained different levels of starter bacteria. Exp1 contained starter cultures at standard inoculum levels, i.e., 0.055 % *L. helveticus* DPC6865 (10^8 cfu ml^{-1}), 0.11 % *S. thermophilus* (10^8 cfu ml^{-1}), 0.00088 % *P. freudenreichii* DPC6451 (10^8 cfu ml^{-1}). Exp2 differed from Exp1 by virtue of containing higher than normal inoculum levels of *L. helveticus* (0.11 %) while Exp3 also contained high inoculum levels of *L. helveticus* (0.11 %) but with lower inoculum levels of *S. thermophilus* (0.055 %) (Table 1). The numbers of the respective *S. thermophilus*, *Lb. helveticus*,

PAB and non-starter LAB (NSLAB) in the cheese were monitored throughout the cheese production and ripening (116 day) process and were in line with expectations (SI Text; SI Appendix, Table S5/S6, Figures S5-S9).

Visual examination of the cheeses revealed that the pinking defect was strongly evident in Exp 2 cheese. The defect was quantified using a Chroma Meter to determine Hunter ***a*** values, which determine the level of redness (+) to greenness (-) (40). Through the centre of the Exp 2 cheese there was a shift towards a more positive average value (i.e., more red) that was not evident in the control cheese (Table 2). These differences were first noted after day 116 of ripening and the relative difference in redness became more apparent by day 144. Indeed, the ***a*** values, at day 144, for Exp2 were significantly less negative than those of the control cheese (p=0.0009) cheese.

4.4. Discussion

Metagenomic sequencing revealed a potential association between higher levels of the genus *Thermus* and cheeses exhibiting a pink defect. *T. thermophilus* is a Gram negative, extremely thermophilic, aerobic, non-pathogenic microorganism (41). It has been associated strongly with hot water sources, including springs (42) and tap water (43, 44). The identification of *Thermus* sp. as a major component of the pink defect cheese microbiota highlights the merits of employing culture-independent strategies to investigate the biological basis for food defects. Representatives from this species can be difficult to culture and do not grow on the microbiological media routinely used to study or test cheese microbiota, thus explaining why this population has not previously been associated with the pinking phenomenon.

Bacteria from the phylum Deinococcus-Thermus are known for their resistance to extreme stresses, including radiation, oxidation, desiccation and high temperature. When cultured, they typically have a red or yellow pigment because of their ability to synthesize carotenoids (41), which often act as non-enzymatic antioxidants and may thereby play a role as cellular protectants (41). Interestingly, members of this phylum, *Deinococcus* species and *Meiothermus* species, have been associated with pink hue formation in various environments, including undesirable discolouration of paper in paper manufacture industries (45, 46). Also, an ancient terrace, referred to as “The Pink Terraces” which were recently re-discovered by geoscientists in New Zealand (Woods Hole Oceanographic Institution, MA, USA), emit a pink hue which has been attributed to the presence of *Thermus ruber* bacterium (47). Analysis of shotgun metagenomic data revealed the presence of *Thermus* genes involved in carotenoid biosynthesis in defect cheeses. More specifically, genes are

involved in the formation of lycopene, a red coloured pigment, and include *crtB* (phytoene synthase) and *crtI* (phytoene desaturase). Carotenoid production is a common feature of *Thermus* species and there have been a number of studies in which carotenoid biosynthesis homologs in Deinococcus-Thermus species, including *T. thermophilus* HB8 and *T. thermophilus* HB27 have been characterised (41, 48, 49). Notably, these observations are consistent with our detection, through Raman analysis, of a carotenoid-associated peak within the pink region of defect cheeses.

Following the detection of *Thermus* at higher levels in cheeses with a pink defect, a series of cheese trials were carried out to determine if *T. thermophilus* bacterium is indeed responsible for this phenomenon. Here we inoculated cheese with *T. thermophilus*, and with thermophilic starter bacteria at various levels. The levels of *T. thermophilus* introduced were consistent with that of a previous study which established that the inoculation of a milk supply with *T. thermophilus* N8, itself a dairy isolate, in the range of 5 - 100 CFU/ml milk prior to passaging through a tube heat exchanger resulted in the strain both adhering to and growing within the tube heat exchanger to levels in excess of 1.2×10^7 CFU/cm^2 even at high temperatures (83 °C). This study also describes heat exchangers as potential reservoirs for milk contamination (50). In addition to the high levels encountered in the aforementioned study, 10^6 CFU/ml of milk was chosen in order to promote 'pinking' to a greater extent than previously observed in commercially sourced cheeses.

Following production of the cheeses, no differences were noted in the chemical composition of the various cheeses. This is consistent with previous studies which

also failed to find a correlation between cheese compositional profiles, including profiles relating to moisture, salt, soluble nitrogen and free amino acids, and the development of the pink defect (5, 12, 51). Through an assessment based on colorimetric analysis, and from visual examination, greater levels of “pinking” were apparent in the cheeses in which *T. thermophilus* is present. Notably, in situations where the levels of starter cultures were adjusted, particularly where *Lb. helveticus* was increased, the pink colour formation was more intense.

The biological basis for the contribution of increased proportions of lactobacilli to the pinking phenomenon has yet to be determined but may be that other components of the cheese microbiota influence carotenoid production or modification to intensify the associated pink discolouration. This will be addressed in future studies. Further to this, improvements in sequencing databases may, in the future, result in a more detailed analysis of shotgun sequencing data. Regardless, these findings have the potential to lead to the development of strategies to understand the exact mechanism involved in *Thermus* mediated pink defect formation in cheese, the eventual goal being to eliminate the problem of pink discolouration in cheese and the associated economic loss.

4.5. References

1. **Daly DFM, McSweeney PLH, Sheehan J.** 2012. Pink discolouration defect in commercial cheese: a review. Dairy Science & Technology **92:**439-453.

2. **Morgan G.** 1933. 60. Discoloration in New Zealand Cheddar Cheese. Muddy, Pink and Bleached Defects. I. Bacteriological Investigations. Journal of Dairy Research **4:**226-237.

3. **Carini S, Lodi R, Giussani G.** 1979. Pink discoloration in Fontina cheese. Latte **4:**914-920.

4. **Giuliano G, Rosati C, Bramley PM.** 2003. To dye or not to dye: biochemistry of annatto unveiled. TRENDS in Biotechnology **21:**513-516.

5. **Martley FG, Michel V.** 2001. Pinkish colouration in Cheddar cheese–description and factors contributing to its formation. Journal of Dairy Research **68:**327-332.

6. **Park H, Reinbold G, Hammond E.** 1967. Role of propionibacteria in split defect of Swiss cheese. Journal of Dairy Science **50:**820-823.

7. **Pelaez C, Northolt MD.** 1988. Factors leading to pink discolouration of the surface of Gouda cheese. Netherlands Milk and Dairy Journal **42:**323-336.

8. **Shannon E, Olson N.** 1969. Rapid screening test to predict the tendency of lactic starter cultures to produce pink discoloration in Italian cheese. Journal of Dairy Science **52:**1678-1680.

9. **Govindarajan S, Morris H.** 1973. Pink discoloration in Cheddar cheese. Journal of Food Science **38:**675-678.

10. **Paramita A, Broome M.** 2008. 5th IDF Symposium on Cheese Ripening - Cheese 2008, Bern, Switzerland.

11. **Shumaker E, Wendorff W.** 2007. Factors affecting pink discoloration in Annatto-colored pasteurized process cheese. Journal of Food Science **63:**828-831.

12. **Betzold N.** 2004. Pink discolouration of Mozzerella cheese. University of Wisconsin.

13. **Bottazzi V, Cappa F, Scolari G, Parisi M.** 2000. Occurring of pink discolouration in Grana cheese made with one-strain starter culture / Comparsa di colorazione rossa in formaggio Grana prodotto con starter monocoltura. . Scienza e Tecnica Lattiero Casearia **51:**67-74.

14. **Shokralla S, Spall JL, Gibson JF, Hajibabaei M.** 2012. Next-generation sequencing technologies for environmental DNA research. Molecular Ecology **21:**1794-1805.

15. **Ercolini D.** 2013. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology. Applied and environmental microbiology **79:**3148-3155.

16. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2012. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. Applied and environmental microbiology **78:**5717-5723.

17. **Wolfe Benjamin E, Button Julie E, Santarelli M, Dutton Rachel J.** Cheese Rind Communities Provide Tractable Systems for In Situ and In Vitro Studies of Microbial Diversity. Cell **158:**422-433.

18. **Bokulich NA, Mills DA.** 2013. Facility-Specific “House” Microbiome Drives Microbial Landscapes of Artisan Cheesemaking Plants. Applied and environmental microbiology **79:**5214-5223.

19. **O'Sullivan DJ, Cotter PD, Sullivan OO, Giblin L, McSweeney PLH, Sheehan JJ.** 2015. Temporal and spatial differences in microbial composition during the manufacture of a Continental-type cheese. Applied and Environmental Microbiology.

20. **Quigley L, O'Sullivan O, Beresford T, Paul Ross R, Fitzgerald G, Cotter P.** 2012. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. Journal of Applied Microbiology **113:**96-105.

21. **Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, O'Toole PW.** 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res **38:**e200.

22. **Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM.** 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res **37:**D141-145.

23. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Tumbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods **7:**335-336.

24. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-410.

25. **Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, Peplies J, Glockner FO.** 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res **35:**7188-7196.

26. **Huson DH, Auch AF, Qi J, Schuster SC.** 2007. MEGAN analysis of metagenomic data. Genome Res **17:**377-386.

27. **Urich T, Lanzen A, Qi J, Huson DH, Schleper C, Schuster SC.** 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. PloS one **3:**e2527.

28. **Kruskal WH, Wallis AW.** 1952. Use of Ranks in One-Criterion Variance Analysis. Journal of the American Statistical Association **Vol. 47:**pp. 583-621.

29. **Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M.** 2014. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res **42:**D199-205.

30. **Quigley L, McCarthy R, O'Sullivan O, Beresford TP, Fitzgerald GF, Ross RP, Stanton C, Cotter PD.** 2013. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. Journal of Dairy Science **96:**4928-4937.

31. **IDF.** 1988. Cheese and processed cheese: Determination of chloride content (potentiometric titration method). International Standards 4a Brussels, Belgium: International Dairy Federation.

32. **IDF.** 1982. Determination of total solids content (cheese and processed cheese), , International Standards 4a Brussels, Belgium: International Dairy Federation.

33. **IDF.** 1993. Milk: Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content., International Standards 20b Brussels, Belgium: International Dairy Federation.

34. **STANDARDS IB.** 1976. British Standard Methods for Chemical Analysis of Cheese: Determination of pH value., London: British Standards Institute.

35. **Sheehan JJ, Fenelon MA, Wilkinson MG, McSweeney PL.** 2007. Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. International Dairy Journal **17:**704-716.

36. **Fenelon M, O'Connor P, Guinee T.** 2000. The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. Journal of Dairy Science **83:**2173-2183.

37. **Marshall CP, Olcott Marshall A.** 2010. The potential of Raman spectroscopy for the analysis of diagenetically transformed carotenoids, vol. 368.

38. **Brock TD, Freeze H.** 1969. Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile. Journal of bacteriology **98:**289-297.

39. **Wadhwani R, McMahon D.** 2012. Color of low-fat cheese influences flavor perception and consumer liking. Journal of Dairy Science **95:**2336-2346.

40. **Tian B, Hua Y.** 2010. Carotenoid biosynthesis in extremophilic Deinococcus–Thermus bacteria. Trends in Microbiology **18:**512-520.

41. **Pěčková M.** 1991. Properties of a hyperthermophilic bacterium (*Thermus* sp.) isolated from a Carlsbad spring. Folia microbiologica **36:**515-521.

42. **Pask-Hughes R, Williams R.** 1975. Extremely thermophilic Gram-negative bacteria from hot tap water. Journal of General Microbiology **88:**321-328.

43. **Brock TD, Boylen KL.** 1973. Presence of Thermophilic Bacteria in Laundry and Domestic Hot-Water Heaters. Applied Microbiology **25:**72-76.

44. **Ekman J, Kosonen M, Jokela S, Kolari M, Korhonen P, Salkinoja-Salonen M.** 2007. Detection and quantitation of colored deposit-forming Meiothermus spp. in paper industry processes and end products. Journal of Industrial Microbiology & Biotechnology **34:**203-211.

45. **Kolari M, Nuutinen J, Rainey F, Salkinoja-Salonen M.** 2003. Colored moderately thermophilic bacteria in paper-machine biofilms. Journal of Industrial Microbiology and Biotechnology **30:**225-238.

46. **Mountain B.** 2012. Life in Hotsprings, The Encyclopedia of New Zealand - Sinter, TeAra.

47. **Tabata K, Ishida S, Nakahara T, Hoshino T.** 1994. A carotenogenic gene cluster exists on a large plasmid in Thermus thermophilus. FEBS Letters **341:**251-255.

48. **Fujita A, Misumi Y, Honda S, Sato T, Koyama Y.** 2013. Construction of new cloning vectors that employ the phytoene synthase encoding gene for color screening of cloned DNA inserts in Thermus thermophilus. Gene **527:**655-662.

49. **Langeveld L.P.M, R.M.G.E vM-Q, A.H. W, R. W, J.S. W.** 1995. Adherence, growth and release of bacteria in a tube heat exchanger for milk. Netherlands Milk and Dairy Journal **49:**201 - 220.

50. **Chang S-F, Ayres JW, Sandine WE.** 1985. Analysis of cheese for histamine, tyramine, tryptamine, histidine, tyrosine, and tryptophane. Journal of Dairy Science **68:**2840-2846.

Table 1: Details and differences between manufacture of continental-type spiked cheese trials.

Treatment	Control Cheese	Experiment 1 Cheese	Experiment 2 Cheese	Experiment 3 Cheese
Milk Volume	454 kg	454 kg	454 kg	454 kg
Starter Culture (% inoculum)				
Streptococcus thermophilus	0.11%	0.11%	0.11%	0.055%
Lactobacillus helveticus	0.055%	0.055%	0.11%	0.11%
Propionibacterium freudenreichii	0.00088%	0.00088%	0.00088%	0.00088%
Test Bacterium cfu ml^{-1}				
Thermus thermophilus	0	10^6	10^6	10^6
Curd Formation	As Standard			
Cook	0.5°C min to 45°C 1°C min to 53°C			
Drain pH	pH 6.30			
Curd Handling	Pre-press and mould			
Salting Method	Brine			
Cheese Size	10kg			
Cool Room Ripening	8.5°C x 10 days			
Hot Room Ripening	22°C x 7 weeks			
Ripening Regime	4.5°C after hot room step			

Table 2: Effect of treatment on colour properties as determined by Hunter *L, a, b*, dimensions

Cheese Sample	Area Assessed	a value 144 d
Control	Top	-2.22
	Side	-2.17
	Base	-2.32
	Centre	-2.38
Exp 1	Top	-2.21
	Side	-2.28
	Base	-2.21
	Centre	-1.95
Exp 2	Top	-2.18
	Side	-2.16
	Base	-2.10
	Centre	-1.34*
Exp 3	Top	-2.14
	Side	-2.35
	Base	-2.13
	Centre	-1.82

a values indicate formation of redness colour. The results are those taken from 144 d old cheeses

* Statistically significant difference compared to control cheese p= 0.0009.

Data presented in this table are means for three replicate trials.

Figure 1: Bacterial composition of defect and control cheeses as determined by 16S rDNA sequencing. 16S rDNA sequences assigned according to MEGAN using the Silva database at the (a) phylum, (b) family and (c) genus levels in continental-type cheese affected by the pink discolouration defect and corresponding control cheeses (n=18).

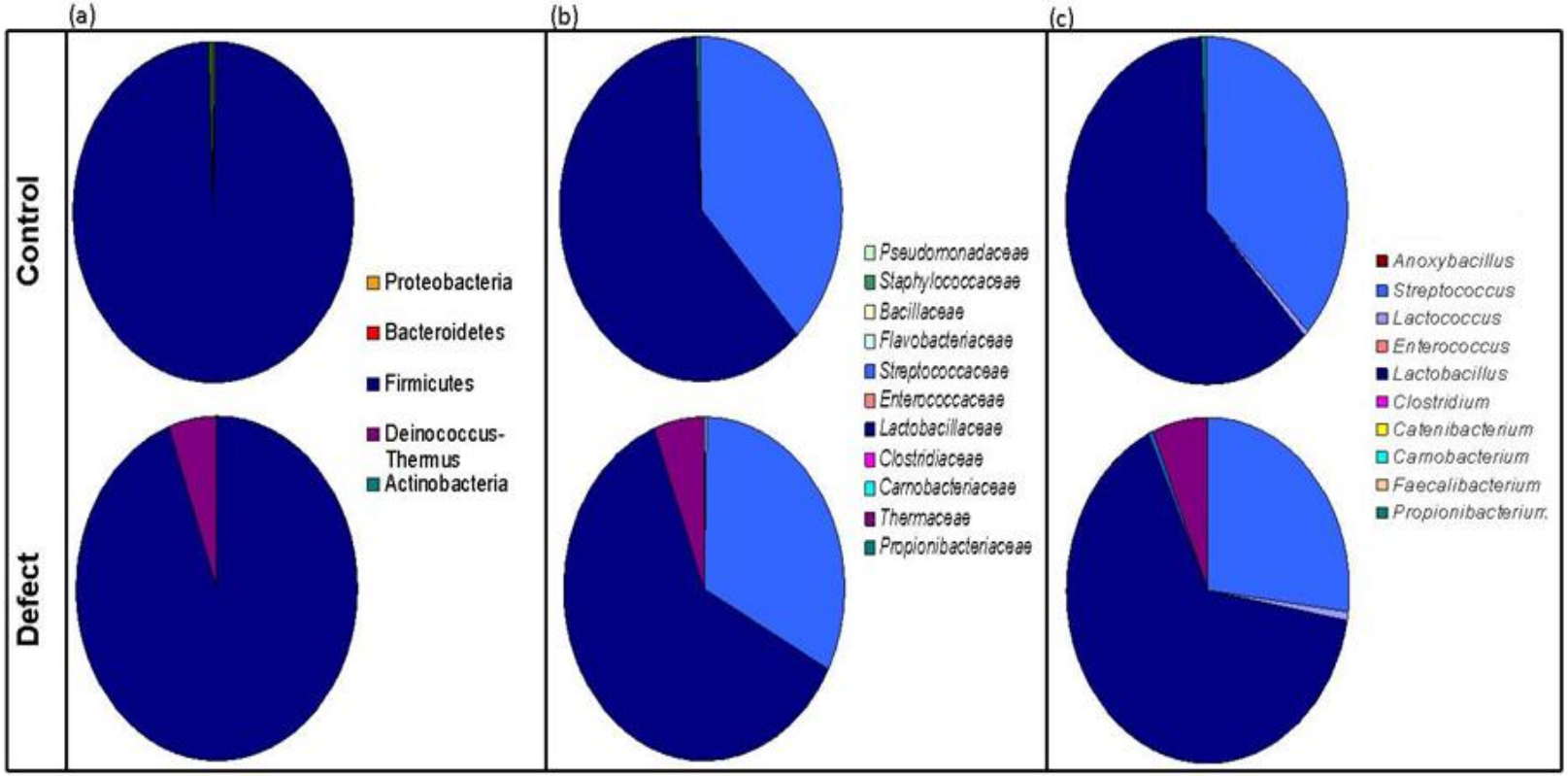
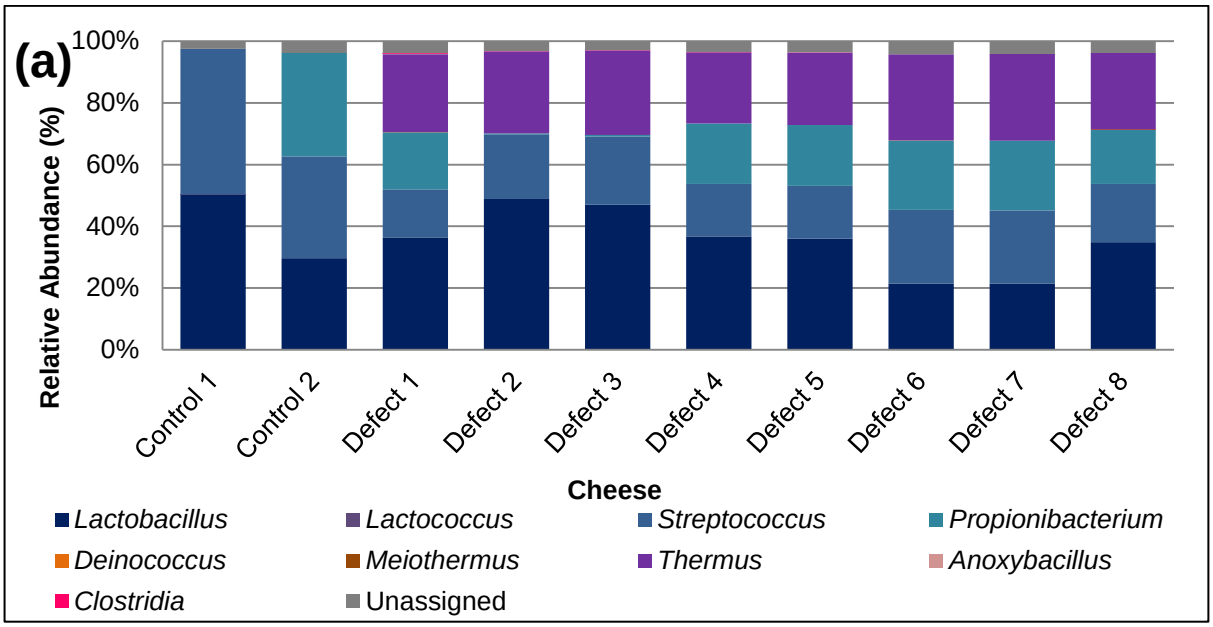


Figure 2: Bacterial composition of defect and control cheeses as determined by shotgun metagenomic sequencing. Sequences assigned according to MEGAN at the (a) genus and (b) species levels for cheeses affected by the pink discolouration defect and corresponding control cheeses (n=10). At species level, unassigned populations have been omitted.



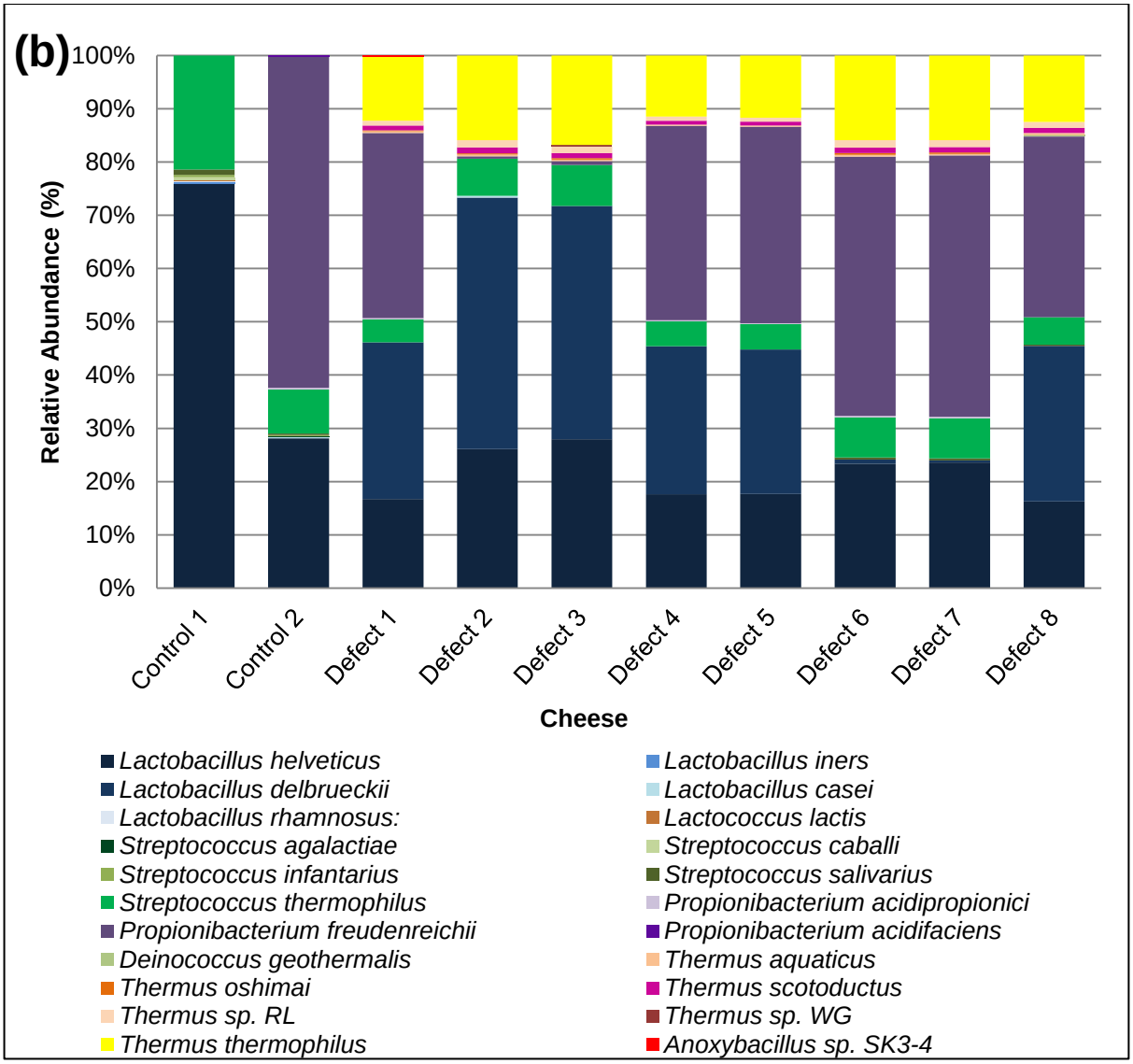
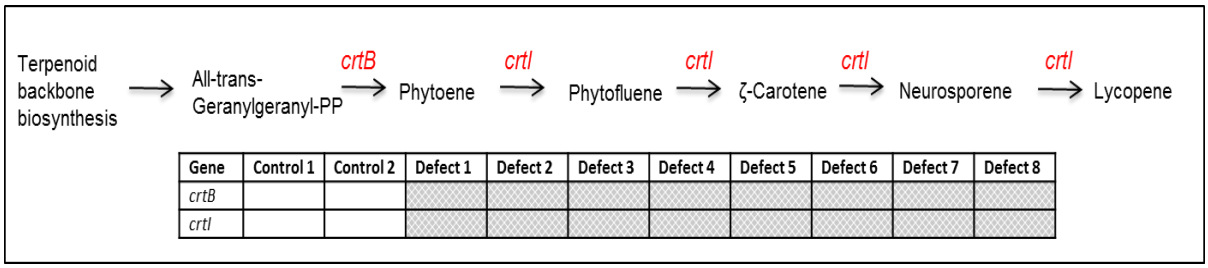
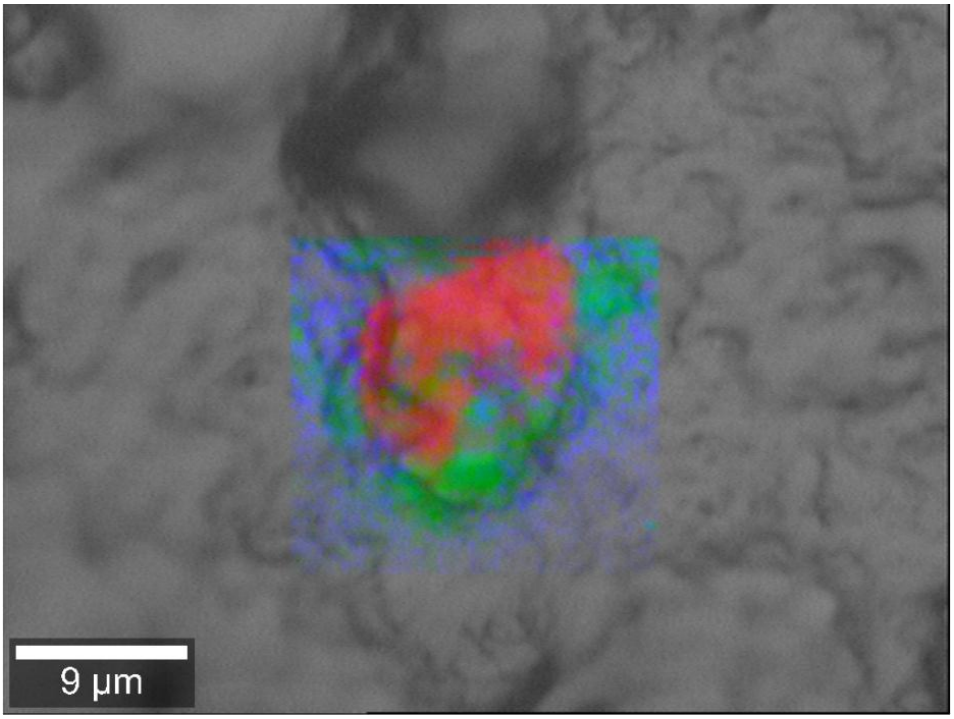

(b)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Abundance (%)
Control 1
Control 2
Defect 1
Defect 2
Defect 3
Defect 4
Defect 5
Defect 6
Defect 7
Defect 8
Cheese
Lactobacillus helveticus
Lactobacillus delbrueckii
Lactobacillus rhamnosus:
Streptococcus agalactiae
Streptococcus infantarius
Streptococcus thermophilus
Propionibacterium freudenreichii
Deinococcus geothermalis
Thermus oshimai
Thermus sp. RL
Thermus thermophilus
Lactobacillus iners
Lactobacillus casei
Lactococcus lactis
Streptococcus caballi
Streptococcus salivarius
Propionibacterium acidipropionici
Propionibacterium acidifaciens
Thermus aquaticus
Thermus scotoductus
Thermus sp. WG
Anoxybacillus sp. SK3-4

Figure 3: Carotenoid biosynthesis pathway genes detected in cheeses exhibiting a pinking defect. The detection of reads corresponding to the *crtB* and *crtI* genes in specific cheeses is indicated by the shaded boxes



Gene	Control 1	Control 2	Defect 1	Defect 2	Defect 3	Defect 4	Defect 5	Defect 6	Defect 7	Defect 8
crtB										
crtI										

Figure 4: Overlay of intensity image of the studied cheese matrix (grey) and the maps of the chemical composition obtained from local Raman spectral analysis of a pink discolored region of a defect cheese: red - carotenoid (lycopane); blue - proteins; green - lipids



4.7. Appendices

Supplementary Tables:

Table S1: List of 16S rRNA reads assigned at genus level to control and defect cheeses

Genus	Control	Defect
Anoxybacillus	0	36
Streptococcus	17635	12842
Lactococcus	245	284
Enterococcus	17	7
Lactobacillus	29291	31187
Clostridium	17	0
Catenibacterium	0	138
Carnobacterium	19	6
Thermus	23	3063
Propionibacterium	228	35

Table S2: BLAST of degenerate primers used in 454 compositional sequencing against *P. freudenreichii* subsp. *shermanii* genome. Partial identity of the reverse primer to the 16S rRNA sequence of *P. freudenreichii* subsp. *shermanii* may affect primer recognition and consequently reduce detection capabilities. This explains the differences between *Propionibacteria* populations detected *via* compositional and shotgun sequencing.

Primer	Sequence	BLAST Template	Max Score	Total Score	Query Cover	E-value	Identity	Accession
Forward Primer	AYTGGGYDTAAAGNG	*P. freudenreichii*	No Similarity					
V5-Reverse	CCGTCAATTYYTTTRAGTTT	*P. freudenreichii*	31.2	47.1	100%	0.01	85%	LN624749.1

Table S3: Shotgun metagenomic sequences assigned at genus level to control and defect cheeses

Genus	**Control 1**	**Control 2**	**Defect 1**	**Defect 2**	**Defect 3**	**Defect 4**	**Defect 5**	**Defect 6**	**Defect 7**	**Defect 8**
Lactobacillus	1936	1963	4112	4193	3826	3897	3791	1993	1970	4145
Lactococcus	4	0	0	0	0	0	0	0	0	0
Streptococcus	1819	2180	1756	1794	1800	1802	1798	2220	2178	2248
Propionibacterium	0	2211	2094	29	35	2085	2084	2085	2087	2087
Deinococcus	0	0	0	0	0	0	0	0	0	21
Meiothermus	0	0	11	0	0	0	0	10	0	0
Thermus	0	0	2843	2274	2231	2444	2465	2589	2582	2947
Anoxybacillus	0	0	33	0	0	0	0	0	0	0
Clostridia	0	0	13	10	9	10	10	0	0	0
Unassigned	95	254	439	274	238	378	383	398	381	457
Sum	3759	6354	10862	8300	7901	10238	10148	8897	8817	11448

Table S4: Shotgun metagenomic sequences assigned at species level to control and defect cheese

Species	Control 1	Control 2	Defect 1	Defect 2	Defect 3	Defect 4	Defect 5	Defect 6	Defect 7	Defect 8
Lb. helveticus	958	959	965	962	955	964	958	957	958	959
Lb. iners	4	0	0	0	0	0	0	0	0	0
Lb. delbrueckii	0	0	1695	1739	1498	1518	1467	33	16	1716
Lb. casei	0	7	0	11	0	0	0	0	0	0
Lb. rhamnosus	3	0	0	0	0	0	0	0	0	0
L. lactis	3	0	0	0	0	0	0	0	0	0
S. agalactiae	0	6	0	0	0	0	0	0	0	0
S. caballi	5	0	0	0	0	0	0	0	0	0
S. infantarius	6	8	0	0	0	0	0	0	0	0
S. salivarius	13	10	0	0	0	0	0	16	16	16
S. thermophilus	270	280	252	256	263	256	256	309	307	301

P. acidipropionici	0	11	11	0	0	11	11	11	11	0
P. freudenreichii	0	2119	2004	18	24	1995	1995	1995	1994	1996
P. acidifaciens	0	8	0	0	0	0	0	0	0	0
D. geothermalis	0	0	0	0	0	0	0	0	0	17
T.aquaticus	0	0	19	12	11	12	13	13	12	25
T. oshimai	0	0	12	8	9	0	0	14	11	0
T. scotoductus	0	0	50	42	33	40	38	43	42	56
T. sp. RL	0	0	54	48	43	44	40	56	51	64
T. sp. WG	0	0	0	0	8	0	0	0	0	0
T. thermophilus	0	0	692	587	575	626	633	653	648	736
Anoxybacillus sp. SK3-4	0	0	14	0	0	0	0	0	0	0
Unassigned	2592	3200	5533	4891	4720	5150	5120	5195	5132	6019
Sum	1262	3408	5768	3683	3419	5466	5411	4100	4066	5886

Table S5: Assessment carried out at different stages of manufacture and ripening

Ripening Time (days)	Stages of Ripening	Sample Type	Microbiological Analysis	Compositional Analysis
0	Day of manufacture	Milk, Wey, Curd	Tt	pH
1	After Brining	Cheese	Tt, St, Lh, PAB	pH, Moisture, Salt, Proteins, pH4.6SN, FAA
11	After 10 days at cool room ripening (8.5°C)	Cheese	Tt, St, Lh, PAB, NSLAB	pH, Moisture, Salt, Proteins, pH4.6SN, FAA
46	After 5 weeks at warm room ripening (22°C)	Cheese	Tt, St, Lh, PAB, NSLAB	pH, pH4.6SN, FAA, visual examination
60	End of warm room ripening (22°C)	Cheese	Tt, PAB, NSLAB	pH, pH4.6SN, FAA, visual examination
88	After 1 month in cold room (4.5°C)	Cheese	Tt, NSLAB	pH, pH4.6SN, FAA, visual examination
116	After 2 months in cold room (4.5°C)	Cheese	Tt, NSLAB	pH, pH4.6SN, FAA, visual examination
144	After 3 months in cold room (4.5°C)	Cheese	Tt	pH, pH4.6SN, FAA, visual examination

Tt - Thermus thermophilus; St – *Streptococcus thermophilus*; Lh – L*actobacillus helveticus*; PAB – Propionic Acid Bacteria; NSLAB – Non-starter lactic acid bacteria; pH4.6SN – pH4.6 soluble nitrogen FAA – Free Amino Acid.

Table S6: Composition of cheeses at 11 days post manufacture

	pH	% Moisture	% Salt	% Protein
Control	5.21	41.10	1.36	24.931
Exp 1	5.24	40.80	1.25	25.271
Exp 2	5.21	41.50	1.22	25.723
Exp 3	5.23	40.94	1.28	24.804

Supplementary Figures:

Figure S1: 16S rRNA sequencing reads analysis. 16S reads per cheese ≥3,500 (average number of reads per sample was 3960). Rarefaction curve of α-diversity, represented by Shannon indices, for all samples sequenced confirmed that satisfactory coverage was achieved (Figure S1). Sequence data has been uploaded to European Nucleotide Archive (ENA) accession number PRSEB6952.

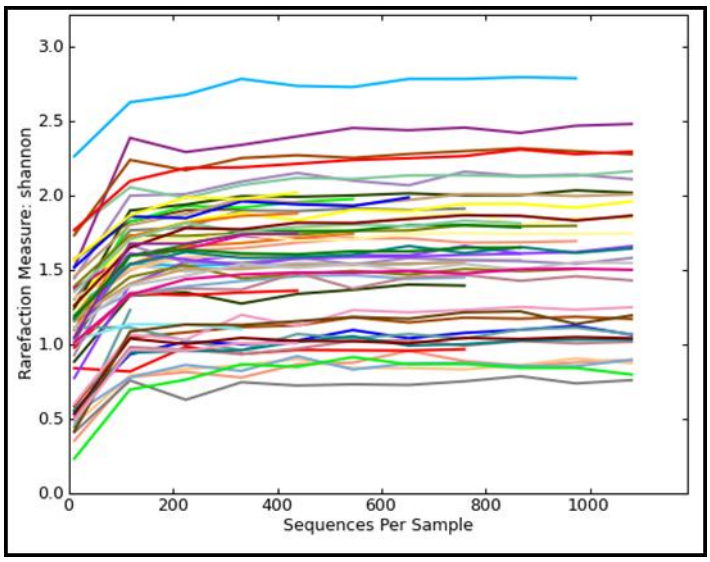


Figure S2: Bacterial composition of defect and control cheese as determined by shotgun metagenomic sequencing. Sequences assigned according to MEGAN at the Phylum level for cheese affected by the pink discolouration defect and corresponding control cheeses.

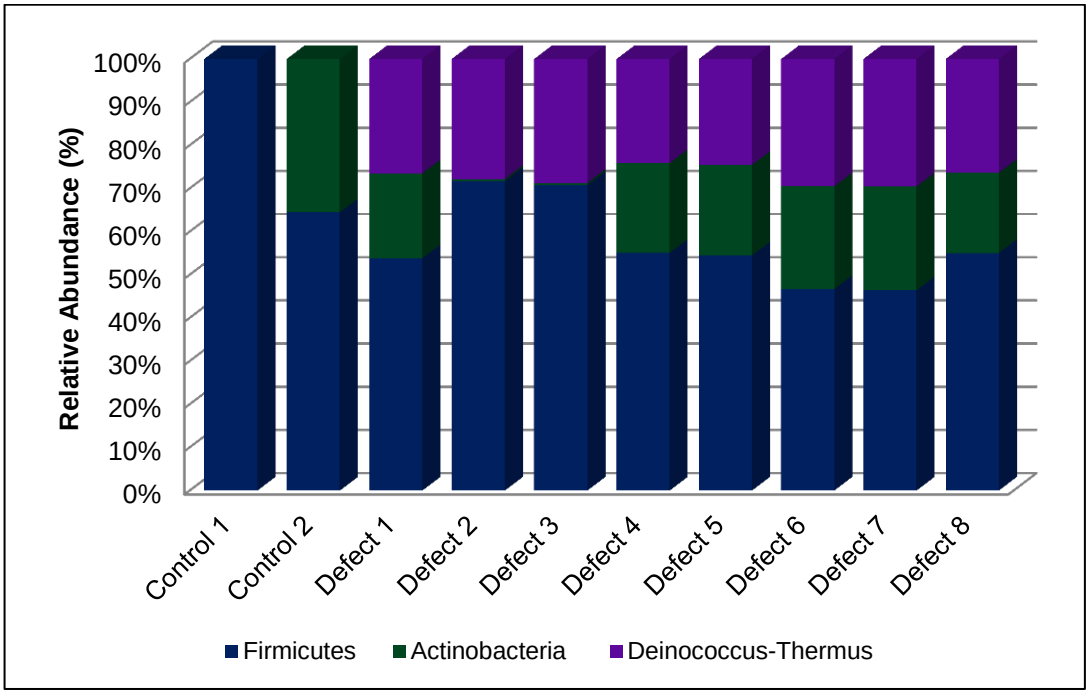


Figure S3: Breakdown of KEGG pathways present. Bar graph data is represented in percentage of assigned reads

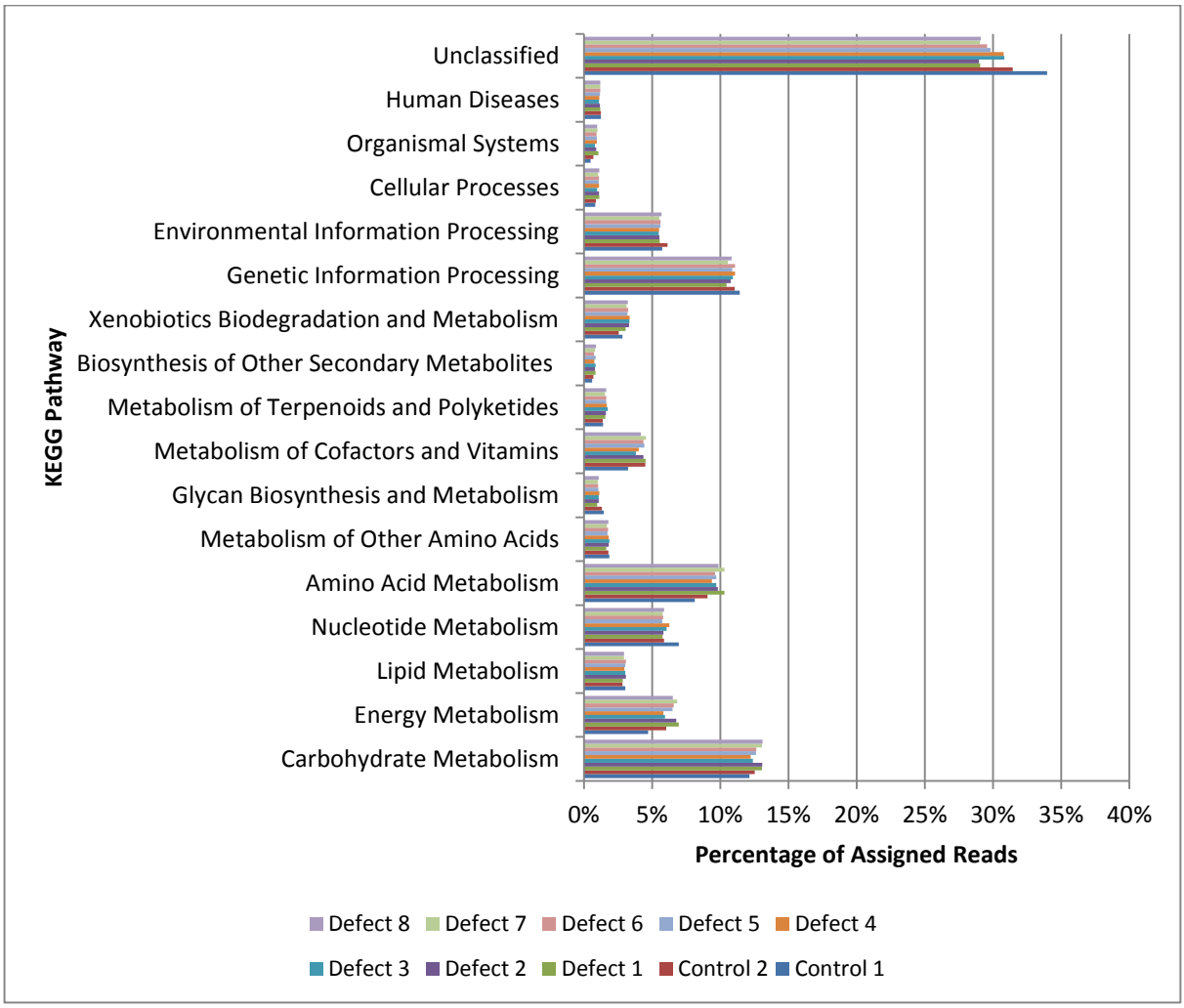
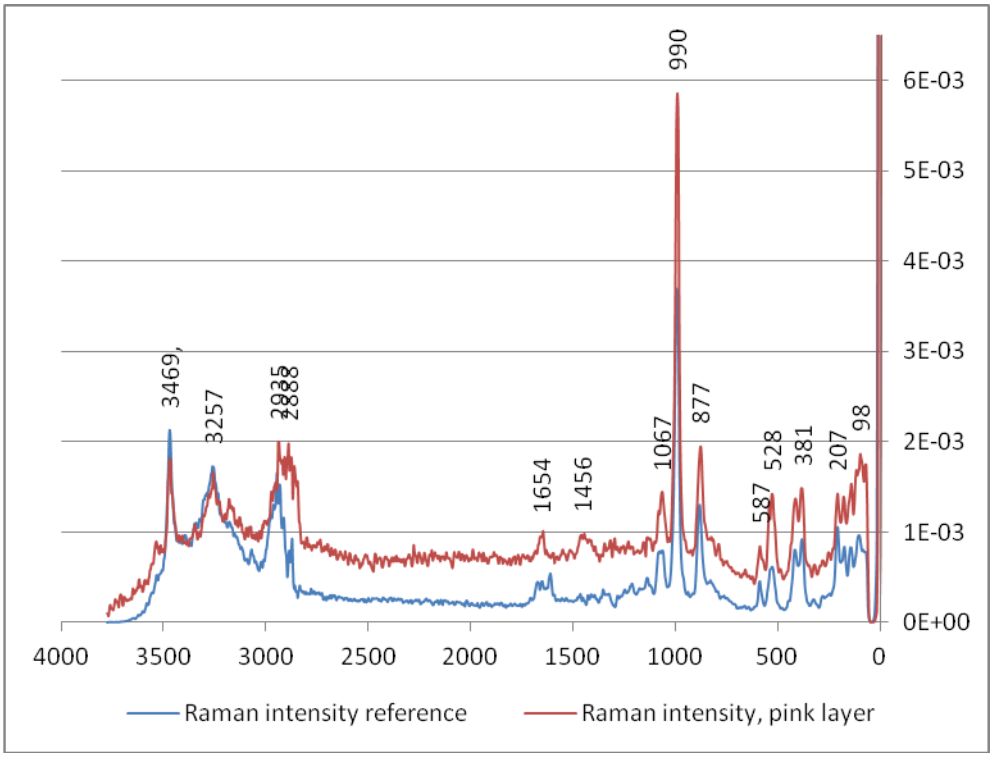


Figure S4: Vibrational characteristics of biomolecules in natural cheese in the pink area (red line) and outside the pink area (blue) line, Raman spectra recorded at 532 nm.



SI Text

Results:

Microbiological content and composition of cheeses and proteolysis were measured at various stages of ripening as detailed in Table S5.

Starter, PAB and NSLAB viability during cheese ripening

Mean viable cell numbers of *S. thermophilus* were determined to be 10^7 cfu g^{-1} at day 1 of ripening in control, exp 1 and exp 2 cheeses and at 10^6 cfu g^{-1} in Exp3 cheese, which correlates with levels of starter *S. thermophilus* inoculated into the cheese milk. There was a significant increase in numbers of *S. thermophilus* between 1 da and 11 d of ripening (p=0.0063), however, thereafter there was no significant change (Figure S5), but there were no significant differences between treatments. *Lb. helveticus* numbers were 1 x 10^6 cfu g^{-1} at 1 d ripening, in control and exp 1 cheese, while Exp2 and Exp3 cheese contained 5 x 10^6 cfu g^{-1}, again reflecting the different levels of *Lb. helveticus* starter added. The changes observed in levels of *Lb. helveticus* during cheese production were not significant. Counts of PAB increased significantly until 46 d ripening (p<0.0001) (Figure S5), however they did not differ significantly between treatments. Viable NSLAB numbers increased significantly until the end of warm room ripening (Figure S5) (p<0.0001). We observed a significant difference in the levels of NSLAB between control cheese and exp 2 cheese (p=0.0438) and control cheese and exp 3 cheese at 60 d ripening (p=0.0225). Using culture-independent qPCR, we determined the levels of *T. thermophilus* present in the inoculated milk, lost in whey, and retained in curd, as well as throughout ripening (Figure S6). We established that *Thermus* was present at 10^6 cfu ml^{-1} in milk after 1 h inoculation (sampled prior to rennet addition). There

was some loss of *T. thermophilus* in whey, i.e. 10^2 cfu ml^{-1}, however, considerable levels were retained within the curd (10^5 cfu g^{-1}). Control cheeses, which were not spiked with *T. thermophilus*, were also assessed and were found not to contain *Thermus* (data not shown), establishing that no natural contamination, or cross-contamination, occurred during production. Slight numerical increases in the levels of *T. thermophilus* were noted during hot room ripening, however these were not significant. Following transfer to the cold room for continued ripening, we observed a slight decrease in the levels of *T. thermophilus* to 10^4 cfu g^{-1}. This was consistent across all three experimental cheeses (Figure S6).

Composition of cheeses

The gross composition of cheeses at 11 d ripening was assessed and is summarised in Table S1. All cheeses had statistically similar pH values, levels of moisture, salt distribution and protein. The consistency of these results between cheeses and cheese trials indicate good repetition across each day of manufacture i.e. no significant differences were detected between these variables. Significant increases in pH (Figure S7), pH 4.6SN (soluble nitrogen) (Figure S8) and total FAA (p<0.0001 for all three parameters assessed) were observed throughout ripening. The concentrations of individual FAAs (mg kg^{-1} of cheese) in all cheeses at 144 d of ripening are shown in Figure S9. The FAAs present at greatest concentrations in the cheeses at most ripening times were glutamic acid, valine, leucine, lysine and proline, and were in line with that expected in Continental-type cheeses (1).

Figure S5: Counts of ripening bacteria, *Lactobacillus helveticus* (Lh), *Streptococcus thermophilus* (St), propionic acid bacteria (PAB) and non-starter lactic acid bacteria (NSLAB) throughout ripening ■ 1d, ▩11d, ▧46 d, ◫60 d, ▨88 d, ▤ 116 d.

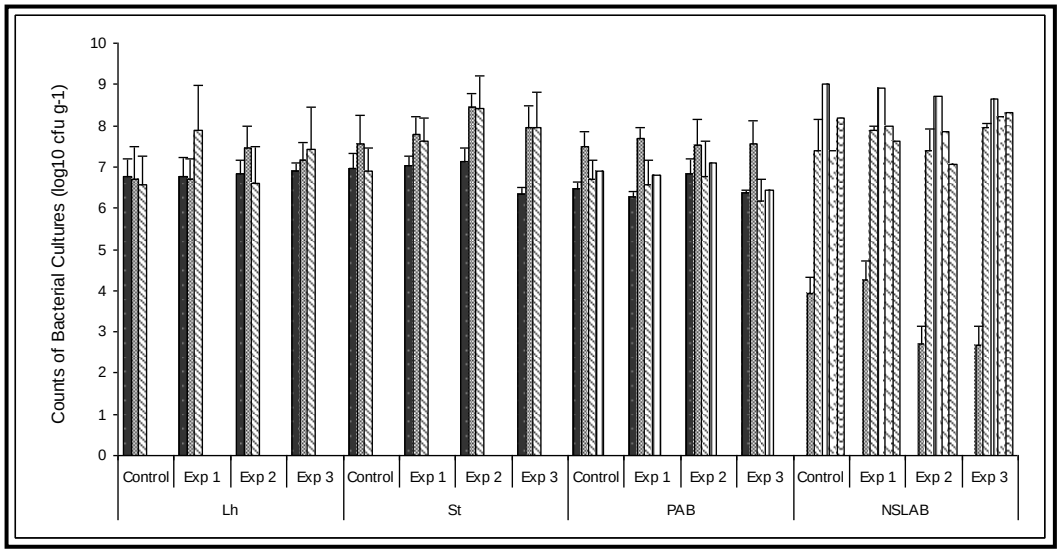


Figure S6: *Thermus thermophilus* levels, as determined by qPCR, throughout manufacture. M-inoculated milk, W-whey, C-curd. Experimental cheese 1 ▩, experimental cheese 2 ▨, experimental cheese 3 ▩.

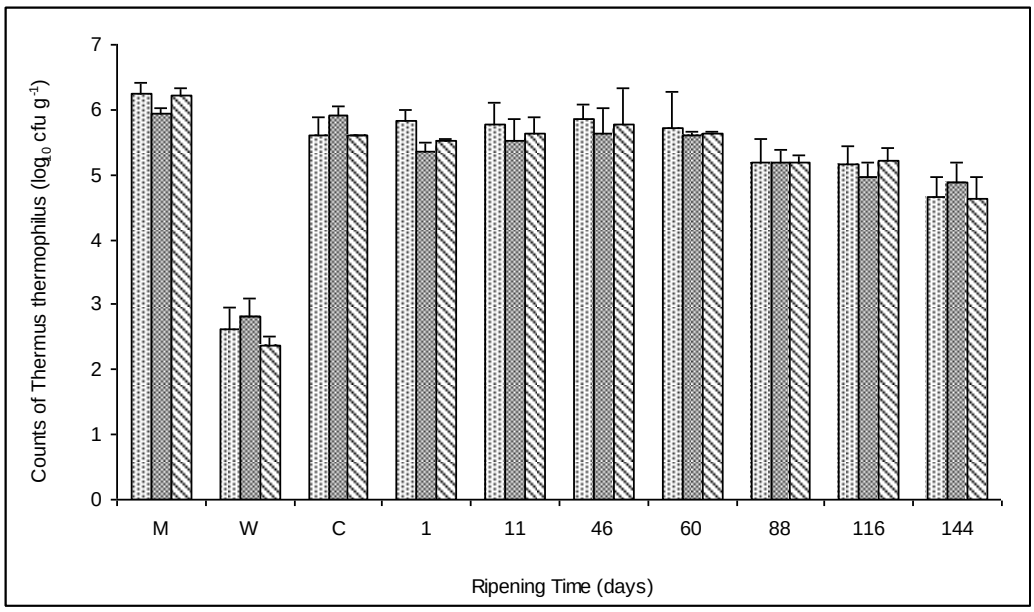


Figure S7: The effect of different treatments on cheese pH over ripening. Control cheese —◆—, experiment 1 cheese ---■---, experiment 2 cheese —▲- - and experiment 3 cheese —✕—.

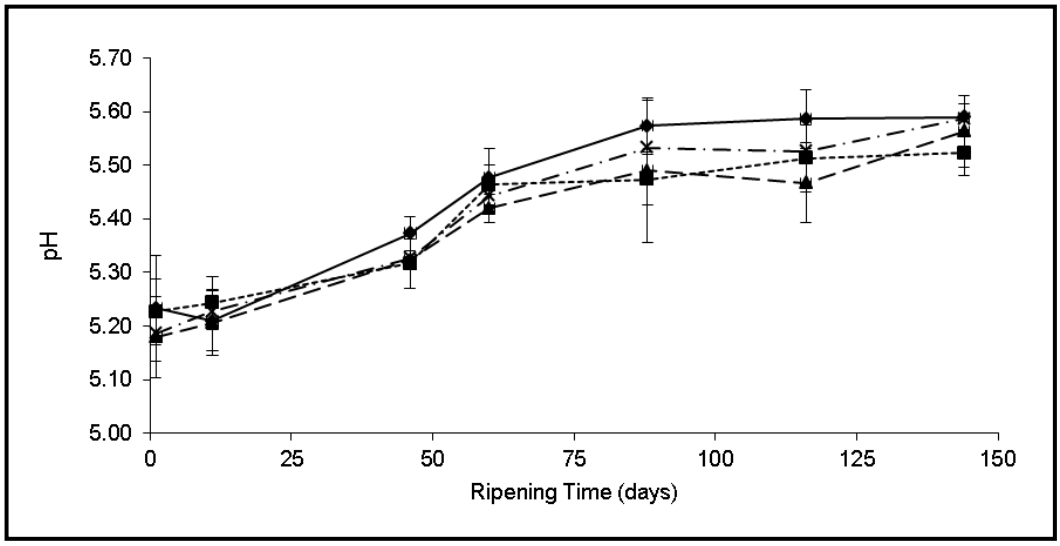


Figure S8: The effect of different experimental set-up on cheese % pH4.6 soluble nitrogen over ripening time. Control cheese —◆—, experiment 1 cheese ---■---, experiment 2 cheese —▲- and experiment 3 cheese —×—.

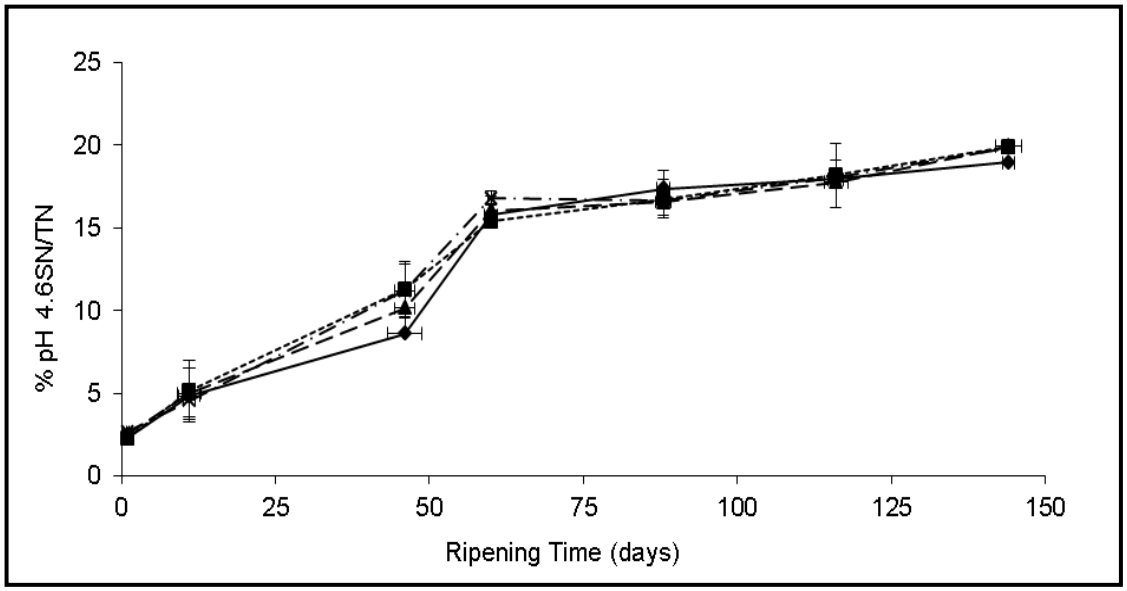
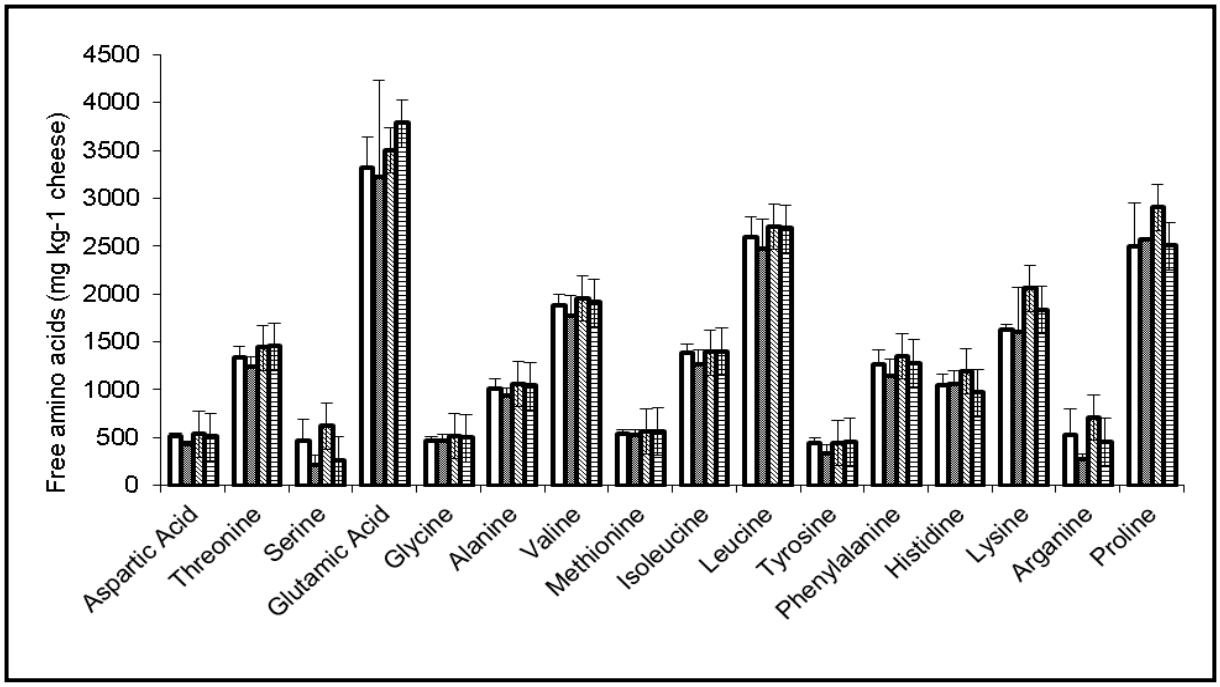


Figure S9: The effect of different experimental set-up on free amino acid levels after 144 days ripening. Control cheese ☐, experiment cheese 1 ▩, experiment cheese 2 ▧, experiment cheese 3 ▤.



Supplementary References

1. **Daly DFM, McSweeney PLH, Sheehan J.** 2012. Pink discolouration defect in commercial cheese: a review. Dairy Science & Technology **92:**439-453.
2. **Morgan G.** 1933. 60. Discoloration in New Zealand Cheddar Cheese. Muddy, Pink and Bleached Defects. I. Bacteriological Investigations. Journal of Dairy Research **4:**226-237.
3. **Carini S, Lodi R, Giussani G.** 1979. Pink discoloration in Fontina cheese. Latte **4:**914-920.
4. **Giuliano G, Rosati C, Bramley PM.** 2003. To dye or not to dye: biochemistry of annatto unveiled. TRENDS in Biotechnology **21:**513-516.
5. **Martley FG, Michel V.** 2001. Pinkish colouration in Cheddar cheese–description and factors contributing to its formation. Journal of Dairy Research **68:**327-332.
6. **Park H, Reinbold G, Hammond E.** 1967. Role of propionibacteria in split defect of Swiss cheese. Journal of Dairy Science **50:**820-823.
7. **Pelaez C, Northolt MD.** 1988. Factors leading to pink discolouration of the surface of Gouda cheese. Netherlands Milk and Dairy Journal **42:**323-336.
8. **Shannon E, Olson N.** 1969. Rapid screening test to predict the tendency of lactic starter cultures to produce pink discoloration in Italian cheese. Journal of Dairy Science **52:**1678-1680.
9. **Govindarajan S, Morris H.** 1973. Pink discoloration in Cheddar cheese. Journal of Food Science **38:**675-678.
10. **Paramita A, Broome M.** Pink discolouration in Romano style cheese – role of a-dicarbonyls, p. *In* (ed),
11. **Shumaker E, Wendorff W.** 2007. Factors affecting pink discoloration in Annatto-colored pasteurized process cheese. Journal of Food Science **63:**828-831.
12. **Betzold N.** 2004. Pink discolouration of Mozzerella cheese.University of Wisconsin.
13. **Bottazzi V, Cappa F, Scolari G, Parisi M.** 2000. Occurring of pink discolouration in Grana cheese made with one-strain starter culture / Comparsa di colorazione rossa in formaggio Grana prodotto con starter monocoltura. . Scienza e Tecnica Lattiero Casearia **51:**67-74.
14. **Shokralla S, Spall JL, Gibson JF, Hajibabaei M.** 2012. Next-generation sequencing technologies for environmental DNA research. Molecular Ecology **21:**1794-1805.
15. **Ercolini D.** 2013. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology. Applied and Environmental Microbiology **79:**3148-3155.
16. **Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.** 2012. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. Applied and Environmental Microbiology **78:**5717-5723.
17. **Wolfe Benjamin E, Button Julie E, Santarelli M, Dutton Rachel J.** Cheese Rind Communities Provide Tractable Systems for In Situ and In Vitro Studies of Microbial Diversity. Cell **158:**422-433.
18. **Bokulich NA, Mills DA.** 2013. Facility-Specific "House" Microbiome Drives Microbial Landscapes of Artisan Cheesemaking Plants. Applied and Environmental Microbiology **79:**5214-5223.
19. **O'Sullivan DJ, Cotter PD, Sullivan OO, Giblin L, McSweeney PLH, Sheehan JJ.** 2015. Temporal and spatial differences in microbial composition during the

manufacture of a Continental-type cheese. Applied and Environmental Microbiology doi:10.1128/aem.04054-14.
20. **Quigley L, O'Sullivan O, Beresford T, Paul Ross R, Fitzgerald G, Cotter P.** 2012. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. Journal of Applied Microbiology **113:**96-105.
21. **Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, O'Toole PW.** 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Research **38:**e200.
22. **Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM.** 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research **37:**D141-145.
23. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Tumbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods **7:**335-336.
24. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. Journal of Molecular Biology **215:**403-410.
25. **Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, Peplies J, Glockner FO.** 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research **35:**7188-7196.
26. **Huson DH, Auch AF, Qi J, Schuster SC.** 2007. MEGAN analysis of metagenomic data. Genome Research **17:**377-386.
27. **Urich T, Lanzen A, Qi J, Huson DH, Schleper C, Schuster SC.** 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. PloS one **3:**e2527.
28. **Kruskal WH, Wallis AW.** 1952. Use of Ranks in One-Criterion Variance Analysis. Journal of the American Statistical Association **Vol. 47:**pp. 583-621.
29. **Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M.** 2014. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res **42:**D199-205.
30. **Wang Y, Freedman Z, Lu-Irving P, Kaletsky R, Barkay T.** 2009. An initial characterization of the mercury resistance (mer) system of the thermophilic bacterium Thermus thermophilus HB27. FEMS Microbiology Ecology **67:**118-129.
31. **Quigley L, McCarthy R, O'Sullivan O, Beresford TP, Fitzgerald GF, Ross RP, Stanton C, Cotter PD.** 2013. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. Journal of Dairy Science **96:**4928-4937.
32. **IDF.** 1988. Cheese and processed cheese: Determination of chloride content (potentiometric titration method). International Standards 4a Brussels, Belgium: International Dairy Federation.
33. **IDF.** 1982. Determination of total solids content (cheese and processed cheese), , International Standards 4a Brussels, Belgium: International Dairy Federation.
34. **IDF.** 1993. Milk: Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content., International Standards 20b Brussels, Belgium: International Dairy Federation.

35. **STANDARDS IB.** 1976. British Standard Methods for Chemical Analysis of Cheese: Determination of pH value., London: British Standards Institute.
36. **Sheehan JJ, Fenelon MA, Wilkinson MG, McSweeney PL.** 2007. Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. International Dairy Journal **17:**704-716.
37. **Fenelon M, O'Connor P, Guinee T.** 2000. The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. Journal of Dairy Science **83:**2173-2183.
38. **Marshall CP, Olcott Marshall A.** 2010. The potential of Raman spectroscopy for the analysis of diagenetically transformed carotenoids, vol 368.
39. **Brock TD, Freeze H.** 1969. Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile. Journal of Bacteriology **98:**289-297.
40. **Wadhwani R, McMahon D.** 2012. Color of low-fat cheese influences flavor perception and consumer liking. Journal of Dairy Science **95:**2336-2346.
41. **Tian B, Hua Y.** 2010. Carotenoid biosynthesis in extremophilic Deinococcus–Thermus bacteria. Trends in Microbiology **18:**512-520.
42. **Pěčková M.** 1991. Properties of a hyperthermophilic bacterium (*Thermus* sp.) isolated from a Carlsbad spring. Folia microbiologica **36:**515-521.
43. **Pask-Hughes R, Williams R.** 1975. Extremely thermophilic Gram-negative bacteria from hot tap water. Journal of General Microbiology **88:**321-328.
44. **Brock TD, Boylen KL.** 1973. Presence of Thermophilic Bacteria in Laundry and Domestic Hot-Water Heaters. Applied Microbiology **25:**72-76.
45. **Ekman J, Kosonen M, Jokela S, Kolari M, Korhonen P, Salkinoja-Salonen M.** 2007. Detection and quantitation of colored deposit-forming Meiothermus spp. in paper industry processes and end products. Journal of Industrial Microbiology & Biotechnology **34:**203-211.
46. **Kolari M, Nuutinen J, Rainey F, Salkinoja-Salonen M.** 2003. Colored moderately thermophilic bacteria in paper-machine biofilms. Journal of Industrial Microbiology and Biotechnology **30:**225-238.
47. **Mountain B.** 2012. Life in Hotsprings, p *In* (ed), The Encyclopedia of New Zealand - Sinter, TeAra, ed vol
48. **Tabata K, Ishida S, Nakahara T, Hoshino T.** 1994. A carotenogenic gene cluster exists on a large plasmid in Thermus thermophilus. FEBS Letters **341:**251-255.
49. **Fujita A, Misumi Y, Honda S, Sato T, Koyama Y.** 2013. Construction of new cloning vectors that employ the phytoene synthase encoding gene for color screening of cloned DNA inserts in Thermus thermophilus. Gene **527:**655-662.
50. **Langeveld L.P.M, R.M.G.E vM-Q, A.H. W, R. W, J.S. W.** 1995. Adherence, growth and release of bacteria in a tube heat exchanger for milk. Netherlands Milk and Dairy Journal **49:**201 - 220.
51. **Chang S-F, Ayres JW, Sandine WE.** 1985. Analysis of cheese for histamine, tyramine, tryptamine, histidine, tyrosine, and tryptophane. Journal of Dairy Science **68:**2840-2846.

Chapter 5

Compromised *Lactobacillus helveticus* starter activity in the presence of facultative heterofermentative *Lactobacillus casei* DPC6987 results in atypical eye formation in Swiss-type cheese

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5.0. Abstract

Non-starter lactic acid bacteria (NSLAB) are commonly implicated in undesirable gas formation in several varieties, including Cheddar, Dutch- and Swiss-type cheeses, primarily due to their ability to ferment a wide variety of substrates. This effect can be magnified due to factors that detrimentally affect the composition and/or activity of starter bacteria, resulting in the presence of greater than normal amounts of fermentable carbohydrates and citrate. The objective of this study was to determine the potential for a facultatively heterofermentative *Lactobacillus* (*Lactobacillus casei* DPC6987) isolated from a cheese plant environment to promote gas defects in the event of compromised starter activity. A Swiss-type cheese was manufactured, at pilot scale and in triplicate, containing a typical starter culture (*Streptococcus thermophilus* and *Lactobacillus helveticus*) together with propionic acid bacteria. *Lb. helveticus* populations were omitted in certain vats to mimic starter failure. *Lb. casei* DPC6987 was added, to each experimental vat, at 10^4 cfu g^{-1}. Cheese compositional analysis and X-ray computed tomography revealed that the failure of starter bacteria, in this case *Lb. helveticus*, coupled with the presence of a faculatively heterofermentative *Lactobacillus* (*Lb. casei*) led to excessive eye formation during ripening. The availability of excess amounts of lactose, galactose and citrate, during the initial ripening stages, likely provided the heterofermentative *Lb. casei* with sufficient substrates for gas formation. The accrual of these fermentable substrates was notable in cheeses lacking the *Lb. helveticus* starter population. The results of this study are commercially relevant as

they demonstrate the importance of viability of starter populations and the control of specific NSLAB to ensure appropriate eye formation in Swiss-type cheese.

5.1. Introduction

Swiss- and Dutch-type cheeses are hard/semi-hard, brine salted cheeses, containing characteristic eyes resulting from the metabolism of various substrates (1-3). With respect to Swiss-type cheeses, propionic acid fermentations, due to the presence of environmental or, more typically, deliberate inoculation of propionic acid bacteria (PAB), results in the production of propionate and acetate, which contribute to the characteristic nutty flavour, and CO_2, which is responsible for eye formation (4, 5). CO_2 production, via lactate metabolism, typically occurs during the hot-room (20 - 23°C) phase of ripening when the cheese curd is sufficiently elastic to accommodate stretching (6). Contrastingly, in Dutch-type cheese, eye formation is primarily due to citrate metabolism by mesophilic lactic acid bacteria (LAB)(7, 8).

Factors essential for desirable eye formation, in both Dutch and Swiss-type cheese, include sufficient quantities of gas producing microbiota, the presence of fermentable substrates, favourable environmental conditions (pH, salt in moisture, temperature), the presence of nuclei as well as a suitably elastic cheese texture (9, 10). Regular eye formation is dependent on the amount of CO_2 produced and its diffusion throughout the cheese matrix, which in turn depends on the solubility and pressure of the gas (solubility is temperature and pH dependant) within the cheese (2, 5).

Undesirable or overproduction of gas, in brine salted cheeses, can manifest as splits, cracks, secondary fermentations or excessive eye formation within the cheese. This generally results in downgrading and/or rejection of the product (5, 11). The extent to which brine salted cheese suffers from excessive gas production depends on the gas type (CO_2 or H_2), amount and the solubility of the gas

produced, the texture of the cheese and the ripening temperatures employed. Gas formation can be further sub-divided depending on the stage of ripening that it occurs i.e. early gas production (24 – 48 h) or late gas (later stage ripening) (11). The presence of coliforms, yeast and citrate-positive starter bacteria are common causes of early gas defects, due primarily to the lactose metabolism (12). For late gas formation, butyric acid bacteria such as *Clostridium* spp. are of particular concern because of their ability to produce H_2/CO_2 which is poorly soluble in the cheese matrix. Adventitious streptococci, and in particular CO_2 producing, heat resistant strains that survive pasteurisation and colonise heat exchangers, can also contribute to openness defects in several cheese varieties (11).

Lactobacilli and PAB are of particular interest as culprits of gas defects in Swiss-type cheese. Non-starter lactic acid bacteria (NSLAB) including obligately and facultatively heterofermentative lactobacilli (O/FHLb), while recognised as contributors to ripening and flavour development, are commonly implicated in undesirable gas formation in several varieties including Cheddar, Dutch- and Swiss-type cheeses (9, 12-14). NSLAB populations contaminate cheese *via* survival of pasteurisation and/or through manufacturing equipment/personnel and by the end of ripening, are the dominant microbiota present in the cheese matrix (15, 16). Salt tolerant O/FHLb, such as those contaminating brine tanks, are further sources of adventitious NSLAB capable of CO_2 formation from substrates present late in ripening such as amino acids (3, 11). Of NSLAB populations, FHLb are commonly encountered in Dutch and Swiss-type cheeses and include *Lb. casei*, *Lb. curvatus,* and *Lb. plantarum*. These lactobacilli occur at high numbers (up to $>10^7$ cfu g^{-1}) during cheese ripening (9, 15, 17). Carbohydrates, particularly lactose and

galactose, as well as lactate, citrate and urea have all been proposed as potential substrates utilised by these microbes for gas formation (16, 17). Lactose is usually rapidly metabolised by starter bacteria at the start of the ripening, liberating glucose and galactose which together with lactose can provide the carbohydrate source, for the growth of gas producing FHLb (18). For this reason *Lb. helveticus* is frequently added with *S. thermophilus*, as a mixed starter to metabolise residual carbohydrates and thereby prevent the growth of undesirable gas producing microbes (15). Factors such as bacteriophage activity, inadequate starter storage or elevated salt concentrations may, however, affect the composition and/or activity of starter bacteria, resulting in the presence of greater than normal amounts of fermentable carbohydrates (9, 17, 19). In addition to carbohydrates, citrate can also be metabolised by various FHLb to produce gas (17, 20, 21).

Excessive propionic acid fermentation, either during the hot-room stage or near the end of ripening, may also result in secondary or late fermentation defects particularly in Swiss-type cheeses (2). PAB species with high aspartase activity are capable of producing more CO_2 per mole of lactate than those with lower activity (5, 22). Certain PAB are also capable of growth at low temperatures allowing for further gas production during the later phase (6 – 8 °C) of ripening (23). Evidence of an interactive effect between LAB, and thermophilic LAB in particular, and PAB also exists. Prior studies, using various experimental conditions, have examined the stimulatory effect of various LAB on the growth and metabolism of PAB strains (24-27).

The size, shape and distribution of eyes within the cheese matrix is of key importance (6, 28, 29). Assessment of eye formation in Swiss-type cheese is

generally done by experienced cheese graders and involves a visual examination of the cheese using a cheese trier, tapping of the cheese surface for a hollow sound or by cutting the cheese into sections for visual examination. These methods are subjective or involve destructive sampling of the cheese and are often not indicative of eye formation throughout the entire block (6, 29). Non-invasive/non-destructive imaging technology, relying on methods such as ultrasound, magnetic resonance imaging (MRI), X-ray and X-ray computed tomography (X-ray CT) have recently been applied to profile eye formation in Swiss-type cheeses (6). A prior study to determine the quantitative power of CT led to cheese manufacture using hollow balls to represent artificial eyes. In this study, an accurate correlation between actual, and determined volume, via CT analysis was observed (30).

The objective of this study was to determine the potential for a facultatively heterofermentative *Lb. casei* isolated from a cheese plant environment to promote gas defects in the event of compromised starter activity. The combined impact of *Lb. casei* and PAB populations on the pattern of openness in the cheeses was also investigated. X-ray Computed Tomography (X-ray CT) was employed as a non-destructive method of imaging defective gas formation.

5.2. Materials and Methods

5.2.1. Starter Cultures

A mixed culture of *S. thermophilus* (DPC6986) was selected from the Teagasc Moorepark culture collection for the purpose of this study. DPC6986 was grown on heat treated 10% RSM (100 °C for 90 min) and incubated at 42 °C until a pH of 4.5 was reached, prior to inoculation into cheese milk. *Lb. helveticus* DPC6865 was sourced from the culture collection of Teagasc Moorepark and grown on heat treated 10% RSM, at 42 °C until a pH of 5.1 was reached, prior to inoculation into cheese milk. *P. freudenreichii* DPC6451, from the Teagasc Moorepark Culture Collection, was grown in sodium lactate broth (1 L containing; 10 g of tryptone [Oxoid, Hampshire, U.K.], 10 g of yeast extract [Merck, Cork, Ireland], 5 g KH_2PO_4 [VWR, Dublin, Ireland], 18.9 g 50% w/w sodium lactate solution [Merck, Cork, Ireland] and 5 ml NaOH [VWR, Dublin, Ireland]) for 7 d at 30 °C, under anaerobic conditions prior to inoculation into cheese milk. *Lb. casei* DPC6987 was isolated, using MRS (BD, Oxford, UK) supplemented with 6% NaCl, from a cheese plant environment. Species verification was carried out via 16S rDNA sequencing prior to use. *Lb. casei* DPC6987 was maintained on MRS agar. DPC6987 cultures were grown in MRS broth and concentrated by centrifugation (4000 g, 20 mins, 4 °C) prior to cheese manufacture. Cell concentrations of 10^4 cfu g^{-1} of cheese milk was selected, to achieve $10^{3.8}$ cfu g^{-1} cheese 1 d post production (31). *Lb. casei* DPC6987 was also tested for carbohydrate utilisation using the API CH50 kit (BioMerieux, Basingstoke, Hampshire, U.K.).

5.2.2. Cheese Manufacture

Three replicate cheese-making trials were undertaken over a 12 month period. Raw milk was obtained from a local dairy farm and standardised to a protein:fat ratio of 1.01:1. Milk was held overnight at <10 °C before being pasteurised at 72° C for 15 s and pumped into cylindrical, jacketed vats. Each vat contained automated variable speed cutting and stirring equipment (APV Schweig AG, Worb, Switzerland). Milk (454 kg vat^{-1}) was inoculated, as per experimental protocols (Table 1), with 500 ml *S. thermophilus*, 25 ml *Lb. helveticus*, 4 ml *P. freudenreichii* and 10^4 cfu g^{-1} *Lb. casei* where indicated. Calcium chloride (34% w/v) was added at 100 ml/454 kg to each respective vat. Rennet (Thermolase from *Cryphonectria parasitica*, Chr. Hansens Ltd.) was added at 16.85 ml (diluted in 2 L of water) per 454 kg milk after a 40 min ripening period at 30° C. Coagulation was achieved over 30 min prior to a 5 min cut programme producing a curd size of approximately 5 mm^2. The curd/whey mixture was then allowed to heal for 5 min prior to stirring and cooking at a rate of 1 °C/3 min from 31 - 33°C and at 2 °C/3 min from 33 °C to a maximum scald of 50 °C. After cooking, curds were pre-pressed under whey with the resultant curds placed in 10 kg moulds. The moulded cheeses were then pressed under increasing pressure to 4 to 6 bar. Cheese were held under pressure until a pH of 5.3 was reached before being transferred to a saturated brine solution (23% w/w NaCl, 0.56% $CaCl_2$, pH 5.2 and 18 °C) for 24 hours. After brining, cheese were dried at room temperature, for 4 hours, before being vacuum packed, in CO_2 permeable bags, and transferred to the ripening room

5.2.3. Cheese Ripening

Cheeses were ripened at 9 – 10 °C for 10 days before being transferred to a hot-room (22 °C) for 35 days. Finally, cheeses were matured at 6 °C for a further 50 days.

5.2.4. Enumeration of Starter, Non-Starter, Propionic Acid Bacteria and *Lb. casei*

Cheese was sampled, aseptically using a cheese trier, at 1, 10, 35, 45 and 95 d of ripening. The samples were placed in a sterile stomacher bag, diluted 1:10 with sterile 2% trisodium citrate buffer (VWR, Dublin, Ireland) and homogenised using a stomacher (Iul Instruments, Barcelona, Spain) for 10 min. Independent duplicate samples were taken at each time point and dilutions were prepared as required. Viable *S. thermophilus* cells were enumerated, aerobically, on Ellikers (BD, Oxford, UK) agar supplemented with 0.5% beef extract (BD, Oxford, UK) after 3 days incubation at 42 °C. *Lb. helveticus* cells were enumerated, anaerobically, on MRS agar (BD, Oxford, UK) pH 5.4 after 3 days at 45 °C. *Lb. casei* cells were plated on MRS media supplemented with vancomycin (Sigma, Arklow, Ireland) as per Ong et al. 2005 (32). Total NSLAB were enumerated, anaerobically, on LBS agar (BD, Oxford, UK) for 5 d at 30 °C. Coliforms were plated on VRBA (BD, Oxford, UK) at 30 °C for 1 d. Propionic acid bacteria were enumerated on sodium lactate agar after 7 d incubation at 30 °C (33).

5.2.5. Cheese Compositional and Biochemical Analysis

Cheese samples were taken at 1, 10, 35, 45 and 95 d of ripening and stored at -20 °C for biochemical analysis. Fresh samples, at 10 d post manufacture, were grated for salt, protein, moisture and calcium as described by Sheehan et al. 2007 (34).

Primary proteolysis was determined using the macro-Kjeldahl method (35) as described by Kuchroo and Fox (1982), and was expressed as a percentage of total

nitrogen soluble at pH 4.6. Secondary proteolysis was determined by measuring the free amino acid (FAA) content of the pH 4.6 soluble extracts according to the methods described by Fenelon et al. 2000 (36) and expressed as a percentage of total nitrogen. FAAs were separated using ion-exchange chromatography with post column ninhydrin derivitisation and colourimetric detection. Represented values are means of triplicate trials.

Citrate content of the cheeses was determined using an enzyme assay kit (Megazyme International, Wicklow, Ireland). D-, L- and total lactic acid contents were also determined using enzymatic kits (Megazyme International, Wicklow, Ireland). Samples were prepared for analysis as per the method described by Bouzas et al.1993 (37). Short chain volatile acids (acetate, propionate and *n*-butyrate) were determined using the ligand exchange, ion-exclusion HPLC method as described by Kilcawley et al. 2001 (38).

5.2.6. X-ray Computed Tomography (CT) Measurement & Image Analysis of CT Data

X-Ray CT measurement of control and experimental cheeses was carried out at 95 d of ripening using a CT scanner (VTOMEX L 300 – Microfocus (300kV), General Electric Company, Wunstorf, Germany) with the following scan parameters; 255kV, 180µA, 105.5µm (voxel resolution) and 10.5 mm slice thickness.

Image analysis of CT data was carried out, using the VG StudioMax 2.2 (Volume Graphics, Heidelberg, Germany) using the defect detection module and default parameters.

5.2.7. Statistical Analysis

Three replicate cheese trials were conducted in which the effects of four treatments were tested. A randomised complete block design incorporating the

four treatments and 3 blocks (replicate trials) was used for data analysis. Analysis of variance (ANOVA) was carried out using a SAS (SAS version 9.3) protocol. Tukey's multiple comparison test was used as described by Hou et al. 2012 (39) and the level of significance was determined at $P < 0.05$.

A split-plot design was used to determine the effects of the experimental variations on response variables including; *L. helveticus* counts, *S. thermophilus* counts, pH4.6 soluble nitrogen (S/N), total plus free amino acids, pH, L-, D- and total lactate, citrate levels and short chain volatile acids. ANOVA was carried out using SAS version 9.3 (SAS Institute, 2004) as per Hou et al. 2012 (39).

5.3. Results and Discussion

In this study a Swiss-type cheese was manufactured in order to investigate the potential for a facultatively heterofermentative *Lb. casei* to promote gas defects in the event of compromised starter activity. Experimental cheeses were produced, in triplicate, and corresponded to 4 treatment groups; control (containing *S. thermophilus, Lb. helveticus, P. freudenreichii* and designated CTL), treatment 1 (without *Lb. helveticus*, designated SPC), treatment 2 (without *P. freudenreichii,* designated SLC) and treatment 3 (containing all the aforementioned cultures designated SLPC). A description of treatments, cultures and ripening regimes is present in Table 1.

5.3.1. Growth and Viability of *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Propionibacterium freudenreichii* during Cheese Manufacture.

Mean viable counts of *S. thermophilus*, *Lb. helveticus*, and *P. freudenreichii* 1 d post production, are presented in Table 2. Viable counts of *S. thermophilus* remained constant up to 10 d of ripening before decreasing significantly ($P<0.0001$), to approximately $10^{7.2}$ cfu g^{-1} at day 95 (Fig. 1A). There was no significant effect of treatment or interaction between treatment and time on *S. thermophilus* levels (Table 3). In addition, viable *S. thermophilus* numbers were similar to those encountered in Swiss-type cheeses manufactured using similar starter bacteria and ripening conditions (40).

Mean viable numbers of *Lb. helveticus*, enumerated on MRS pH 5.4 agar, were $10^{6.3}$ cfu g^{-1}, after 1 d of ripening, in the CTL as well as the SLC and SLPC cheeses (Fig. 1B). As expected, no *Lb. helveticus* was detected in the SPC cheeses. A significant effect ($P<0.05$) was observed with respect to both treatment and time over the 35 days

monitored. Between d 10 and 35, viable counts increased from zero to $10^{5.8}$ cfu g^{-1} in the SPC cheese. This was unexpected and is most likely due to *Lb. casei* growth on MRS pH 5.4 agar which is not solely selective for *Lb.helveticus* and can support the growth of *Lb.casei* (data not shown). Viable counts in the SLC and SLPC cheeses decreased to $10^{2.3}$ and $10^{2.2}$ cfu g^{-1} respectively, possibly indicating lysis of *Lb. helveticus*. Alternatively, prior studies have shown that *Lb. delbrueckii*, often used as an alternative to *Lb. helveticus*, cell numbers decrease in the presence of FHLb adjuncts (41). With respect to this a similar effect may have impacted *Lb. helveticus* populations. In the control cheeses, counts at day 10 of $10^{7.3}$ cfu g^{-1} were observed and decreased to $10^{6.0}$ cfu g^{-1} by day 35. Cell counts of *Lb. helveticus* were not enumerated beyond 35 days as increased NSLAB numbers, and *Lb. casei* in particular, precluded accurate counts on MRS pH 5.4 agar. Viable counts, on MRS agar, were lower than those previously encountered in Swiss-type cheese (40).

Mean viable counts of *P. freudenreichii* were $10^{4.2}$ cfu g^{-1} in the CTL, SPC and SPLC cheeses after 1 day of ripening (Fig. 1C). *P. freudenreichii* populations increased significantly ($P<0.0001$) during hot-room ripening to reach $10^{7.9}$ cfu g^{-1} by day 35 and eventually to $10^{8.5}$ cfu g^{-1} by the end of ripening. As expected, *P. freudenreichii* was not detected, throughout ripening, in the SLC cheeses. PAB growth was comparable to that seen in similar studies (40, 42, 43). Although prior studies have reported that in cases where adjunct cultures, such as *Lb. casei*, are added, PAB growth is reduced by 0.4 to 1 log cycles (42), this effect was not observed in this study as PAB growth was consistent in control, SPC and SLPC cheeses.

5.3.2. Growth and Viability of *Lactobacillus casei* and Total Lactobacilli during Cheese Manufacture

A citrate positive strain of *Lb. casei* (DPC6987) was added to each treatment vat at approximately 10^4 cfu g^{-1}. It was established, using a BioMerieux Api 50 CH kit, that the strain used in this study was capable of metabolising a variety of carbohydrates including lactose, galactose, glucose, fructose, mannose and ribose (data not shown). As expected, *Lb. casei* was not detected in the CTL cheeses, at the early stages of ripening (1 d – 10 d) (Fig. 2A/Table 2). Mean viable numbers of *Lb. casei* increased significantly ($P<0.0001$), in all cheeses, during hot-room ripening, eventually reaching levels of $10^{8.6}$ cfu g^{-1} in the SPC, SLC and SLPC cheeses, by day 95. Increased cell numbers observed during hot-room ripening resembled that of total *Lactobacillus* counts. Mean levels of *Lb. casei* were significantly lower ($P<0.0001$) in the CTL cheeses in comparison to the treatment cheeses in the initial stages of ripening (days 1 – 10), where *Lb. casei* was not detected. Levels of *Lb. casei* were consistently lower in the CTL cheeses, although not significantly for the remainder of ripening. This 1 – 2 log cfu g^{-1} difference between control and cheeses manufactured with a mesophilic adjunct has been observed in similar studies (42). Viable cells were isolated, in CTL cheeses, at day 35 and eventually reached levels of $10^{7.4}$ cfu g^{-1} by the end of ripening. The detection of *Lb. casei* in the CTL cheese is likely to be as a result of environmental contamination. Further to this, previous studies have indicated that some *Lb. casei* isolates show particular resistance to pasteurisation temperatures (15). Although not significantly so, *Lb. casei* cell numbers were observed to be consistently higher in the SPC cheeses, in comparison to all other cheeses, possibly due to the presence of higher levels of lactose and galactose encountered, in those cheeses, at the early stages of ripening.

Mean NSLAB counts were similar in CTL, SPC, SLC and SLPC cheeses at day 1 of ripening ($10^{5.8}$ cfu g^{-1}) (Fig. 2B). NSLAB counts were higher than observed in similar studies (42) and this most likely reflects post pasteurisation contamination (i.e. from equipment and/or environment) and/or as a result of failure of pasteurisation to fully inactivate lactobacilli populations (14-16). A significant ($P<0.0001$) increase in viable counts was evident throughout the ripening process and particularly when the cheeses were transferred to the hot-room. This effect was most obvious in cheeses with added *Lb. casei*. As NSLAB numbers are heavily influenced by temperature, significant increases in cell numbers would be expected to occur during hot-room ripening, as previously described (44, 45). As expected, mean viable counts were consistently lower in the control, throughout ripening, than in cheeses to which *Lb. casei* was intentionally added. The highest viable counts were noted in the SPC cheeses, particularly at days 45 and 95 ($10^{8.8}$ cfu g^{-1} at d 95), although not significantly different to those in other cheeses. Total lactobacilli counts were higher (~ 10^{6} cfu g^{-1} immediately after production) than encountered in similar studies (Swiss and semi-hard cheeses manufactured using thermophilic starters and PAB) (34, 40, 46). Final viable cell counts in the control were similar to those encountered in the aforementioned studies.

Plating was also carried out to determine coliform numbers present in the cheeses, however no viable cells were recovered.

5.3.3. Changes in pH

In Swiss-type cheese pH decreases in the initial stages of ripening due to the metabolism of residual sugars (lactose and galactose), before increasing in the later stages of ripening due to proteolytic liberation of short peptides and amino acids

(18). In this study, there was a significant (P<0.01) effect of ripening time on pH (Fig 3). pH was higher than observed in similar studies during initial stages of ripening but was similar to that of Emmental (pH 5.5 – 5.7) towards the end of ripening (2). This reflects the continual metabolism of residual lactose and galactose present during the early stages of ripening by *Lb. helveticus* or *Lb. casei*/NSLAB populations. Furthermore, the higher average pH levels in the SPC cheeses, 1 d post production, (although not significant) likely reflect the absence of the *Lb. helveticus* starter.

5.3.4. Cheese Composition

5.3.4.1. Moisture, protein, salt, calcium and pH levels

The addition of *Lb. casei* as well as the omission of *Lb. helveticus* (SPC) and *P. freudenreichii* (SLC) had no significant effect on mean levels of protein (%), salt, calcium and pH (10 d) (Table 4). Differences (P<0.05) were, however, observed with respect to moisture, as the SLC cheeses were significantly higher than that of the CTL and SPLC cheeses, likely due to reduced acidification during cheese manufacture. This is surprising as PAB are not considered to impact on rates of acidification during cheese manufacture. It is, however, noticeable that, although significantly different, the magnitude of the difference was not large (~ 1%) and may therefore have little biological significance. Compositional indices were similar to those encountered in similar studies (40). No significant difference in salt in moisture levels was observed.

5.3.4.2. Lactose and Galactose

A significant (P<0.0001) reduction in lactose levels was observed in the CTL, SPC, SLC and SLPC cheeses throughout ripening (Fig 4A). This effect was expected as

lactose is rapidly metabolised by *S. thermophilus* in the first few hours of ripening with residual lactose being metabolised by starter and non-starter lactobacilli (47). A significant ($P<0.05$) effect of treatment was noted as lactose levels were observably higher in the SPC than in the CTL or SPLC cheeses. This effect is attributed to the absence of *Lb. helveticus* in the SPC cheeses. Lactose levels were not significantly different in the SLC cheeses compared to the CTL or SPLC cheeses. A significant ($P<0.01$) interactive (treatment by time) effect was also observed between the CTL and SPC cheeses, 1 d post production. This is, again, likely due to the absence of *Lb. helveticus* populations. Similarly, a significant ($P<0.01$) interactive difference was observed between SPC and SLPC cheeses, 1 d post production. In this case, the presence of both *Lb. helveticus* and *Lb. casei* in the SLPC cheeses likely resulted in a significant and rapid reduction in lactose levels. Low residual levels of lactose (<0.0005 g $100g^{-1}$) were present in control and SLPC cheeses at 10 d of ripening while lactose was undetectable in all cheeses by 35 d post production.

Galactose is metabolised primarily by lactobacilli (starter lactobacilli). Therefore absence or failure of a galactose fermenting starter such as *Lb. helveticus* can allow for galactose accumulation, leading to undesirable bacterial growth and/or fermentations (48, 49). In this study, galactose levels declined significantly ($P<0.0001$), as expected, throughout ripening (Fig 4B). A significant ($P<0.05$) interactive effect was observed with respect to galactose levels, 10 d post production, in the SPC cheeses when compared to the control. This effect is likely due to the absence of *Lb. helveticus* populations. Additionally, a significant ($P<0.01$) interactive effect was also observed between the SPC and SLPC cheese, 10 d post

production. Galactose levels were lowest in the control cheeses 1 d post production. Upon entering the hot-room ripening phase, galactose was rapidly metabolised in all cases, and was not detected by day 35 in all cheeses with added *Lb. casei* (SPC, SLC, SLPC cheeses). It is feasible that the additional galactose present in the SPC cheeses provides a suitable substrate for *Lb. casei* populations, particularly upon transfer to the hot-room, resulting in the production of gas prior to propionic acid fermentation.

5.3.4.3. D-, L-Lactate and Total Lactate

Starter bacteria, including *S. thermophilus* and *Lb. helveticus,* produce L-lactate and a mixture of D- and L-lactate, respectively, during Swiss-cheese production (47). Levels of both D-, L-lactate, and total lactate were monitored throughout the course of ripening (Fig 5 A - C). There was a significant effect of time ($P<0.05$) and treatment ($P<0.05$), observed throughout ripening, on levels of total lactate. Due to the absence of PAB, which metabolise lactate to propionate, acetate and CO_2, total lactate levels were highest in the SLC cheeses. Differences were observed between the control and SLC cheeses from day 35 until the end of ripening and were significant ($P<0.05$) at d 45 and d 95. Total lactate levels were similar in SPC and SLPC cheeses, both of which contained PAB and *Lb. casei*. This effect has also been noted in previous studies where lactate levels were higher in cheeses produced with FHLb and may be due to the competition/inhibition of PAB by FHLb (41, 48, 50). Total lactate levels were similar in our control cheeses to those reported to levels encountered in similar Swiss-type cheeses (1200 – 1500 mg $100g^{-1}$) (34).

There was a significant effect of both time ($P<0.0001$) and treatment ($P<0.05$) on levels of D-lactate throughout ripening (Fig. 5B). D-lactate levels were

low in the early stages of ripening due to the slower metabolism of lactose by *Lb. helveticus* in comparison to that of *S. thermophilus*. As *Lb. helveticus* was not present in the SPC cheeses, no D-lactate was detected 1 d post production and only slight increases were observed 10 d post production, possibly due to metabolism of residual lactose by FHLb. Levels of D-lactate increased significantly ($P<0.0001$), across all treatments, once the cheeses entered the hot-room ripening phase, as previously described (47). Levels then decreased due to metabolism by PAB. No consequent reduction of D-lactate was observed in SLC cheese due to the absence of PAB. A significant ($P<0.05$) treatment by time interactive difference was observed, in D-lactate levels, between the control and SLC cheese at days 45 and 95 of ripening. Low levels of D-lactate were observed in the control cheeses (~ 0.2 g $100g^{-1}$ cheese) at the end of ripening, while cheese containing PAB and *Lb. casei* displayed similar D-lactate levels, again likely due to the inhibitory action of FHLb on PAB activity.

Levels of L-lactate were similar across all cheeses and are considerably higher than that of D-lactate, 1 d post production, due to the presence of *S. thermophilus,* which produces L-lactate from lactose. Thereafter a significant ($P<0.01$) reduction was observed in levels of L-lactate throughout ripening (Fig 5C). Similar to total and D-lactate levels, L-lactate was highest in the SLC cheeses due to the absence of PAB, which preferentially metabolise L-lactate. L-Lactate levels were significantly ($P<0.05$) lower in the control cheese, at day 45 of ripening than the SLC cheeses on that day. L-lactate reduced considerably in the control throughout ripening, while similar levels of L-lactate were again observed in both the SPC and SLPC cheeses. A noticeable reduction in L-lactate levels together with a

corresponding increase in D-lactate, both at d 35 of ripening, may be due in part to racemisation of L-lactate to D-lactate by NSLAB/*Lb. casei* present, numbers of which increase considerably during hot-room ripening (47).

5.3.4.4. Citrate Levels

Citrate metabolism is responsible for eye formation in Dutch-type cheeses (e.g., Edam and Gouda which are made without added PAB) (47), and acts as a potential substrate for gas formation by FHLb in both Cheddar and Swiss-type cheeses. (9). Initially, citrate levels averaged 0.13 mg kg^{-1} 1 d post production across all cheeses, and decreased significantly ($P<0.0001$) thereafter throughout the ripening process (Fig 6). Once the cheeses entered the hot-room, a significant ($P<0.0001$) reduction in citrate levels occurred, in all cheeses. A significant ($P<0.0001$) interactive effect (treatment by time) was observed between the CTL and all other cheeses from day 35 until the end of ripening (d 95). SPC, SLC, SLPC cheeses containing *Lb. casei* displayed lower levels of citrate (0.01 mg kg^{-1} at the end of ripening) than were observed in the CTL cheese (0.06 mg kg^{-1} at the end of ripening). As NSLABs such as *Lb. casei* are capable of metabolising citrate (17) to produce CO_2, it is feasible that the addition of this adjunct resulted in the differences in levels observed between the control and experimental cheeses. Furthermore, significantly reduced levels of citrate have been observed in cheeses manufactured with FHLb such as *Lb. paracasei* and *Lb. rhamnosus* as has previously been reported (41).

5.3.4.5. Short Chain Volatile Carboxylic Acids (SCVCA)

Acetic acid (acetate) is produced by propionic acid fermentation by PAB as well as metabolism of citrate by members of the LAB (40). Initial levels of acetate were low in all cheeses (215 mg kg^{-1}) and increased significantly ($P<0.01$) upon transfer to

the hot-room (Fig. 7A). This is likely due to the metabolism of citrate by *Lb. casei* as well as the metabolism of lactate by PAB. No significant differences were observed with respect to treatment. As shown previously, viable numbers of *Lb. casei*, NSLAB and PAB all increased significantly when the cheeses were transferred to the hot-room, likely resulting in the observed increase in levels of acetate produced. As hot-room ripening progressed into cold storage, acetate levels were similar in CTL, SPC and SPLC cheeses, while levels were noticeably lower in SLC cheeses. The latter effect is most likely due to the absence of PAB. Therefore, acetate levels present were likely as a result of NSLAB and *Lb. casei* populations. The levels of acetate produced were similar to those in similar Swiss-type cheese studies (40).

Propionic acid (propionate) is produced *via* the metabolism of lactate by PAB, primarily during the hot-room phase of ripening (20 – 24 °C) (48). As no PAB were present in the SLC cheeses, no propionate was detected. A significant effect of time ($P<0.01$) was observed throughout the ripening process in all other cheeses (Fig 7B). No significant effect of treatment was observed. A significant increase in viable cell counts of PAB occurred once the cheeses were transferred to the hot-room and this correlated with an increase in levels of propionate detected. By the end of ripening (d 95) the highest levels of propionate were observed in the control cheeses. Propionate levels were similar in the SPC and SLPC cheeses, providing further evidence for an inhibitory effect of FHLb on PAB activity. This effect may be due to the production of acetate, which inhibits PAB growth. Similarly, the presence of complexed copper, released during metabolism of citrate also has an inhibitory effect on PAB growth (31). Propionate levels, in the control cheeses at the end of ripening, were similar to those encountered by Fröhlich-Wyder and

Bachmann (2004) (5000 mg kg^{-1}), however levels encountered in SPC and SPLC cheeses were considerably lower.

The stoichiometric equation of PAB lactate metabolism describes 2 molecules of propionate produced for every 1 molecule of acetate (51). As NSLAB populations can produce acetate rather than propionate, the contribution of both PAB and NSLAB to acetate and propionate production can be roughly ascertained by deducing the ratio of propionate to acetate. In this case, ratios of propionate to acetate averaged 1.59 in the control, 1.05 in the SPC cheeses, 0 in the SLC, and 0.81 in the SPLC cheeses (Table 5). This indicated that the SPC and SPLC cheeses, i.e., those containing PAB and *Lb. casei*, displayed considerably lower ratios than that of the control, likely due to acetate production by NSLAB populations. As SLC cheese contained no PAB, no propionate was produced. The highest ratios were observed in the control cheeses, due to the absence of added *Lb. casei*.

In this study, butyrate levels were low in the control, SLC and SLPC cheeses, respectively, and in line with levels previously reported in Swiss-type cheese (150 mg kg^{-1}) (Fig. 7C) (31, 52). A significant ($P<0.01$) effect of time, particularly between 35 d and 45 d post production, was observed. Additionally, there was a significant ($P<0.01$) treatment by time interactive effect observed in butyrate levels between the SPC and all other cheeses at d 45 and d 95 of ripening. The reason for the accumulation of butyrate in the SPC cheeses is, at this stage, unknown. However it may be due to bacterial lipases, such as those from PAB, or amino acid catabolism (53). PAB are among the major contributors to lipolysis in Swiss-type cheeses, however thermophilic bacteria including *S. thermophilus* and *Lb. helveticus* have previously been shown to exhibit lipolytic and esterolytic capabilities (54). Butyrate

can also be formed by clostridia, and is responsible for blowing of Swiss-type cheeses (2), however, no evidence of blowing was detected in this study.

5.3.5. Proteolysis

5.3.5.1. pH4.6SN/TN

Levels of pH 4.6SN/TN increased significantly ($P<0.0001$) throughout ripening (data not shown) with a marked increase occurring when cheeses were transferred to the hot-room. No effect of treatment was observed. The increase in pH 4.6 SN observed is as expected and is similar to trends seen in studies on Swiss-type cheeses (40). Levels of soluble N as a percentage of total nitrogen were similar to those described in the literature (55).

5.3.5.2. Total and Individual Free Amino Acids

Levels of total free amino acids (TFAA) increased significantly ($P<0.0001$) throughout the ripening process (Fig. 8A), particularly when the cheeses entered the hot-room ripening phase. A significant ($P<0.05$) treatment by time interactive effect was also observed at d 95 where SPC cheeses had significantly lower levels of total FAA in comparison to all other cheeses. This significant difference between SPC and the other cheeses is likely due to the absence of highly proteolytic *Lb. helveticus* populations in the SPC cheeses (15). Highest levels of TFAA were encountered at d 95 in the control cheeses (9063 mg kg^{-1}), while the lowest levels were observed in the SPC cheeses (3168 mg kg^{-1}).

Levels of individual free amino acids at 95 d post production are shown in Figure 8B. A significant ($P<0.05$) effect of treatment was observed as individual FAA levels were lower in SPC cheeses than in all other cheeses. The FAAs detected at highest

concentrations at d 95 included glutamate, leucine, valine, lysine and proline with proportions similar to those commonly observed in Swiss-type cheeses such as Emmental (40, 52, 55). Levels of glutamate, leucine, lysine and proline were significantly ($P<0.01$) higher, at d 95, in the CTL than in the SPC cheeses.

5.3.6. Eye Formation in Swiss-type Cheeses as Determined by X-ray Computed Tomography (CT)

Swiss-type cheeses were investigated, using non-destructive X-ray CT, to allow for examination of the 3-D spatial distribution of eyes produced by the various treatments as well as the size of the eyes present (Fig. 9). With respect to the physical appearance (shape, distribution, size and number) of eyes formed during the ripening process, the control cheese resembled most closely a standard Swiss-type cheese. As the control cheese was manufactured at pilot scale and not in an industrial setting, eye formation would still be regarded as somewhat irregular. However, marked physical differences were observed in the control compared to the other cheeses. In the SPC cheeses a large number of small eyes were distributed throughout the cheese wheel. This observation is consistent with prior studies which describe the presence of FHLb (such as *Lb. casei*) providing conditions conducive to the production of a large number of small eyes, likely due to citrate and carbohydrate metabolism (6, 50, 56). Several eyes with a very large volume were also present. In the SLC cheeses 'normal' eye formation did not occur, due to the absence of PAB. However, a large number of minute eyes were distributed throughout the cheese wheel. It is likely that these are small eyes produced as a result of CO_2 production by FHL present in the cheese but were not enlarged due to

the absence of a PAB fermentation. In the SLPC cheeses a large number of eyes, with varying volumes, were observed. These eyes are distributed throughout the cheese wheel and are observably larger than those present in the SPC cheeses.

With respect to void percentage, at 95 d post production, the greatest ($P<0.05$) void volume occurred in the SPC cheeses (22.6%) (Table 6). Following this, SLPC and control cheeses (14.6% and 12.6%, respectively) displayed similar void percentages. The SLC cheeses displayed the lowest void percentage at 1.5%. Defect volume is represented, in mm^3, by the colouration of the void spaces.

5.4. Conclusions

The results of this study demonstrate that the failure of starter bacteria (*Lb. helveticus*) coupled with the presence of faculatively heterofermentative lactobacilli (*Lb. casei*) leads to a greater propensity for excessive eye formation in Swiss-type cheeses, during ripening. The availability of residual amounts of lactose, galactose and citrate, present during the initial stages of ripening due to the absence of *Lb. helveticus*, likely provided the heterofermentative *Lb. casei* with sufficient substrates for gas formation. The accrual of these fermentable substrates was notable in cheeses lacking the *Lb. helveticus* starter population (SPC cheeses) and consequently excessive eye formation occurred. With particular respect to galactose, accumulation is commonly associated with textural defects in cheeses, due to CO_2 production by non-starter bacteria (conventional starters such as *S. thermophilus* and *Lactococcus lactis* do not metabolise galactose) (57). The presence of citrate, accepted as a fermentable substrate responsible for gas production in Cheddar cheeses (17), likely provided a further substrate for CO_2 accumulation. As the cheese body can only accommodate a certain amount of gas, it is conceivable that increased amounts of fermentable substrates, coupled with the presence of heterofermentative microbial populations, resulted in build-up of CO_2 within the cheese prior to propionic acid fermentation. Once propionic acid fermentation occurred, towards the end of hot-room ripening, an additional accumulation of gas resulted in the excessive eye formation observed. Previously, evidence to suggest a stimulatory effect of LAB on PAB has been proposed in the literature (5, 24). A stimulatory effect of LAB on PAB was not evident in this study but rather, contrastingly, indicators of PAB activity, such as propionic acid

production, were lower in cheeses containing both *Lb. casei* and *P. freudenreichii*. This suggested an inhibitory effect of *Lb. casei* metabolism on PAB activity. Heterofermentative adjuncts such as *Lb. casei* are often intentionally added to artisanal Swiss-type cheeses to control and reduce the occurrence of secondary fermentation defects (2). This effect is thought to be *via* production of acetate, competition for nutrients and even through liberation of copper during citrate metabolism (50). While *Lb. casei* addition has proved a successful method for controlling excessive gas formation, this study has shown that the addition of FHLb, such as *Lb. casei,* can promote gas defects particularly in situations where starter cultures fail. X-ray CT analysis of the various cheese treatments provided an accurate, non-invasive, overall image, not only of eye formation, but eye size, distribution and overall void percentage. This method also allows for the establishment of relationships between the biochemical characteristics of the cheese and the physical manifestation of eyes.

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5.6. References

1. **Reinbold GW.** 1972. Swiss Cheese Varieties. Pfizer Incorporated.

2. **Fröhlich-Wyder M-T, Bachmann H-P.** 2007. Swiss cheese. frequency Several times yearly at irregular intervals ISSN 1660-7856 (online).

3. **van den Berg G, Meijer WC, Düsterhöft EM, Smit G.** 2004. Gouda and related cheeses, p. 103-140. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed.), Cheese: Chemistry, Physics and Microbiology, vol. Volume 2. Academic Press.

4. **Kerjean J-R, Condon S, Lodi R, Kalantzopoulos G, Chamba J-F, Suomalainen T, Cogan T, Moreau D.** 2000. Improving the quality of European hard-cheeses by controlling of interactions between lactic acid bacteria and propionibacteria. Food Research International **33:**281-287.

5. **Daly DM, McSweeney PH, Sheehan J.** 2010. Split defect and secondary fermentation in Swiss-type cheeses — A review. Dairy Sci. Technol. **90:**3-26.

6. **Guggisberg D, Schuetz P, Winkler H, Amrein R, Jakob E, Fröhlich-Wyder M-T, Irmler S, Bisig W, Jerjen I, Plamondon M, Hofmann J, Flisch A, Wechsler D.** 2015. Mechanism and control of the eye formation in cheese. International Dairy Journal **47:**118-127.

7. **Walstra P, Noomen A, Geurts TJ.** 1993. Dutch-Type Varieties, p. 39-82. *In* Fox PF (ed.), Cheese: Chemistry, Physics and Microbiology. Springer US.

8. **Smit G, Smit BA, Engels WJM.** 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products, vol. 29.

9. **Porcellato D, Johnson ME, Houck K, Skeie SB, Mills DA, Kalanetra KM, Steele JL.** 2015. Potential of Lactobacillus curvatus LFC1 to produce slits in Cheddar cheese. Food microbiology **49:**65-73.

10. **McSweeney PL.** 2007. Cheese problems solved. Woodhead Publishing Ltd.

11. **Sheehan JJ.** 2011. Cheese | Avoidance of Gas Blowing, p. 661-666. *In* Fuquay JW (ed.), Encyclopedia of Dairy Sciences (Second Edition). Academic Press, San Diego.

12. **O Sullivan D, Cotter P, Giblin L, Sheehan J, McSweeney P.** 2013. Nucleic acid-based approaches to investigate microbial-related cheese quality defects. Frontiers in Microbiology **4**.

13. **Fröhlich-Wyder M-T, Guggisberg D, Badertscher R, Wechsler D, Wittwer A, Irmler S.** 2013. The effect of Lactobacillus buchneri and Lactobacillus parabuchneri on the eye formation of semi-hard cheese. International Dairy Journal **33:**120-128.

14. **Ortakci F, Broadbent JR, Oberg CJ, McMahon DJ.** 2015. Growth and gas production of a novel obligatory heterofermentative Cheddar cheese nonstarter lactobacilli species on ribose and galactose. Journal of dairy science **98:**3645-3654.

15. **Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM.** 2001. Recent advances in cheese microbiology. International Dairy Journal **11:**259-274.

16. **Martley FG, Crow VL.** 1993. Interactions between non-starter microorganisms during cheese manufacture and repening. International Dairy Journal **3:**461-483.

17. **Mullan WMA.** 2000. Causes and control of early gas production in cheddar cheese. International Journal of Dairy Technology **53:**63-68.

18. **McSweeney PL.** 2004. Biochemistry of cheese ripening. International Journal of Dairy Technology **57:**127-144.

19. **Spus M, Li M, Alexeeva S, Wolkers-Rooijackers JCM, Zwietering MH, Abee T, Smid EJ.** 2015. Strain diversity and phage resistance in complex dairy starter cultures. Journal of dairy science **98:**5173-5182.

20. **Adamberg K, Antonsson M, Vogensen FK, Nielsen EW, Kask S, Møller PL, Ardö Y.** 2005. Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semi-hard Danish cheese. International Dairy Journal **15:**873-882.

21. **Gagnaire V, Thierry A, Léonil J.** 2001. Propionibacteria and facultatively heterofermentative lactobacilli weakly contribute to secondary proteolysis of Emmental cheese. Lait **81:**339-353.

22. **Wyder M-T, Bosset J, Casey M, Isolini D, Sollberger H.** 2001. Influence of two different propionibacterial cultures on the characteristics of Swiss-type cheese with regard to aspartate metabolism. Milchwissenschaft **56:**78-81.

23. **Hettinga DH, Reinbold GW, Vedamuthu ER.** 1974. Split Defect of Swiss Cheese: I. Effect of Strain of Propionibacterium and Wrapping Material. Journal of Milk and Food Technology (JMFT) **37:**322-328.

24. **Piveteau P, Condon S, Cogan T.** 1995. Interactions between lactic and propionic acid bacteria. Le Lait **75:**331-343.

25. **Thierry A, Salvat-Brunaud D, Maubois J-L.** 1999. Influence of thermophilic lactic acid bacteria strains on propionibacteria growth and lactate consumption in an Emmental juice-like medium. Journal of dairy research **66:**105-113.

26. **Baer A, Ryba I.** 1999. Interactions between propionic acid bacteria and thermophilic lactic acid bacteria. Lait **79:**79-92.

27. **White SR, Broadbent JR, Oberg CJ, McMahon DJ.** 2003. Effect of Lactobacillus helveticus and Propionibacterium freudenrichii ssp. shermanii Combinations on Propensity for Split Defect in Swiss Cheese1. Journal of dairy science **86:**719-727.

28. **Caccamo M, Melilli C, Barbano D, Portelli G, Marino G, Licitra G.** 2004. Measurement of gas holes and mechanical openness in cheese by image analysis. Journal of dairy science **87:**739-748.

29. **Lee K, Uegaki K, Nishii C, Nakamura T, Kubota A, Hirai T, Yamada K.** 2012. Computed tomographic evaluation of gas hole formation and structural quality in Gouda-type cheese. International Journal of Dairy Technology **65:**232-236.

30. **Guggisberg D, Fröhlich-Wyder M-T, Irmler S, Greco M, Wechsler D, Schuetz P.** 2013. Eye formation in semi-hard cheese: X-ray computed tomography as a non-invasive tool for assessing the influence of adjunct lactic acid bacteria. Dairy Sci. Technol. **93:**135-149.

31. **Fröhlich-Wyder M-T, Bachmann H-P, Casey MG.** 2002. Interaction between propionibacteria and starter / non-starter lactic acid bacteria in Swiss-type cheeses. Lait **82:**1-15.

32. **Ong L, Henriksson A, Shah NP.** 2006. Development of probiotic Cheddar cheese containing Lactobacillus acidophilus, Lb. casei, Lb. paracasei and Bifidobacterium spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal **16:**446-456.

33. **Drinan FD, Cogan TM.** 1992. Detection of propionic acid bacteria in cheese. Journal of Dairy Research **59:**65-69.

34. **Sheehan JJ, Fenelon MA, Wilkinson MG, McSweeney PLH.** 2007. Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. International Dairy Journal **17:**704-716.

35. **IDF.** 1993. Milk: Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content., International Standards 20b Brussels, Belgium: International Dairy Federation.

36. **Fenelon MA, O'Connor P, Guinee TP.** 2000. The effect of fat content on the microbiology and proteolysis in cheddar cheese during ripening dairy foods. Journal of dairy science **83:**2173-2183.

37. **Bouzas J, Kantt CA, Bodyfelt FW, Torres JA.** 1993. Time and Temperature Influence on Chemical Aging Indicators for a Commercial Cheddar Cheese. Journal of Food Science **58:**1307-1313.

38. **Kilcawley KN, Wilkinson MG, Fox PF.** 2001. A Survey of Lipolytic and Glycolytic End-Products in Commercial Cheddar Enzyme-Modified Cheese. Journal of dairy science **84:**66-73.

39. **Hou J, Hannon JA, McSweeney PLH, Beresford TP, Guinee TP.** 2012. Effect of curd washing on composition, lactose metabolism, pH, and the growth of non-starter lactic acid bacteria in full-fat Cheddar cheese. International Dairy Journal **25:**21-28.

40. **Sheehan JJ, Wilkinson MG, McSweeney PLH.** 2008. Influence of processing and ripening parameters on starter, non-starter and propionic acid bacteria and on the ripening characteristics of semi-hard cheeses. International Dairy Journal **18:**905-917.

41. **Bouton Y, Buchin S, Duboz G, Pochet S, Beuvier E.** 2009. Effect of mesophilic lactobacilli and enterococci adjunct cultures on the final characteristics of a microfiltered milk Swiss-type cheese. Food microbiology **26:**183-191.

42. **Kocaoglu-Vurma NA, Harper WJ, Drake MA, Courtney PD.** 2008. Microbiological, Chemical, and Sensory Characteristics of Swiss Cheese Manufactured with Adjunct Lactobacillus Strains Using a Low Cooking Temperature. Journal of dairy science **91:**2947-2959.

43. **Noel Y, Boyaval P, Thierry A, Gagnaire V, Grappin R.** 1999. 8 Eye formation and Swiss-type cheeses. Technology of cheesemaking**:**222.

44. **Gilles J, Turner K, Martley F.** 1983. Swiss-type cheese. I. Manufacturing and sampling procedures. New Zealand Journal of Dairy Science and Technology (New Zealand).

45. **Fox PF, Law J, McSweeney PLH, Wallace J.** 1993. Biochemistry of Cheese Ripening, p. 389-438. *In* Fox PF (ed.), Cheese: Chemistry, Physics and Microbiology. Springer US.

46. **Thierry A, Salvat-Brunaud D, Madec M-N, Michel F, Maubois J-L.** 1998. Swiss cheese ripening: dynamics of bacterial populations and evolution of the aqueous phase composition for three industrial cheeses. Lait **78:**521-542.

47. **McSweeney PLH, Fox PF.** 2004. Metabolism of residual lactose and of lactate and citrate, p. 361-371. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed.), Cheese: Chemistry, Physics and Microbiology, vol. Volume 1. Academic Press.

48. **Fröhlich-Wyder MT, Bachmann HP.** 2004. Cheeses with propionic acid fermentation, p. 141-XV. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed.), Cheese: Chemistry, Physics and Microbiology, vol. Volume 2. Academic Press.

49. **Turner K, Morris H, Martley F.** 1983. Swiss-type cheese. II. The role of thermophilic lactobacilli in sugar fermentation. New Zealand Journal of Dairy Science and Technology.

50. **Weinrichter B, Sollberger H, Ginzinger W, Jaros D, Rohm H.** 2004. Adjunct starter properties affect characteristic features of Swiss-type cheeses. Die Nahrung **48:**73-79.

51. **Piveteau P.** 1999. Metabolism of lactate and sugars by dairy propionibacteria: a review. Le Lait **79:**23-41.

52. **Lawlor JB, Delahunty CM, Wilkinson MG, Sheehan J.** 2002. Relationships between the gross, non-volatile and volatile compositions and the sensory attributes of eight hard-type cheeses. International Dairy Journal **12:**493-509.

53. **Christensen JE, Dudley EG, Pederson JA, Steele JL.** 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek **76:**217-246.

54. **Chamba J-F, Perreard É.** 2002. Contribution of propionic acid bacteria to lipolysis of Emmental cheese. Lait **82:**33-44.

55. **Upadhyay VK, McSweeney PLH, Magboul AAA, Fox PF.** 2004. Proteolysis in cheese during ripening, p. 391-VIII. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed.), Cheese: Chemistry, Physics and Microbiology, vol. Volume 1. Academic Press.

56. **Law BA, Tamime AY.** 2011. Technology of Cheesemaking. Wiley.

57. **Wu Q, Cheung CKW, Shah NP.** 2015. Towards galactose accumulation in dairy foods fermented by conventional starter cultures: Challenges and strategies. Trends in Food Science & Technology **41:**24-36.

Table 1: Description of the treatments, starter cultures and ripening regimes used in the study

Treatment	**CTL cheese**	**SPC cheese**	**SLC cheese**	**SLPC cheese**
Milk volume:	454 kg	454 kg	454 kg	454 kg
Starter cultures:	*S. thermophilus*	*S. thermophilus*	*S. thermophilus*	*S. thermophilus*
	Lb. helveticus	-	*Lb. helveticus*	*Lb. helveticus*
	P. freudenreichii	*P. freudenreichii*	-	*P. freudenreichii*
	-	*Lb. casei* @ 10^4	*Lb. casei* @ 10^4	*Lb. casei* @ 10^4
		cfu/g	cfu/g	cfu/g
Manufacturing	Rindless Swiss-	Rindless Swiss-	Rindless Swiss-	Rindless Swiss-
method:	type	type	type	type
Ripening	10°C x 10 d	10°C x 10 d	10°C x 10 d	10°C x 10 d
regime:				
	22°C x 35 d	22°C x 35 d	22°C x 35 d	22°C x 35 d
	6°C x 45 d	6°C x 45 d	6°C x 45 d	6°C x 45 d

CTL cheese: control cheese containing *S. thermophilus*, *Lb. helveticus* and *P. freudenreichii*, SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lb. casei* and no *Lb. helveticus,*

SLC cheese: contains no *P. freudenreichii* populations

SLPC cheese: contains *S. thermophilus*, *Lb. helveticus*, *P. freudenreichii* and *Lb. casei*

Table 2: Mean viable counts of cultures inoculated to vats at 1 d after manufacture.

Culture	CTL (1 d)	SPC (1 d)	SLC (1 d)	SLPC (1 d)
S. thermophilus	$10^{8.9}$ cfu g^{-1}	$10^{8.9}$ cfu g^{-1}	$10^{8.9}$ cfu g^{-1}	$10^{8.9}$ cfu g^{-1}
Lb. helveticus	$10^{6.3}$ cfu g^{-1}	0	$10^{6.4}$ cfu g^{-1}	$10^{6.3}$ cfu g^{-1}
P. freudenreichii	$10^{4.3}$ cfu g^{-1}	$10^{3.9}$ cfu g^{-1}	0	$10^{4.2}$ cfu g^{-1}
Lb. casei	0	$10^{4.7}$ cfu g^{-1}	$10^{4.7}$ cfu g^{-1}	$10^{4.5}$ cfu g^{-1}

CTL cheese: control cheese containing *S. thermophilus*, *Lb. helveticus* and *P. freudenreichii*, SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lb. casei* and no *Lb. helveticus,*

SLC cheese: contains no *P. freudenreichii* populations

SLPC cheese: contains *S. thermophilus*, *Lb. helveticus*, *P. freudenreichii* and *Lb. casei*

Table 3: Statistical summary for the effect of respective treatment, time and their interaction in a Swiss-type cheese[a,b]

Parameter	Treatment	Time	Interactive Effect (Treatment * Time)
S. thermophilus	NS	***	NS
Lb. helveticus	*	*	***
PAB	***	***	***
Lb. casei	***	***	***
NSLAB[c]	**	***	NS
pH	NS	**	NS
Lactose	*	***	*
Galactose	*	***	**
Citrate	***	***	***
Total Lactate	*	*	NS
D-lactate	*	***	*
L-lactate	NS	**	NS
Propionate	NS	**	NS
Acetate	NS	***	NS
Butyrate	***	***	***
Total FAA[c]	**	***	NS
Individual FAA	*	***	**
%pH4.6SN/TN[c]	NS	***	NS

[a]Significance levels: *; P<0:05, **; P<0:01, ***; P<0:001, NS; not significant (P>0.05)

[b]Description of the various treatments given in Table 1

[c]Non-starter lactic acid bacteria (NSLAB), free amino acids (FAA), soluble nitrogen at pH 4.6 as a percentage of total nitrogen (SN/TN)

Table 4: Cheese composition (protein, moisture, salt, calcium and salt in moisture), 1 d after manufacture, and pH at 10 d after manufacture.

Compositional Indices	CT	SPC	SLC	SLPC
Protein (%)	25.78[a]	25.13[a]	25.02[a]	25.81[a]
Moisture (%)	39.63[a]	40.37[ab]	40.7[b]	39.91[a]
Salt (%)	1.19[a]	1.12[a]	1.24[a]	1.04[a]
Calcium (mg/100g)	895[a]	886[a]	880[a]	887[a]
pH day 10	5.42[a]	5.46[a]	5.40[a]	5.45[a]
Salt in moisture% SM	3.0[a]	2.77[a]	3.04[a]	2.63[a]

Means sharing a common letter (a) are not statistically significant ($P<0.05$). Values presented are means of three replicate trials

CTL cheese: control cheese containing *S. thermophilus*, *Lb. helveticus* and *P. freudenreichii*, SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lb. casei* and no *Lb. helveticus,*

SLC cheese: contains no *P. freudenreichii* populations

SLPC cheese: contains *S. thermophilus*, *Lb. helveticus*, *P. freudenreichii* and *Lb. casei*

Table 5: Ratio of propionate to acetate, during the later stages of ripening (d 35 – 95) in the control and 3 treatment cheeses. Ratios displayed are an average of mean propionate and acetate production cross replicate trials. Ratios are not included before d 35 as no propionate was produced.

Ripening (d)	CTL	SPC	SLC	SLPC
35 d	1.23	0.98	0.00	0.41
45 d	1.68	1.10	0.00	1.03
95 d	1.87	1.07	0.00	0.99

CTL cheese: control cheese containing *S. thermophilus*, *Lb. helveticus* and *P. freudenreichii*, SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lb. casei* and no *Lb. helveticus,*

SLC cheese: contains no *P. freudenreichii* populations

SLPC cheese: contains *S. thermophilus*, *Lb. helveticus*, *P. freudenreichii* and *Lb. casei*

Table 6: Void percentage summary for each treatment, at 95 d of ripening. Three sections were analysed per treatment group.

CT Section	CTL	SPC	SLC	SLPC
Section 1 (%)	16.6	25.48	1.76	17.49
Section 2 (%)	12.59	25.64	1.1	20.31
Section 3 (%)	8.54	16.62	1.55	5.97
Average (%)	12.6^{ab}	22.6^{a}	1.5^{b}	14.6^{ab}

^{a,b}Means with the same letter are not significant (P<0.05).

CTL cheese: control cheese containing *S. thermophilus*, *Lb. helveticus* and *P. freudenreichii*, SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lb. casei* and no *Lb. helveticus,*

SLC cheese: contains no *P. freudenreichii* populations

SLPC cheese: contains *S. thermophilus*, *Lb. helveticus*, *P. freudenreichii* and *Lb. casei*

Figure 1: Effect of the respective treatments on mean viable counts of (A) *Streptococcus thermophilus*, (B) *Lactobacillus helveticus* and (C) *Propionibacterium freudenreichii*, enumerated on Ellikers agar, MRS pH5.4 and SLA respectively. Control cheese (CTL) ◆, SPC cheese ■, SLC cheese ▲, SLPC cheese ✕. Values presented are means of 3 replicate trials.

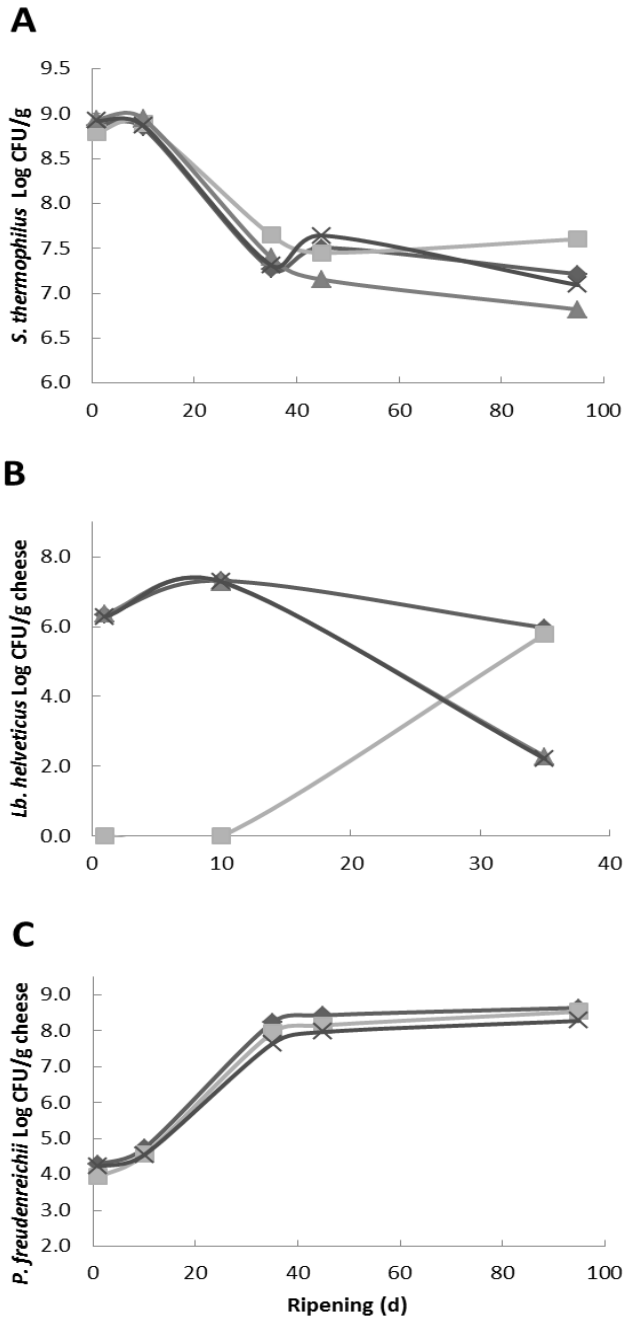


Figure 2: Effect of the respective treatments on mean viable counts of (A) *Lactobacillus casei* and (B) Total Lactobacilli, enumerated on MRS supplemented with Vancomycin and LBS agar respectively. Control cheese (CTL) ◆, SPC cheese ■, SLC cheese ▲, SLPC cheese ✕. Values presented are means of 3 replicate trials.

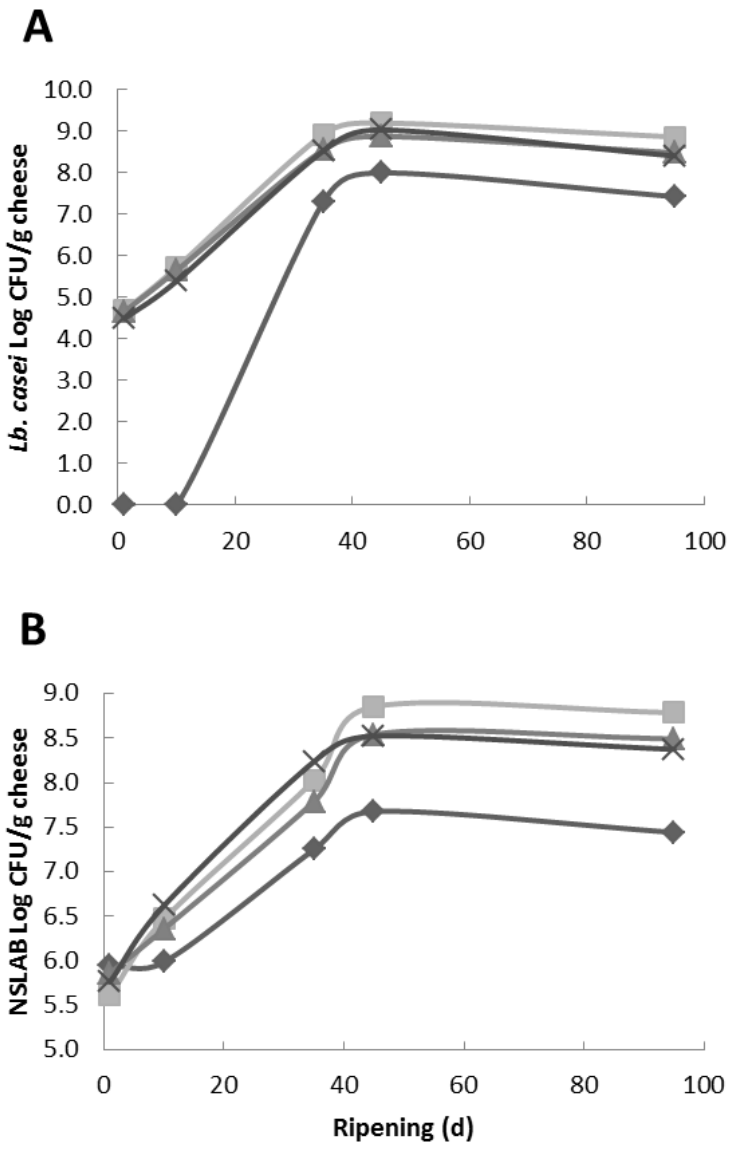


Figure 3: pH values throughout ripening for all cheeses. Control cheese (CTL) , SPC cheese , SLC cheese , SLPC cheese . Values presented are means of 3 replicate trials.

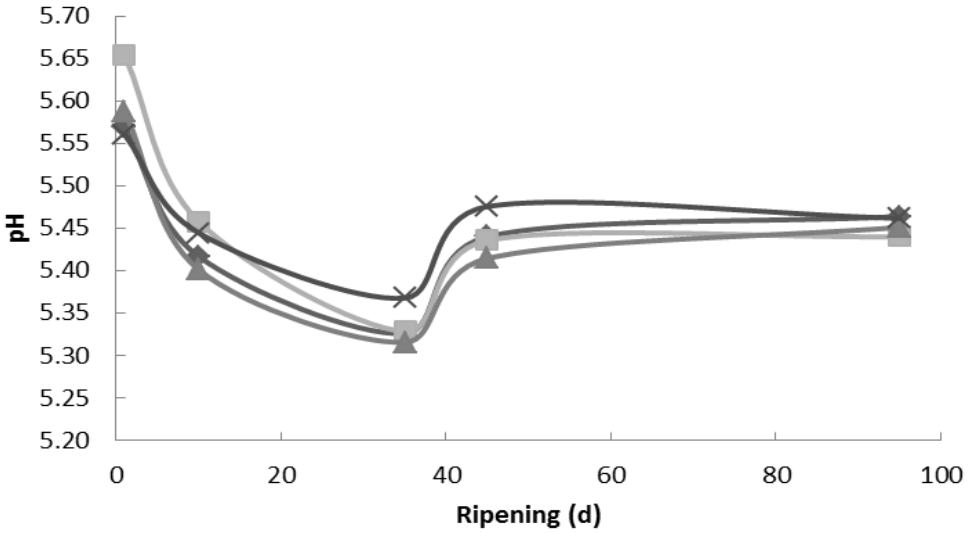


Figure 4: Levels of (A) lactose and (B) galactose expressed in g/100g cheese. Control cheese (CTL) ◆, SPC cheese ■, SLC cheese ▲, SLPC cheese ×. Values presented are means of 3 replicate trials.

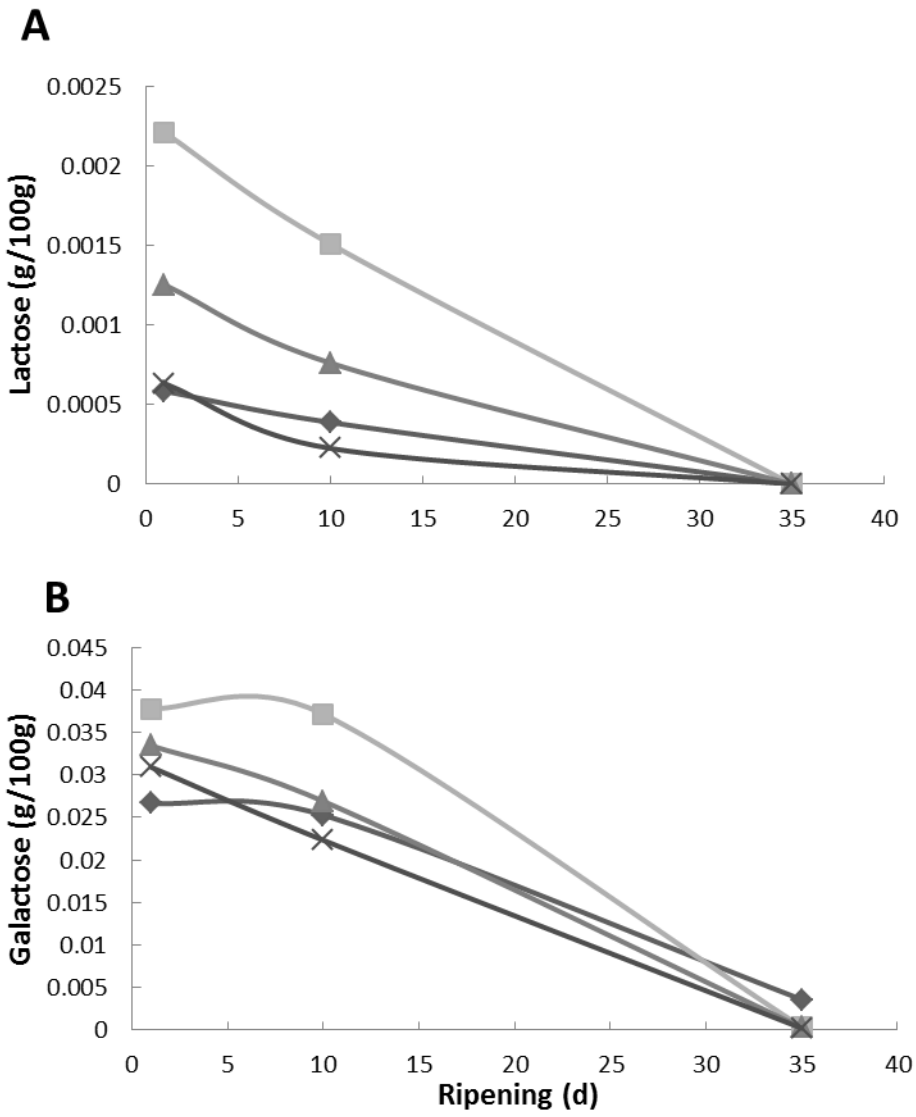


Figure 5: Levels of (A) D-lactate, (B) L-lactate and (C) Total lactate (g/100g) present in the control and treatments 1 – 3. Control cheese (CTL) , SPC cheese , SLC cheese , SLPC cheese . Values presented are means of 3 replicate trials

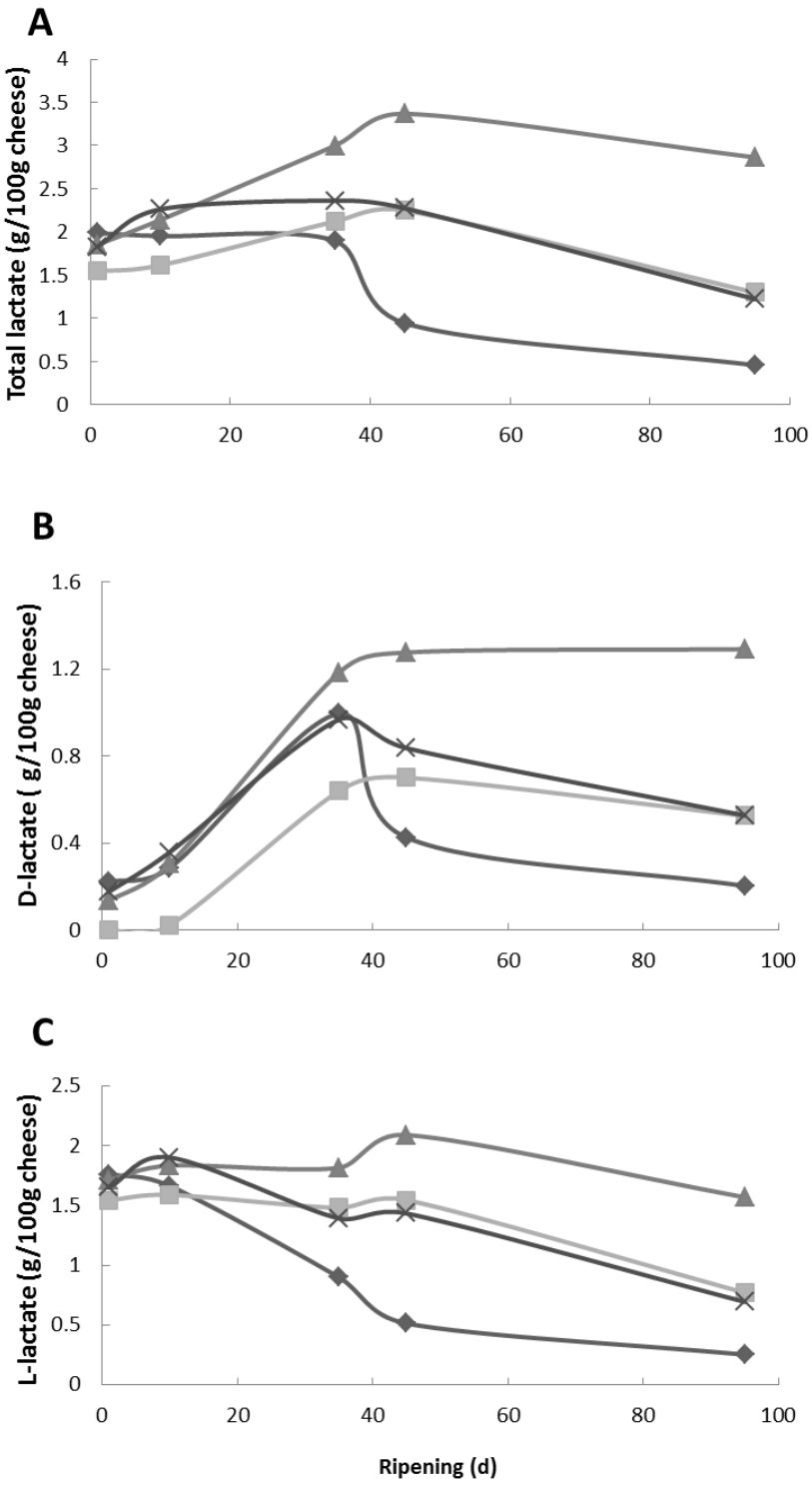


Figure 6: Citrate levels (g/100g) present in the control and treatments 1 – 3 throughout ripening. Control cheese (CTL) , SPC cheese , SLC cheese , SLPC cheese . Values presented are means of 3 replicate trials.

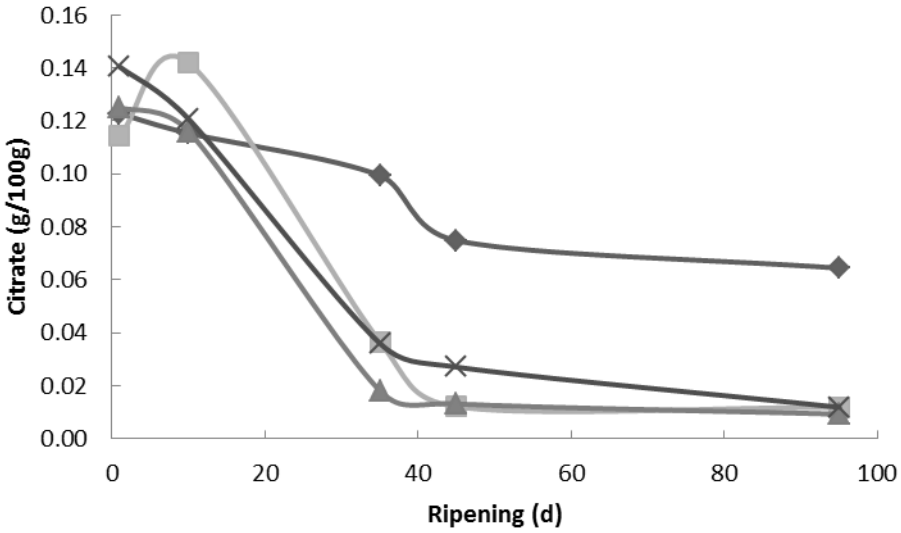


Figure 7: Short chain volatile carboxylic acids including (A) acetic acid, (B) propionic acid and (C) butyric acid, presented in mg/kg cheese. Control cheese (CTL) ◆, SPC cheese ■, SLC cheese ▲, SLPC cheese ✕. Values presented are means of 3 replicate trials.

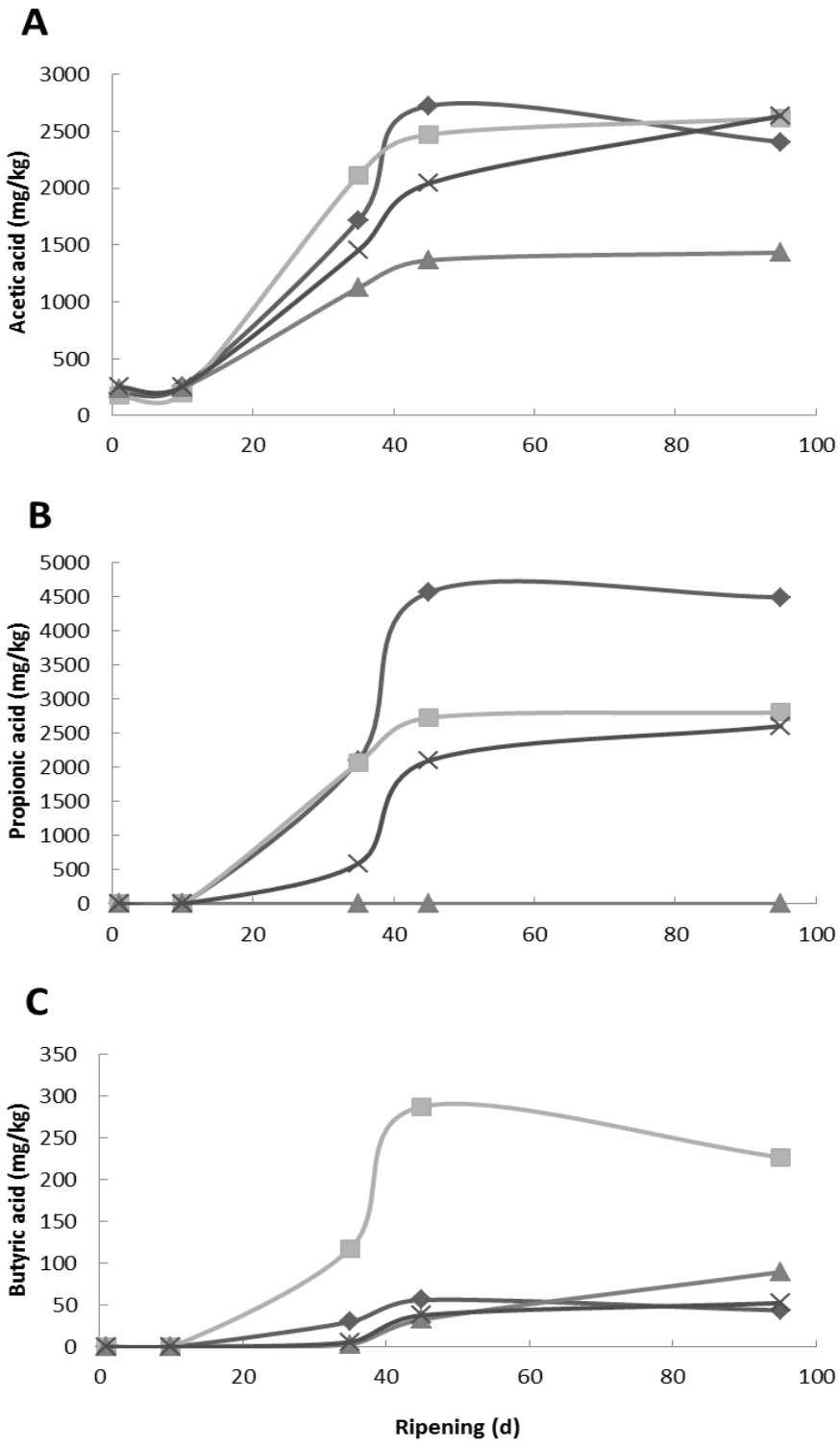


Figure 8: Total free amino acids (FAA) expressed in mg kg^{-1} cheese (A) and individual free amino acids (FAA), at 95 d (B) post production, in the control and treatments 1 – 3. Control cheese CTL , , SPC cheese , , SLC cheese , , SPLC cheese , . Values presented are means of 3 replicate trials.

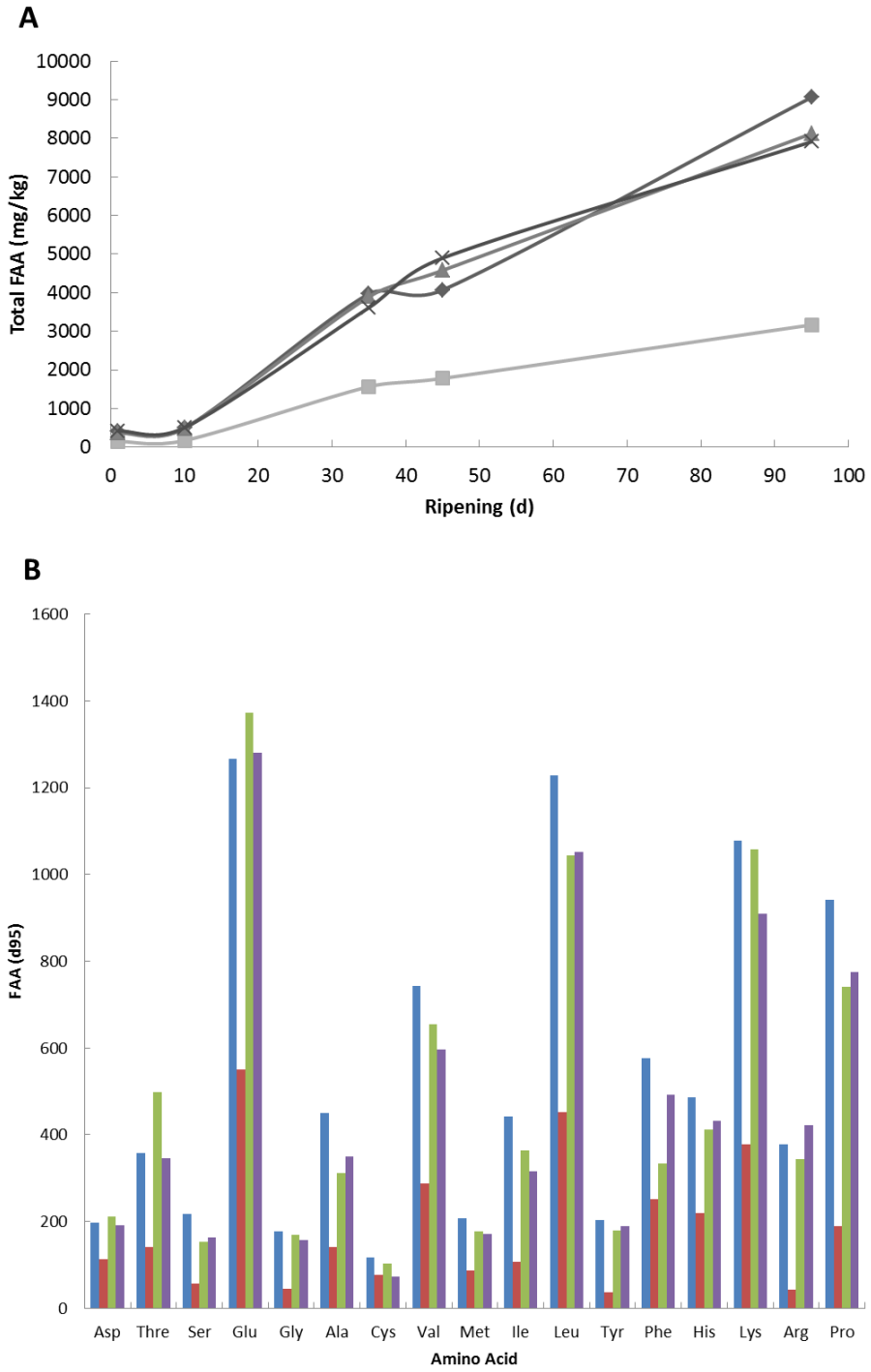
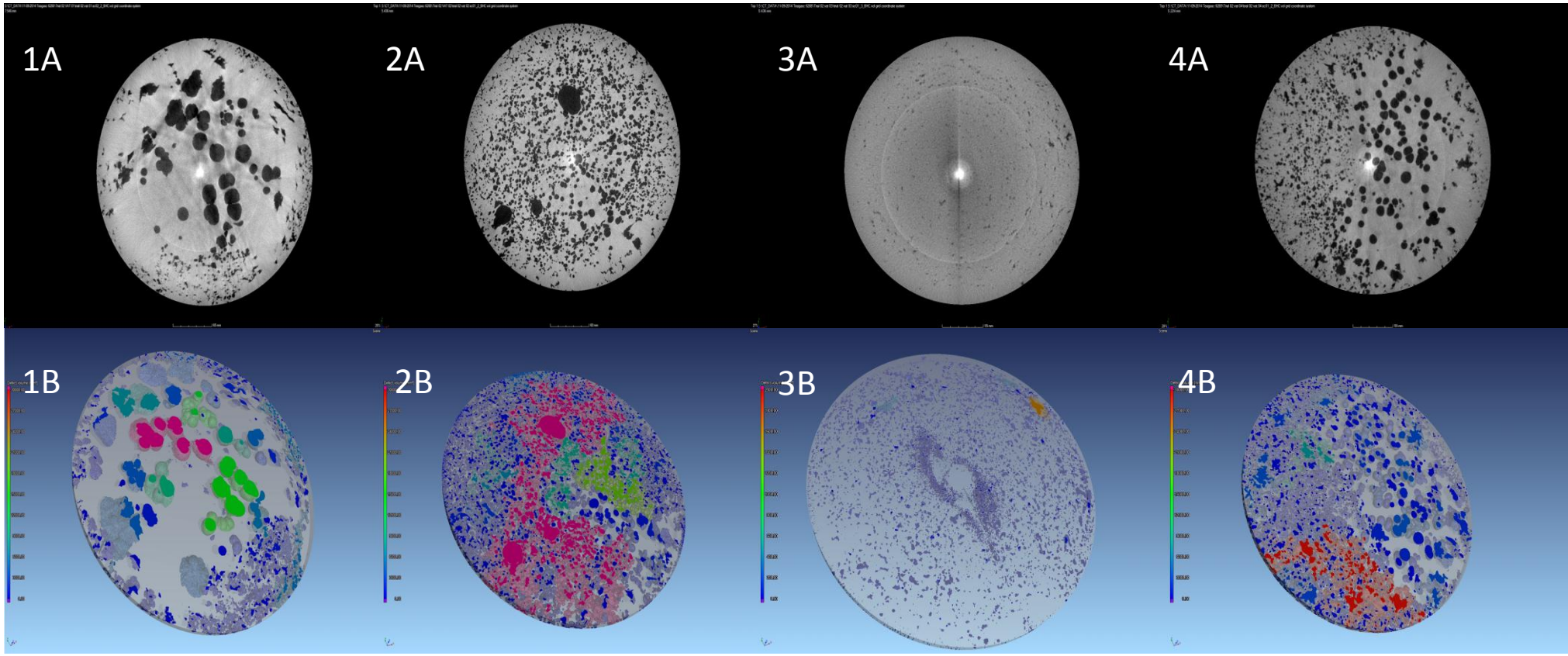


Figure 9: Eye formation in Swiss-type cheeses as determined by X-ray computed tomography (CT). CT images are represented, in the particular sections of the cheeses, are represented with an A while a void overview is represented by a B. Control cheese (1A & B), SPC cheese (2A & B), SLC cheeses (3A & B), SLPC cheese (4A & B). Images were taken from trial 2 at 95 d post production and are representative of trials 1 and 3. Colours in the blue spectrum represents voids of 0 – 6000 mm^3, green represents 9000 – 21000 mm^3, while red represents 24000 – 30000 mm^3.


1A
2A
3A
4A
1B
2B
3B
4B

Chapter 6

General Discussion

6.1. General Discussion

Fermentation represents the oldest and most effective form of food preservation and has likely been practiced by man for thousands of years. The fermentation process, which is conducted by several families of bacteria, yeast and fungi, impacts on foods in several ways. In addition to preservation, these include an enhanced nutritional content, increased digestibility and improved organoleptic properties. Notably, fermented foods can also act as a source of beneficial bacteria and metabolites (1-3). In the last 100 years, the roles of microbes, both beneficial and detrimental, in food fermentations has been the focus of in-depth studies relying on the use of classical and, more recently, molecular-based approaches. This has led to marked improvements in food quality/safety (4). Indeed, the recent advent of the molecular biology age has revealed that fermented food products are active, diverse microbial ecosystems rather than simple food products. With respect to this thesis, the fermented product of interest is cheese. Cheese is thought to have originated in the Middle-East some 8000 years ago, having been developed in order to preserve the constituents of milk (5, 6). The microbial populations present in cheese occur either intentionally (through starter and adjunct culture addition) or incidentally (*via* environmental contamination), and are the least controllable factor in cheese production (7). Microorganisms confer a significant effect on the characteristics and flavour of the respective varieties (8) and, as a result, are a primary determinant of cheese quality. Moreover, microorganisms can contribute to aroma and taste defects, form biogenic amines, cause gas and secondary fermentation defects, and can contribute to cheese pinking and mineral deposition

issues (6). Previously, cheese microbiota has been studied using classical microbiological methods. Indeed, these methods are still commonly used particularly in commercial cheese production plants (7). Classical methods, however, have numerous limitations, including their inability to detect un-culturable, stressed or weakened microbes, reveal sub-dominant populations or provide genera, species and/or strain level identification (8). Due to such limitations, and with the increased availability of molecular based approaches, classical methods are being replaced with culture independent techniques which can provide early and rapid detection of specific microbes/genes and, ultimately, assist in enhancing cheese quality and reducing costs.

Chapter 1:

Summary:

Chapter 1 of this thesis provided an in-depth analysis of the various molecular methods employed to profile microbial populations in cheese. By doing so, it also highlights the ever-greater insights that are being provided through the application of next generation sequencing (NGS) to study cheese microbiota.

Chapter 2:

Summary:

Chapter 2 built on results of a previous, culture based, study which described increased microbial diversity in cheeses produced later during the cheese production day (9). Our approach was to employ high throughput, 16S rRNA amplicon sequencing to further explore the impact of time of production day on

the successive development of microbial communities, in the respective cheeses, throughout the ripening process.

Outcomes and Impact:

1. For the first time, the spatial distribution of populations in the cheese core and rind was investigated, using an NGS based approach. In agreement with the previous study, higher microbial diversity, as determined by diversity matrices such as the Shannon Index, Chao1 and observed OTUs, was observed in cheese produced later during the cheese production day, throughout ripening.

2. Analysis of spatial variation indicated that cheese rinds were initially (1 d post production) more diverse than that of the core. However, for the remainder of ripening (i.e. after 10 d) the opposite was the case. This effect was likely due to environmental conditions such as the presence of oxygen, salt micro-gradients and pH.

3. As observed in similar studies (10-12), the use of culture independent sequencing identified novel and interesting genera that would not ordinarily be detected using either agar based screening methods or more basic molecular methods. Of particular interest in this study was the identification of Gram-negative halophilic genera such as *Pseudoalteromonas* and *Vibrio* as well as *Thermus*. Indeed, the presence of *Thermus* formed the basis of further studies into the phenomenon of cheese pinking.

4. This study is industrially relevant as it describes how cheese manufacturing practices may impact on the microbiota present in the cheese and, consequently, on cheese quality.

Limitations and Difficulties:

1. The greatest limitation to this study is the quantity of cheeses surveyed. Ideally the study would examine several cheeses, produced across an entire calendar year, in order to address seasonal differences in milk composition and provide a greater overall picture of industrially produced cheeses. The study was, however, carried out in conjunction with an international cheese producer and surveyed cheeses produced from large volumes of milk. In addition, this study simply provides a molecular based follow up to a similar study, carried out in the same production facility, over an extended period of time and is therefore of significant value.

Chapter 3:

Summary:

In Chapter 3, a slightly different approach was taken as, instead of the typical 16S rRNA based approach used in Chapter 2, specific defect causing genes (decarboxylase genes) were selected for amplicon sequencing. Prior studies on biogenic amines in food products have focussed on detecting either the amine present within the food (chromatographic methods) or individual bacteria/genes responsible for their production.

Outcomes and Impact:

1. This study, the first recorded application of the Ion PGM platform to profile a food ecosystem, used degenerate PCR primers were used to amplify segments of the bacterial histidine and tyrosine decarboxylase genes with a view to providing an in-depth analysis of the bacteria present.

2. Next generation sequencing allowed for the identification of common biogenic amine forming species such as *Lactobacillus buchneri*, *Lb. curvatus* and *Enterococcus faecium*.

3. In addition to this, decarboxylase genes from bacteria commonly used as cheese starters such as *Streptococcus thermophilus* and *Lactobacillus delbreueckii* were also identified.

4. This approach may be of particular interest for commercial companies as limits for the concentration of biogenic amines in cheese are expected to be established by the European Food Safety Authority (EFSA) in the coming years.

5. In the future, methods such as this may allow for large scale facility monitoring, providing a valuable tool for microbial modelling, and ultimately leading to safer, better quality products (13).

Limitations and Difficulties:

1. This method cannot determine the transcriptional activity of the respective genes present, it can however be used to establish a risk factor for biogenic amine occurrence, not only in cheese but in a variety of food products as well as the production environment.

2. While the approach used for this study was novel, the study was limited by the number and type of primers used. As decarboxylase genes are well conserved, the design of highly specific primers is particularly challenging.

3. Further studies, using more specific primers could potentially allow for greater resolution with respect to species identification.

4. The development of primers targeting biogenic amine producing yeast populations would have allowed for a more complete analysis of the aminogenic potential in the respective cheeses.

Chapter 4:

Summary:

The focus of Chapter 4 of this thesis was on the cheese pinking phenomenon, which has attracted attention for many years but the cause of which has yet to be comprehensively elucidated (14). In this study, a combined 16S rRNA, whole genome sequencing and quantitative PCR approach was taken in order to characterise this obscure defect

Outcomes and Impact:

1. While 16S rRNA based approaches are invaluable for determining the microbial composition of complex systems (15, 16), the genetic potential of the community in question remains elusive. With respect to this, whole genome shotgun sequencing allows for a more in-depth analysis of community structure by providing information on the functional capacity of a complex community (17).

2. NGS results described the presence of bacteria corresponding to the genus *Thermus*, in defective cheeses, and upon further examination through culture based screening and whole genome sequencing, *T. thermophilus* was identified as the dominant *Thermus* species present.

3. Genes involved in carotenoid production, and specifically that of lycopene were detected.

4. Verification of the presence of carotenoids was provided *via* Raman microscopy.

5. The use of NGS, in this study, played a key role in identifying *Thermus* spp. as key contributors to the pinking phenomenon. While standard 16S rRNA based sequencing successfully highlighted the presence of *Thermus*, whole genome sequencing allowed for identification, at species level, of several members of the *Thermus* clade as well as determining the pathway involved in carotenoid production. This allowed for the establishment of a link between the presence of *Thermus* and the occurrence of cheese pinking.

5. The results of this represent a significant step forward in our understanding of the cause of the pinking defect, however further research is still required on this topic into several key issues.

<u>Limitations and Difficulties:</u>

1. The greatest limitation to this research is the quantity of *T. thermophilus* (10^6 CFU ml^{-1}) used in the cheese trials. Initially, it was thought that adding a significant amount of *T. thermophilus* would allow for the greater manifestation of cheese pinking than was observed in the commercially sourced cheeses. The quantity of

cells used may, however, have impacted on the occurrence of pinking within the experimental cheeses.

2. The ability of an aerobic extremophile such as *Thermus* to grow in a cheese environment, the mechanism by which cheese pinking manifests, the contribution of thermophilic lactobacilli as well as conditions that contribute to and/or inhibit development are among the remaining questions and will form the basis of further research.

Chapter 5

Summary:

Chapter 5 involved a more traditional approach to determining causes of cheese defects. Recently, several studies have reported the effects of the addition of heterofermentative bacteria on defect development, and defective gas formation in particular (18-21). These studies have focussed on the addition of the aforementioned heterofermentative lactobacilli to cheese where any form of gas is regarded as a defect (e.g. Cheddar). With respect to Dutch- and Swiss-type cheeses, eye formation is considered a part of the overall cheese characteristics and therefore, only excessive gas formation, or development of gas at inappropriate times during ripening, resulting in defects such as disproportionate eye formation, splits and cracks are of particular concern (22, 23). In addition, certain studies have focussed on novel, non-destructive, methods for visualisation of gas defects (20, 24) including Magnetic Resonance Imaging and X-Ray Computed Tomography (X-ray CT). In this study the effect of addition of a facultatively heterofermentative

Lactobacillus (*Lactobacillus casei*) was investigated for its ability to promote gas defects in Swiss-type cheeses, where the starter activity was compromised. Patterns of openness were then investigated using non-destructive X-ray CT analysis.

Outcomes and Impact:

1. Results of this study showed that failure of starter bacteria, in this case *Lactobacillus helveticus*, coupled with the presence of a heterofermentative *Lb. casei* strain led to a greater propensity for excessive eye formation in Swiss-type cheeses.

2. This was likely due to the availability of residual lactose, galactose and citrate, which accumulate due to the absence of *Lb. helveticus*.

3. X-ray CT analysis of the various cheese treatments provided an accurate, non-invasive, overall image, not only of eye formation, but eye size, distribution and overall void percentage.

4. The results of this study are commercially relevant as they demonstrate the importance of viability of starter populations and the control of specific NSLAB to ensure appropriate eye formation in Swiss-type cheese.

Limitations and Difficulties:

1. A molecular based methodology i.e. DNA sequencing, could potentially have been employed in order to determine if any other populations present had a significant role to play in gas formation in this study. Furthermore, a molecular

based method, such as quantitative PCR would likely have provided more detail with respect to *Lb. helveticus* populations within the experimental cheeses.

In conclusion, advanced molecular methods, particularly NGS has revolutionised the field of food microbiology. NGS based methods facilitate detailed examination of the microbial community structure, and also allow for inferences on functional potential of the populations present. This thesis has shown how effective sequencing based techniques are, not just for microbial characterisation, but also for determination of the effects of particular populations and even genes. The significant reduction in cost of sequencing, particularly with respect to platforms such as the Ion PGM, has also allowed for NGS to become beneficial not only from an academic standpoint but also potentially industrially relevant. In the future, it is possible that NGS based methods will expedite large scale facility monitoring and microbial modelling allowing for in depth microbial surveillance. In this way, food safety and quality could become inherent to the product, significantly negating potential safety concerns for consumers and consequently reducing product recall. Additionally, in cases where the facility "microbiome" plays a key role in maintaining characteristic properties of a product (i.e. artisanal cheeses), NGS could be used to conserve and further explore microbial consortia, not only ensuring product quality, but allowing for development of new varieties.

6.2. References

1. **Paul Ross R, Morgan S, Hill C.** 2002. Preservation and fermentation: past, present and future. International Journal of Food Microbiology **79:**3-16.
2. **Bokulich NA, Mills DA.** 2012. Next-generation approaches to the microbial ecology of food fermentations. BMB reports **45:**377-389.
3. **Mayo B, Rachid CT, Alegria A, Leite AM, Peixoto RS, Delgado S.** 2014. Impact of next generation sequencing techniques in food microbiology. Current genomics **15:**293-309.
4. **Cocolin L, Ercolini D.** 2015. Zooming into food-associated microbial consortia: a 'cultural' evolution. Current Opinion in Food Science **2:**43-50.
5. **McSweeney PLH, Ottogalli G, Fox PF.** 2004. Diversity of cheese varieties: An overview, p. 1-23. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed.), Cheese: Chemistry, Physics and Microbiology, vol. Volume 2. Academic Press.
6. **O'Sullivan DJ, Giblin L, McSweeney PL, Sheehan JJ, Cotter PD.** 2013. Nucleic acid-based approaches to investigate microbial-related cheese quality defects. Frontiers in microbiology **4**.
7. **Justé A, Thomma BPHJ, Lievens B.** 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiology **25:**745-761.
8. **Jany J-L, Barbier G.** 2008. Culture-independent methods for identifying microbial communities in cheese. Food Microbiology **25:**839-848.
9. **Daly DFM.** 2014. Studies on factors relating to the development of defects in commercial cheese. PhD. University College Cork.

10. **Quigley L, O'Sullivan O, Beresford T, Ross R, Fitzgerald G, Fitzgerald G, Cotter P, Cotter P.** 2012. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. Appl Environ Microbiol **78:**5717 - 5723.

11. **Masoud W, Takamiya M, Vogensen FK, Lillevang S, Al-Soud WA, Sørensen SJ, Jakobsen M.** 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. International Dairy Journal **21:**142-148.

12. **Alegria A, Szczesny P, Mayo B, Bardowski J, Kowalczyk M, Kinde H, Mikolon A, Rodriguez-Lainz A, Adams C, Walker RL F, Cernek-Hoskins S.** 2012. Biodiversity in Oscypek, a traditional Polish cheese, determined by culture-dependent and -independent approaches. Appl Environ Microbiol **78:**1890 - 1898.

13. **Bokulich NA, Mills DA.** 2013. Facility-Specific "House" Microbiome Drives Microbial Landscapes of Artisan Cheesemaking Plants. Applied and Environmental Microbiology **79:**5214-5223.

14. **Daly DFM, McSweeney PLH, Sheehan J.** 2012. Pink discolouration defect in commercial cheese: a review. Dairy Science & Technology **92:**439-453.

15. **Ercolini D.** 2013. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology. Applied and Environmental Microbiology **79:**3148-3155.

16. **Cocolin L, Alessandria V, Dolci P, Gorra R, Rantsiou K.** 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. Int J Food Microbiol **167:**29-43.

17. **Wolfe Benjamin E, Button Julie E, Santarelli M, Dutton Rachel J.** 2014. Cheese Rind Communities Provide Tractable Systems for In Situ and In Vitro Studies of Microbial Diversity. Cell **158:**422-433.

18. **Porcellato D, Johnson ME, Houck K, Skeie SB, Mills DA, Kalanetra KM, Steele JL.** 2015. Potential of Lactobacillus curvatus LFC1 to produce slits in Cheddar cheese. Food microbiology **49:**65-73.

19. **Mullan WMA.** 2000. Causes and control of early gas production in cheddar cheese. International Journal of Dairy Technology **53:**63-68.

20. **Guggisberg D, Schuetz P, Winkler H, Amrein R, Jakob E, Fröhlich-Wyder M-T, Irmler S, Bisig W, Jerjen I, Plamondon M, Hofmann J, Flisch A, Wechsler D.** 2015. Mechanism and control of the eye formation in cheese. International Dairy Journal **47:**118-127.

21. **Fröhlich-Wyder M-T, Guggisberg D, Badertscher R, Wechsler D, Wittwer A, Irmler S.** 2013. The effect of Lactobacillus buchneri and Lactobacillus parabuchneri on the eye formation of semi-hard cheese. International Dairy Journal **33:**120-128.

22. **Fröhlich-Wyder MT, Bachmann HP.** 2004. Cheeses with propionic acid fermentation, p. 141-XV. *In* Patrick F. Fox PLHMTMC, Timothy PG (ed.), Cheese: Chemistry, Physics and Microbiology, vol. Volume 2. Academic Press.

23. **Sheehan JJ.** 2011. Cheese | Avoidance of Gas Blowing, p. 661-666. *In* Fuquay JW (ed.), Encyclopedia of Dairy Sciences (Second Edition). Academic Press, San Diego.

24. **Guggisberg D, Fröhlich-Wyder M-T, Irmler S, Greco M, Wechsler D, Schuetz P.** 2013. Eye formation in semi-hard cheese: X-ray computed tomography as a non-invasive tool for assessing the influence of adjunct lactic acid bacteria. Dairy Sci. Technol. **93:**135-149.

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